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Salt and solvent effects in the microscale chromatographic separation of heparan sulfate disaccharides

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Conflict of interest

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Abstract

The analysis of heparan sulfate disaccharides poses a real challenge both from chromatographic and mass spectrometric point of view. This necessitates the constant improvement of their analytical methodology. In the present study, the chromatographic effects of solvent composition, salt concentration, and salt type were systematically investigated in isocratic HILIC-WAX separations of heparan sulfate disaccharides. The combined use of 75% acetonitrile with ammonium formate had overall benefits regarding intensity, detection limits, and peak shape for all salt concentrations investigated. Results obtained with the isocratic measurements suggested the potential use of a salt gradient method in order to maximize separation efficiency. A 3-step gradient from 14 mM to 65 mM ammonium formate concentration proved to be ideal for separation and quantitation. The LOD of the resulting method was 0.8-1.5 fmol for the individual disaccharides and the LOQ was between 2.5-5 fmol. Outstanding linearity could be observed up to 2 pmol. This novel combination provided sufficient sensitivity for disaccharide analysis, which was demonstrated by the analysis of heparan sulfate samples from porcine and bovine origin.

Keywords: glycosaminoglycan; heparan sulfate; capillary liquid chromatography; HILIC-WAX; salt gradient

1. Introduction

Glycosaminoglycans (GAGs) are long linear polysaccharides consisting of repeating disaccharide units that comprise an amino sugar (N-acetylglucosamine or N-acetylgalactosamine) and a hexuronic acid (HexA; glucuronic acid or iduronic acid) or galactose and are sulfated along the chain which results in highly polar nature. The saccharide units can be sulfated at various positions and epimerization may also occur along the chain. They are localized in the extracellular matrix and on cell surfaces and are involved in numerous biological functions, including organogenesis, cell adhesion, signaling, inflammation, and tumorigenesis [1-3]. GAG chains may interact with different effector proteins (e.g. cytokines and chemokines) and this interaction depends on sulfation motifs within the chain [4, 5].

Heparan sulfate (HS) is the class of GAGs carrying the largest diversity. It consists of HexA and N-acetylglucosamine (GlcNAc) disaccharide units. Following the synthesis of the sugar chain, epimerization of glucuronic acid to iduronic acid occurs in certain regions, and finally, sulfation is carried out by sulfotransferases [6].

Due to their large size (up to 70 kDa), the investigation of intact HS chains is practically impossible by instrumental analytical tools. The structural characterization of the average sulfation pattern is usually performed after enzymatic hydrolysis of the polymeric chain into the constituent disaccharide units [7]. Bacterial polysaccharide lyase enzymes degrade the chains into $\Delta^{4,5}$-unsaturated disaccharides with varying degrees of sulfation. The characteristics of the resulting HS disaccharides are summarized in Table 1. Determining the ratio of these different structures is important in understanding the mechanisms underlying several diseases.

Because of the above-mentioned facts, even the structural characterization of GAG disaccharides poses several challenges [8]. Various chromatographic methods have been reported to analyze $\Delta^{4,5}$-unsaturated and sulfated HS disaccharides. These include derivatization (for retention or detection) followed by reversed-phase chromatography [9-11], reversed-phase ion-pairing chromatography (RPIP) [12-14], size exclusion chromatography (SEC) [15-17], graphitized carbon [18, 19], amide-HILIC [20, 21], or HILIC-WAX [22, 23] chromatography. Most of these separation methods can be on-line coupled to mass spectrometry (MS) thus detailed structural information of GAGs can be acquired [24, 25]. The main disadvantage of the above-mentioned methods is their relatively high detection limits, most have LODs in the picomole, some in the high femtomole range [17, 22]. An excellent

review was written recently, by Solakyildirim, summarizing the recent advances of glycosaminoglycan analysis [26].

We have recently reported an isocratic nanoflow HPLC-MS method [23] using self-packed columns which allowed quantification of as few as 10 fmol HS disaccharides from four out of the six investigated compounds. Sensitivity was an order of magnitude better than that of previously reported methods. A mixed-mode resin combining hydrophilic interaction (HILIC) and weak anion exchange (WAX) retention mechanisms was chosen as packing material, as it enables separation of polar solutes based on charge, size, and polarity [22]. When an ion-exchange column is operated under HILIC elution conditions, electric repulsion hydrophilic interaction chromatography (ERLIC) is created [27], which is an efficient technique in the separation of differently polarized components [28].

Building on our previously reported method [23], several further steps were considered in order to maximize the performance of HS quantitation from limited sample amounts. Lower limits were desirable especially for the non-sulfated D0A0 and the triply sulfated D2S6 disaccharides which showed relatively high quantitation limits (20 fmol and 50 fmol, respectively). To achieve this goal, it was necessary to increase the sensitivity of the individual disaccharides and to obtain better peak shapes. Moreover, in order to increase throughput, a fast chromatographic method with a more robust coupling (normal ESI source) was intended. In our earlier work the pH, acetonitrile content and ammonium formate concentration were optimized independently after one another, and the developed isocratic method was applied for the sample preparation development and characterization of tissue microarrays. We have concluded that acetonitrile gradients could not be applied due to the loss in sensitivity for the late eluting doubly and triply sulfated disaccharides.

Since the above-mentioned parameters were observed to be interdependent, a detailed investigation on the individual and cross-effects of acetonitrile content and salt concentration showed great promise. Thus, we decided to map the individual and interaction effects through a 3-factor experimental design operating with ammonium acetate and ammonium formate salts.

Although frequently used in ion (exchange) chromatography, salt gradients are almost entirely neglected in HILIC-based chromatography, mainly because of the lack of theoretical understanding of the retention mechanisms which still need theoretical and experimental elucidation [29-31]. Using salt gradients in reversed-phase chromatography is also uncommon, even for pH-gradient there are only a few reported examples [32, 33]. However, it was recently

proven that salt gradients can provide different selectivity, this way supplementing traditional solvent gradients [34, 35].

Our aim was to pursue a detailed examination of the salt and solvent effects in the chromatography of HS disaccharides to further improve throughput, quantitation limits, and repeatability. As opposed to its rare previous utilization, we designed a method building on salt gradients for HS disaccharide separation. The developed method proved to be applicable for determining the sulfation pattern of HS chains from biological origin.

2. Materials and Methods

2.1. Chemicals and reagents

The $\Delta^{4,5}$-unsaturated heparan sulfate disaccharide standards (listed in Table 1, 'HS disaccharides' hereinafter) and heparan sulfate from porcine intestinal mucosa (HSPIM) were purchased from Iduron (Cheshire, UK). LC-MS grade water and acetonitrile, crystalline ammonium formate and ammonium acetate, formic acid and acetic acid, and heparan sulfate from bovine kidney (HSBK) were purchased from Sigma-Aldrich (Budapest, Hungary).

2.2. Column packing

A GlycanPac™ AXH-1 1.9 µm analytical HPLC column (2.1x100mm, Thermo Fisher Scientific, Waltham, MA USA) was unpacked and repacked into capillaries. For this purpose, 250 µm i.d. capillaries were cut and fritted as previously reported [36]. Briefly, capillaries were dipped in a solution containing potassium silicate (Kasil® 1624, Kasil 1) and formamide in the ratio of 3:1:1. The capillaries were then placed in an oven at 80 °C for 4 hours. After the fully porous frit was produced, it was trimmed to 0.5 cm to reduce dead volume.

The packing itself was based on a method published recently [23] as follows. The capillary was placed in a pressure injection cell and was washed with 1 mL methanol. A 1 mg/mL suspension was prepared from the GlycanPac™ AXH-1 resin in 75% acetonitrile - 25% water. The slurry was continuously vortexed using a magnetic stir bar and the column was packed using nitrogen at 2000 psi. After reaching the desired 13 cm length, the pressure was carefully released overnight. Finally, a 60-minute-long UPLC compression procedure at 8000 psi was applied to maximize the axial homogeneity of the chromatographic bed.

2.3. Liquid chromatography-mass spectrometry

For microscale chromatography, a 'hybrid' system was assembled. The in-house packed capillary column was mounted on a Waters® nanoAcquity UPLC system (Waters, Milford, MA, USA) coupled to a high-resolution Waters® QTOF Premier™ Mass Spectrometer (Waters, Milford, MA, USA) via normal electrospray ionization source.

2.3.1. MS parameters

The mass spectrometry parameters were optimized for the highest sensitivity avoiding undesirable fragmentation in the ion source by directly infusing Leucine Enkephalin, D0A0, and D2S6 standards and further optimized via HPLC measurements. The capillary voltage was set to 2.4 kV, sampling cone to 20 eV, extraction cone to 4 V, the ion guide to 1.5. The source temperature was 80 °C, the desolvation temperature was 100 °C, the cone gas was 25 L/h and the desolvation gas 300 L/h. The investigated compounds were measured as singly-charged

anions (deprotonated molecules, $[M-H]^-$). Multiply charged ions or adduct forms complicating the analysis were not observed.

2.3.2. UHPLC parameters

For the investigation of the chromatographic behavior, we injected a mixture of the HS standards: 1 pmol of D0A0, D0S0, D2A6; 0.5 pmol of the D2A0 and D0A6 standards (positional isomers, thus resulting in a total of 1 pmol D2A0/D0A6 content); 2.5 pmol of D2S0 and D0S6 (positional isomers, thus resulting in a total of 5 pmol D2S0/D0S6 content) and 5 pmol of the D2S6 standard. The injection of this mixture resulted in similar peak heights.

The flow rate was selected to be 8 µL/min based on the stability of the signal investigated in a flow injection study (details not shown).

The column temperature was adjusted using an AgileSleeve capillary heater with MonoSleeve column heater controller (Analytical Sales and Services Inc, Flanders, NJ USA). The temperature was optimized and thermostating at 45°C was found optimal for the chromatographic performance (details not shown).

2.4. Screening of salt effects with ammonium formate

2.4.1. Mobile phase preparation

Mobile phase solvents were prepared by dissolving ammonium formate in water, then formic acid was added in an acid-to-salt molar ratio of 0.13 to adjust the pH to a previously optimized value of 4.4 [23], and finally, acetonitrile was added.

2.4.2. Solvent composition and salt content in isocratic methods

The effects were investigated by isocratic measurements at the respective salt concentration and solvent composition. The eluents were prepared with 75%, 50% and 25% acetonitrile content, and in each case 5 mM, 10 mM, 15 mM, 20 mM, 25 mM, 30 mM, 35 mM, 45 mM, 55 mM, and 65 mM ammonium formate concentrations were adjusted.

As a further optimization step, isocratic methods using 80%, 75%, 70%, 65% and 60% acetonitrile content were also tested, in order to justify that the formerly established 75% acetonitrile content provides the optimal conditions. The salt concentration for these runs was set to 45 mM.

2.5. Screening of salt effects with ammonium acetate

After optimizing the acid-to-salt molar ratio to reach the necessary pH of 4.4, and minimize ion-suppression, the same experimental design was performed as in the case of ammonium formate.

2.6. Salt gradient method

Based on the results of the isocratic screening, we designed a salt gradient method using the following parameters. Eluent A: 10 mM ammonium formate in 75:25 v/v ACN:water (pH 4.4); Eluent B: 65 mM ammonium formate in 75:25 v/v ACN:water (pH 4.4).

The flow rate was 8 µL/min and the gradient program was the following: after 0.5 minutes isocratic flow at 6% B, then the eluent ratio changed in 3 minutes to 70% B, then in 1 minute to 90% B, finally to 100% B in 4 minutes. 100% B solvent composition was held for 1.5 minutes and was followed by a 9-minute-long equilibration at the initial composition.

2.7. Enzymatic digestion of heparan sulfate

300 ng heparan sulfate from HSPIM/HSBK was dissolved in 45 µL aqueous digestion solution (12.5 mM ammonium bicarbonate, 2.5 mM $Ca(OH)_2$ containing 2.5 mU of Heparan Lyase I, 2.5 mU of Heparan Lyase II, and 1.25 mU of Heparan Lyase III). Following 24 hours of incubation at 37 °C, another cycle of enzymes (2.5 mU of Heparan Lyase I, 2.5 mU of Heparan Lyase II, and 1.25 mU of Heparan Lyase III) in 5 µL volume was added and the mixture was incubated at 37 °C for 24 more hours. The reaction was quenched by heating the sample to 80 °C for 5 minutes. The samples were dried down and re-dissolved in 50 µL ‘gradient starting solvent’ from which 2 µL was injected containing 12 ng HS portions.

2.8. Data evaluation and interpretation

Chromatographic parameters, like resolution, peak area, and intensity values were calculated with the QuanLynx add-in of Waters MassLynx 4.1 software. Then, the data were imported to OriginPro 8 to visualize in a contour plot using default settings of the program.

3. Results and discussion

3.1. Solvent and salt effects

Previous findings suggest that both salt concentration and solvent composition may have crucial effects on the separation efficiency of HS disaccharides in HILIC-WAX chromatography. In these studies, we used an acetonitrile-water solvent system with ammonium formate salt. We had previously tested a methanol-water solvent system as well, but it produced worse results. The combined effects of ammonium formate concentration and acetonitrile-water solvent ratio were mapped using a 3x10 factorial design. Three different acetonitrile contents were studied, 75%, 50%, and 25%. Initial studies have shown that acetonitrile content higher than 75% resulted in wide and shallow peaks, so we did not exceed this range for detailed investigations in the current study. Ammonium formate content was studied in the 5-65 mM range using 10 steps. This way we mapped the whole range that can be conveniently used for HPLC coupled to MS. It is important to note that even at high salt concentration we have observed no contamination of the ion source and the MS signals were stable over several weeks of analysis.

The key objectives in studying the effect of salt concentration and solvent composition were the proper separation of early eluting components, improving the resolution of monosulfated (D0S0-D2A0/D0A6) and disulfated (D2A6-D2S0/D0S6) peak pairs, eluting highly sulfated components in a relatively short time, and providing good sensitivity of analysis for all components. Note, that D2A0/D0A6 and D2S0/D0S6 disaccharides are positional isomers having practically identical hydrophilicity and charge, respectively, thus their separation is not feasible in this setup. The effect of salt concentration on the separation of the HS disaccharides is demonstrated in Fig. 1 (at 75% acetonitrile content), while that of solvent composition is shown in Fig. 2 (at 25 mM ammonium formate concentration). These effects are discussed in detail below in terms of retention factor, selectivity, resolution, sensitivity (peak area), and S/N ratio (intensity) parameters.

The first criterion is, that all the target compounds should be eluted from the column in a reasonable time, presuming appropriate retention and selectivity. Plotting retention factors as a function of salt concentration and solvent composition (Fig. 3), the changes were remarkable. Increasing salt concentration resulted in a fast decrease in the retention factors for all the components at all solvent compositions. Relatively low (10 mM) ammonium formate concentration (Fig. 1A) gave unsatisfactory results: retention factors were too high, thus not all components eluted in the 25-minute elution window. Increasing salt concentration to 25 mM resulted in a remarkable improvement (Fig. 1B). Using even higher (45 mM) salt concentration

(Fig. 1C) further decreased the retention factors, but this also resulted in co-elution of several components, mainly monosulfated (peaks 2, 3) and disulfated (peaks 4, 5) HS disaccharides which are closest to each other in polarity.

Increasing water content also changed the retention factors, although to a lower degree, and unequally for various components. Retention factors of non-sulfated and monosulfated disaccharides (peaks 1, 2, 3) showed a minor decrease with elevated water content, while those for doubly and triply sulfated disaccharides (peaks 4, 5, 6) increased, and co-elution of monosulfated (peaks 2, 3) and doubly sulfated components (peaks 4, 5) occurred (Fig. 2 and Fig. 3).

In highly aqueous solvents ion-exchange mechanism dominates, thus ions are distinguished primarily by the number of their charges, therefore co-elution of similarly charged disaccharides occur.

Second, we considered the resolution (R) of monosulfated and doubly sulfated peak pairs an important indicator for chromatographic separation. The contour plots in Fig. 4 show the change in resolution of the monosulfated (Fig. 4A) and the disulfated (Fig. 4B) peak pairs. As both the peak width and retention time decreased with increased salt concentration (as seen in Fig. 1) it was important to find a balance between these two to maximize resolution. The highest resolution of monosulfated components can be achieved by using relatively low (5-15 mM) ammonium formate concentration, while the resolution of disulfated HS disaccharides increased practically monotonously with increasing salt concentration at 75% acetonitrile content, and elaborately at other solvent compositions. This suggested that using a salt gradient may be optimal for the analysis of sulfated disaccharides.

A further important factor to consider is the overall sensitivity in ESI mass spectrometry. In fact, in most biological applications this is of crucial importance. Although proper quantitation is typically based on peak area, for optimal sensitivity, peak intensity (related to S/N ratio, being best for narrow peaks) may be even more important. The average peak areas, peak heights, and signal-to-noise ratios as a function of salt concentration and solvent composition are shown in the contour plots in Fig. 4C, 4D, and 4E, respectively. These show that sensitivity decreases fast with decreasing acetonitrile content with respect to both peak areas and peak intensities, due to lower ionization efficiency and increasing peak width. Increasing salt concentration (going from bottom to top on the contour plots) influenced peak areas only slightly, but (due to sharper peaks) intensities and the S/N ratio increased substantially. These imply that ion suppression or deterioration of ion source conditions do not happen even at elevated salt concentrations.

The results discussed above show that optimal solvent composition was found at the high edge of the investigated range, so we have performed further experiments (at 45 mM ammonium formate content) to map the range of 65% - 80% acetonitrile ratio in detail. It was found (analogously to that reported earlier [23]) that the best results were obtained at 75% acetonitrile content. Using 65-70% acetonitrile content resulted in a decreased resolution. Increasing acetonitrile content to 80% resulted in approximately 5-times increase in peak widths, resulting in a major loss (3-5-fold) of peak intensities (Fig. A-1). As it is seen in Fig. A-2, S/N ratios had a maximum at 70% ACN, the peak areas had maximum at 75% and 80%, but peak height had a large maximum at 75%. For this reason, we decided not to study solvent compositions over 75% in more detail.

Besides ammonium formate, the other commonly used buffer salt in HILIC-based separations (especially when on-line coupled to mass spectrometry) is ammonium acetate. We repeated the study discussed above using ammonium acetate. This study did not yield any further information: salt concentration and solvent composition had the same effects as obtained with ammonium formate. However, chromatographic peak shapes were more asymmetric, and mass spectrometry sensitivity was also worse. The data are shown in details in Appendix B.

3.2. Discussion of retention mechanism

The GlycanPac AXH-1 column is a HILIC-WAX mixed-mode resin, the chemistry of which is unknown for the public. The HILIC functional group is used to retain very polar compounds, and the WAX property separates based on charge. Discussing the possible interactions governing retention is rather difficult due to the unknown stationary phase chemistry and the mixed-mode operation. The solvation of the analytes and their dissociation states are strongly influenced by the acetonitrile ratio in the mobile phase [37]; and the fact that besides the effective pH [38, 39], the ionic strength may also have an influence [29], further complicating the picture. Instead of the pure HILIC and WAX operation conditions, their resultant determines the retention. Besides, the amino group in the HS disaccharides may be protonated under operating conditions, thus electric repulsion hydrophilic interaction chromatography (ERLIC) mechanism might also play a role [27]. This means that under HILIC conditions, the positively charged WAX functional groups repel the molecules that bear a protonated amino group. Bearing all of these in mind, we would like to propose an explanation for the interactions governing the retention of HS disaccharides on a HILIC-WAX column.

25% acetonitrile (thus high water) content in the mobile phase provides ideal circumstances for ion exchange, while under such high water content the HILIC effect is inoperative. In WAX,

the interaction of the analytes with both the stationary and the mobile phase is based on ionic effects, and therefore separation is mainly due to charge differences. Under such conditions, it is not possible to separate the mono- and the disulfated HS disaccharides, respectively. This mode of action is illustrated in Fig. 3, where at 25% acetonitrile content these components coelute (Fig. 3C).

Increasing acetonitrile content up to 50%, WAX interactions are weakened, and HILIC effects start to modify chromatography. The retention of non-sulfated and monosulfated components are hardly affected, while retention decrease of doubly and triply sulfated components becomes significant (Fig. 3B). Note, that the interactions affected by acetonitrile content may cause further changes [37], but their detailed investigation and discussion is out of the scope of the present paper.

Using 75% acetonitrile content, chromatographic effects are fairly complicated. Under such conditions, the HILIC functionality becomes dominant, while the WAX functionality may switch from ionic interactions to electric repulsion hydrophilic interaction chromatography (ERLIC). Separation is determined by the combination of the two. This is shown by the increased retention of non-sulfated and monosulfated disaccharides compared to lower acetonitrile content (Fig. 3A vs Fig. 3B). On the other hand, the retention of di- and trisulfated disaccharides decreases significantly due to the switch of WAX related strong retention to HILIC and ERLIC. The large selectivity increase between N-sulfated and N-acetylated disaccharides of the same charge can be explained as follows. N-sulfation decreases the electron density of the N-atom, the protonation is less likely, this way the effect of ERLIC is smaller; this is corroborated by the larger retention times of N-sulfated components (D0S0 and D2S0/D0S6) compared to the respective N-acetylated compounds (D2A0/D0A6 and D2A6).

In mixed-mode separations, the orientation of the various molecules may also play a crucial role [40], therefore even small structural differences result in increased separation. At 75% ACN content, planar coordination of the molecules is likely, as the proximity of O-sulfate groups to the amino group has no effect, while modification of the N-acetyl group has a large effect on retention. In contrast, at 50% and 25%, acetonitrile content the column operates mainly as an anion exchanger, and the coordination of the sulfate groups to the stationary phase has no effect on the strength of retention.

In summary, the degree of significance of HILIC, WAX and ERLIC mechanisms on the retention behavior depends on both the sulfation degree (i.e. charge state) of the various

compounds and the experimental conditions. Multiply charged disaccharides exhibit strong ionic interactions with the stationary phase and their retention is governed predominantly by the ion-exchange process in the whole experimental space (mostly WAX, partly ERLIC). HILIC, on the other hand, provides a substantial contribution to the retention of disaccharides with a lower number of sulfate groups present, especially at high acetonitrile content and at low salt concentration. Finally, when the acetonitrile content is increased above 75%, HILIC-type interactions with the stationary phase become very strong, and the various disaccharides do not elute from the column in a reasonable time (see Fig. A-1 and A-2).

3.3. Salt gradient separation of HS disaccharides

Results of the isocratic screening discussed above show that best separation characteristics (in terms of sensitivity, peak shape, and resolution) were obtained using 75% ACN – 25% H_2O solvent composition with ammonium formate buffering salt. However, separation of all components in reasonable run time and at high sensitivity could not be achieved simultaneously for all components using a constant salt concentration. Applying a generally used solvent gradient would have no significant benefits, as this would result either in bad elution characteristics or poor sensitivity (as seen in Fig. 3 and 4). Another alternative, rarely used in HILIC-based separations, is applying a salt gradient. The concept was to start the run at relatively low ammonium formate concentration (10-15 mM) so that the non-sulfated and monosulfated components were baseline-separated, then apply a moderately fast salt gradient to decrease the retention time and to increase intensity/sensitivity of the highly sulfated components. Note that this concept resolved the most important limitations of the previously reported HILIC studies, i.e. low sensitivity, badly resolved, and often irreproducible peaks for highly sulfated HS disaccharides. Detailed information on the development of the 3-step gradient is shown in Appendix C.

We designed a salt gradient method which used a 3-step gradient starting from 14 mM to 65 mM ammonium formate concentration. This way we have obtained a chromatogram (Fig. 5) with close to ideal peak shapes (FWHM of the last peak was practically the same as that of the first peak), good resolution, selectivity, and sensitivity. Note that salt concentration had a crucial effect on the retention and peak shape, but in contrary to usual mass spectrometry experience, it caused no additional ion suppression and gave no problems even in the long run (over several weeks). Resolution for all peak pairs was over 1.5, except that of the D2A6-D2S0/D0S6 peak pair, which was around 1.3. We considered this separation acceptable, especially as the two components have different molecular mass; separating them by mass spectrometry is trivial.

For the D0A0 and the D2S0/D0S6 components, the limit of quantitation (LOQ, defined according to the FDA Bioanalytical Method Validation guidelines [41]) was determined at 5 fmol, while for the D0S0, D0A6/D2A0, D2A6, and D2S6 components the LOQs were below 2.5 fmol. The limit of detection (LOD, estimated as 3-times S/N) was approximately 1.5 fmol for D0A0 and D2S0/D0S6, and below 1 fmol for the other components. These values show that our method is 100 times more sensitive than one of the recent papers on heparan sulfate analysis [22], and approximately 2-10 better than obtained before [23] using the same instrument in isocratic mode. Improved sensitivity may be attributed to gradient focusing and lower noise observed.

The linearity of the method was characterized in a wide range from 2.5 fmol to 2 pmol, each concentration was measured in triplicate. The R^2 values covering the whole range were all above 0.99, except in the case of D0A0, where it was 0.98. Calibration curves are shown in the Supplementary Information plotted both in linear and logarithmic mode (Fig. A-2).

The repeatability of the method was analyzed in 5 consecutive runs (intra-day repeatability) on 3 different days of the week (inter-day repeatability) using a 1 pmol HS disaccharide standard mixture. Intra-day repeatability (relative standard deviation of peak areas) was 3% on average, while inter-day repeatability was 5% (Table A-1). The retention time stability was 0.32 % intra-day, and 0.73% inter-day (RSD values). Long-term robustness of the system was outstanding; around 3 months of problem-free operation was observed when working with these high salt-content methods. Furthermore, no carry-over was experienced, even after injecting as much as 10 pmol samples.

3.4. Analysis of heparan sulfate samples

Performance of the developed method has been tested in the case of two different HS samples, which have been studied before [20, 42]. The samples, derived from porcine intestinal mucosa (HSPIM) and bovine kidney (HSBK), were enzymatically degraded with bacterial lyase enzymes into unsaturated HS disaccharides. Four replicates of digested samples were injected and separated using the developed salt gradient µHPLC-MS method. The sulfation pattern of heparan sulfate was demonstrated by the relative abundance of the HS disaccharides (Fig. 6). The non-sulfated D0A0, the disulfated D2S0/D0S6, and the triply sulfated D2S6 were the dominant disaccharides in both samples, while the disulfated D2A6 disaccharide was present only in a negligible amount. The monosulfated disaccharides, D0S0 and D0A6/D2A0, were present in moderate amounts in both samples. The ratio of the multiply sulfated components

was lower in the bovine than in the porcine sample, thus the degree of sulfation was much lower in HSBK than in HSPIM. This can be numerically described by the average number of sulfate groups on disaccharides, which is 0.96 for HSBK and 1.92 for HSPIM. These results agree well with previously reported data [20, 42]. The relative standard deviation of peak areas for all components in both samples remained under 20%, except for the D2S6 in HSBK (21.79%), present in relatively low amount. These RSD values are considered acceptable contemplating the variability of the biological samples, the difficult sample preparation steps, and the low amounts of the measured disaccharides.

4. Conclusions

The HPLC-MS analysis of heparan sulfate disaccharides poses a challenge from both chromatography and mass spectrometry sides, due to their diverse polarity and unfavorable ionization characteristics. In this paper, we performed a detailed isocratic screening of salt and solvent effects through a factorial design. We found that the acetonitrile-water ratio of the solvent highly influenced both the elution characteristics and ionization efficiency. Altering the salt concentration improved elution characteristics, but did not cause problems on the mass spectrometry side of the analysis.

Based on the above-mentioned results, we developed a salt gradient operating with self-packed HILIC-WAX µHPLC columns coupled to ESI mass spectrometry working in negative ion mode. Using the salt gradient improved sensitivity and repeatability could be achieved, compared to previous methods using the same resin [22, 23]. It was possible to separate and quantify the unsaturated HS disaccharides down to a few femtomoles, using a relatively short, 20-minute-long gradient. Sulfation patterns of heparan sulfates determined using the present method gave analogous results to those determined using other techniques.

The developed salt gradient method on mixed-mode HILIC-WAX resin offers several advantages as compared to previously published methods. First, the method is shorter than any other method reported (20 minutes instead of 30-60 minutes) [15-18, 20, 26]. Second, it provides utmost sensitivity with LOD below 1 fmol for all HS disaccharides (min. 100 fmol with other resins, and 10-50 fmol with HILIC-WAX was possible before). Furthermore, it facilitates proper investigation of non-sulfated and triply sulfated components in a single run.

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Appendices

Appendix A - Supplementary figures and tables

Figure A-1. Effects of solvent ratios surrounding 75% acetonitrile. A: 70% ACN; B: 75% ACN; C: 80% ACN content. The ammonium formate concentration was set to 45 mM, which resulted in sharp peaks but moderate resolution using 75% ACN content.

Figure A-2. Effects of solvent ratios surrounding 75% acetonitrile on average peak area (red), average peak height (blue), and average signal-to-noise ratio (black). The ammonium formate concentration was set to 45 mM at all points.

Figure A-3. The linearity of the method, calibration curves for the individual HS disaccharide standards: D0A0 (A), D0A6/D2A0 (B), D0S0 (C), D2A6 (D), D2S0/D0S6 (E), and D2S6 (F).

Table A-1. Intra-Day and Inter-Day repeatability of analysis characterized by the relative standard deviation of peak areas and retention times for HS disaccharides.

Appendix B - Ammonium acetate isocratic screening

Appendix C - Development of the 3-step gradient

List of tables

Table 1. Structure, nomenclature, and m/z values of the HS disaccharides investigated. Note, that D2A0/D0A6 and D2S0/D0S6 are positional isomers and are not distinguished in the present study.

List of figures

Figure 1. Effect of different salt concentrations using 75 % acetonitrile – 25% water solvent composition. Sums of extracted ion chromatograms (EICs) are shown in the diagram for A: 10 mM (not sufficient elution); B: 25 mM (sufficient resolution); and C: 45 mM (insufficient retention and resolution) ammonium formate salt concentrations.

Figure 2. Sums of EICs indicating the changes caused by different solvent compositions using 25 mM ammonium formate concentration. A: 75% ACN; B: 50% ACN; C: 25% ACN.

Figure 3. Retention factor (k) values of individual disaccharides as a function of ammonium formate salt concentration. Results are shown for three solvent compositions separately (A: 75% ACN; B: 50% ACN; C: 25% ACN content). Missing points mean that the respective component did not elute within the 25-minute elution window.

Figure 4. Resolution values of D0S0 - D2A0/D0A6 (A) and D2A6 - D2S0/D0S6 peak pairs (B), average peak areas (C), average peak intensities (D), and average signal-to-noise ratios (E) of all the HS disaccharides plotted on a contour plot as a function of salt concentration and acetonitrile content of the solvents. Color codes with the corresponding values are incorporated in the figure for each plot.

Figure 5. Extracted ion chromatograms of HS disaccharides obtained by separating 1 pmol of each compound using the developed salt gradient method.

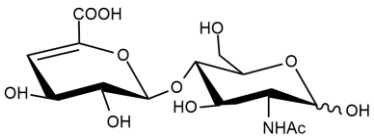
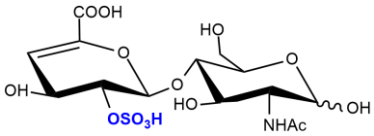
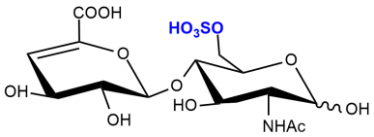
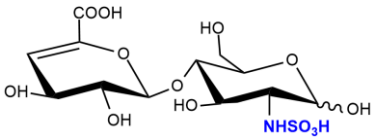
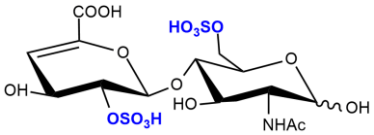
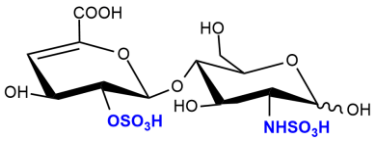
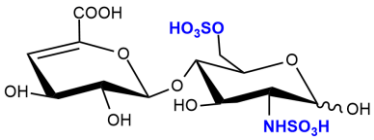
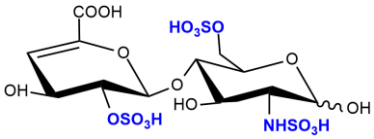
Figure 6. The sulfation patterns of heparan sulfate from porcine intestinal mucosa (HSPIM) and bovine kidney (HSBK). The sulfation pattern of the respective compound is characterized by the relative abundance of the HS disaccharides present following bacterial lyase digestion.

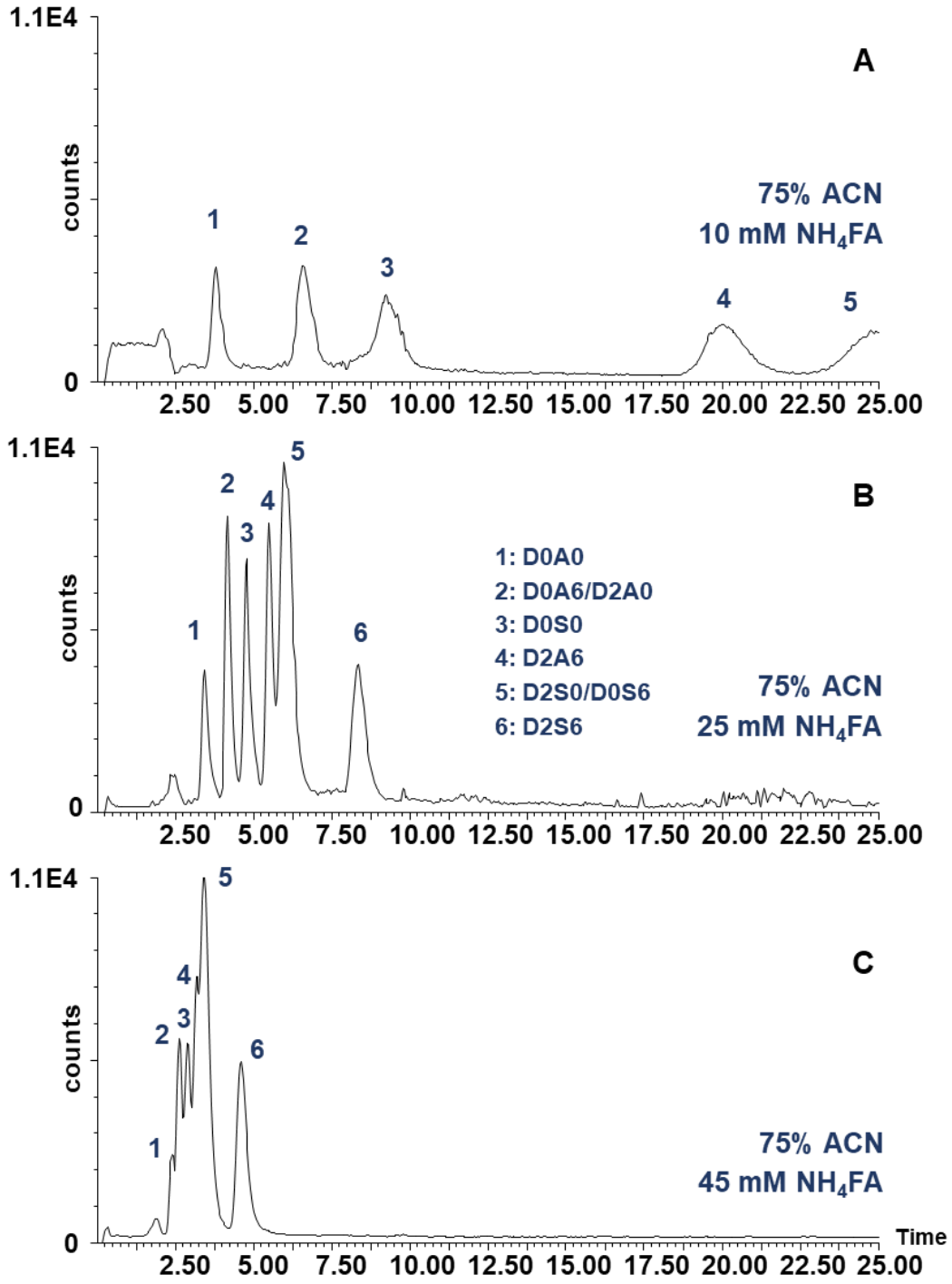
References

1. Handel, T.M., et al., *Regulation of protein function by glycosaminoglycans--as exemplified by chemokines.* Annu Rev Biochem, 2005. **74**: p. 385-410.
2. Karthikeyan, S. and S. Barbara, *Tumor-dependent Effects of Proteoglycans and Various Glycosaminoglycan Synthesizing Enzymes and Sulfotransferases on Patients' Outcome.* Current Cancer Drug Targets, 2019. **19**(3): p. 210-221.
3. Lin, H.-Y., et al., *The relationships between urinary glycosaminoglycan levels and phenotypes of mucopolysaccharidoses.* Molecular Genetics & Genomic Medicine, 2018. **6**(6): p. 982-992.
4. Gibbs, R.V. *Cytokines and Glycosaminoglycans (GAGS)*. 2003. Boston, MA: Springer US.
5. Kjellen, L. and U. Lindahl, *Specificity of glycosaminoglycan-protein interactions.* Current Opinion in Structural Biology, 2018. **50**: p. 101-108.
6. Multhaupt, H.A.B. and J.R. Couchman, *Heparan sulfate biosynthesis: methods for investigation of the heparanosome.* The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society, 2012. **60**(12): p. 908-915.
7. Lawrence, R., et al., *Disaccharide structure code for the easy representation of constituent oligosaccharides from glycosaminoglycans.* Nat Methods, 2008. **5**(4): p. 291-292.
8. Zaia, J., *Principles of mass spectrometry of glycosaminoglycans.* Journal of Biomacromolecular Mass Spectrometry, 2005. **1**(1): p. 3-36.
9. Galeotti, F. and N. Volpi, *Online Reverse Phase-High-Performance Liquid Chromatography-Fluorescence Detection-Electrospray Ionization-Mass Spectrometry Separation and Characterization of Heparan Sulfate, Heparin, and Low-Molecular Weight-Heparin Disaccharides Derivatized with 2-Aminoacridone.* Anal. Chem, 2011. **83**(17): p. 6770-6777.
10. Volpi, N., et al., *Analysis of glycosaminoglycan-derived, precolumn, 2-aminoacridone-labeled disaccharides with LC-fluorescence and LC-MS detection.* Nat. Protoc, 2014. **9**(3): p. 541-558.
11. Pan, Y., et al., *Glycosaminoglycans from fish swim bladder: isolation, structural characterization and bioactive potential.* Glycoconj J, 2017.
12. Yang, B., et al., *Ultra-performance ion-pairing liquid chromatography with on-line electrospray ion trap mass spectrometry for heparin disaccharide analysis.* Anal. Biochem, 2011. **415**(1): p. 59-66.
13. Wang, B., et al., *Characterization of currently marketed heparin products: Analysis of heparin digests by RPIP-UHPLC-QTOF-MS.* J. Pharmaceut. Biomed, 2012. **67-68**: p. 42-50.
14. Zhang, Z.Q., et al., *Quantification of Heparan Sulfate Disaccharides Using Ion-Pairing Reversed-Phase Microflow High-Performance Liquid Chromatography with Electrospray Ionization Trap Mass Spectrometry.* Anal. Chem, 2009. **81**(11): p. 4349-4355.
15. Hitchcock, A.M., C.E. Costello, and J. Zaia, *Glycoform quantification of chondroitin/dermatan sulfate using a liquid chromatography-tandem mass spectrometry platform.* Biochemistry, 2006. **45**(7): p. 2350-2361.
16. Shi, X.F. and J. Zaia, *Organ-specific Heparan Sulfate Structural Phenotypes.* J. Biol. Chem, 2009. **284**(18): p. 11806-11814.
17. Shao, C., et al., *Mass spectral profiling of glycosaminoglycans from histological tissue surfaces.* Anal. Chem, 2013. **85**(22): p. 10984-10991.
18. Barroso, B., M. Didraga, and R. Bischoff, *Analysis of proteoglycans derived sulphated disaccharides by liquid chromatography/mass spectrometry.* J. Chromatogr. A, 2005. **1080**(1): p. 43-48.
19. Karlsson, N.G., et al., *Use of graphitised carbon negative ion LC-MS to analyse enzymatically digested glycosaminoglycans.* J. Chromatogr. B, 2005. **824**(1-2): p. 139-147.
20. Gill, V.L., et al., *Disaccharide Analysis of Glycosaminoglycans Using Hydrophilic Interaction Chromatography and Mass Spectrometry.* Analytical Chemistry, 2013. **85**(2): p. 1138-1145.
21. Takegawa, Y., et al., *Simultaneous Analysis of Heparan Sulfate, Chondroitin/Dermatan Sulfates, and Hyaluronan Disaccharides by Glycoblotting-Assisted Sample Preparation*

538 *Followed by Single-Step Zwitter-Ionic-Hydrophilic Interaction Chromatography.* Anal. Chem,
539 2011. **83**(24): p. 9443-9449.
540 22. Chen, J.H., et al., *Heparan sulfate: Resilience factor and therapeutic target for cocaine abuse.*
541 Sci Rep, 2017. **7**.
542 23. Turiak, L., et al., *Sensitive method for glycosaminoglycan analysis of tissue sections.* J
543 Chromatogr A, 2018. **1544**: p. 41-48.
544 24. Ucakturk, E., et al., *Changes in composition and sulfation patterns of glycoaminoglycans in*
545 *renal cell carcinoma.* Glycoconjugate J, 2016. **33**(1): p. 103-112.
546 25. Bruinsma, I.B., et al., *Sulfation of heparan sulfate associated with amyloid-beta plaques in*
547 *patients with Alzheimer's disease.* Acta Neuropathol, 2010. **119**(2): p. 211-220.
548 26. Solakyildirim, K., *Recent advances in glycosaminoglycan analysis by various mass*
549 *spectrometry techniques.* Analytical and Bioanalytical Chemistry, 2019. **411**(17): p. 3731-
550 3741.
551 27. Alpert, A.J., *Electrostatic Repulsion Hydrophilic Interaction Chromatography for Isocratic*
552 *Separation of Charged Solutes and Selective Isolation of Phosphopeptides.* Analytical
553 Chemistry, 2008. **80**(1): p. 62-76.
554 28. Loroch, S., et al., *Multidimensional electrostatic repulsion-hydrophilic interaction*
555 *chromatography (ERLIC) for quantitative analysis of the proteome and phosphoproteome in*
556 *clinical and biomedical research.* Biochim Biophys Acta, 2015. **1854**(5): p. 460-468.
557 29. Alpert, A.J., *Effect of salts on retention in hydrophilic interaction chromatography.* J
558 Chromatogr A, 2018. **1538**: p. 45-53.
559 30. Buszewski, B. and S. Noga, *Hydrophilic interaction liquid chromatography (HILIC)--a powerful*
560 *separation technique.* Anal Bioanal Chem, 2012. **402**(1): p. 231-247.
561 31. Naidong, W., *Bioanalytical liquid chromatography tandem mass spectrometry methods on*
562 *underivatized silica columns with aqueous/organic mobile phases.* Journal of
563 Chromatography B, 2003. **796**(2): p. 209-224.
564 32. Kaliszan, R., P. Wiczling, and M.J. Markuszewski, *pH Gradient Reversed-Phase HPLC.*
565 Analytical Chemistry, 2004. **76**(3): p. 749-760.
566 33. Vreeker, G.C.M. and M. Wuhrer, *Reversed-phase separation methods for glycan analysis.*
567 Analytical and Bioanalytical Chemistry, 2017. **409**(2): p. 359-378.
568 34. Mant, C.T., et al., *An improved approach to hydrophilic interaction chromatography of*
569 *peptides: Salt gradients in the presence of high isocratic acetonitrile concentrations.* Journal
570 of Chromatography A, 2013. **1277**: p. 15-25.
571 35. Mant, C.T. and R.S. Hodges, *Mixed-mode hydrophilic interaction/cation-exchange*
572 *chromatography: separation of complex mixtures of peptides of varying charge and*
573 *hydrophobicity.* Journal of separation science, 2008. **31**(9): p. 1573-1584.
574 36. Maiolica, A., D. Borsotti, and J. Rappsilber, *Self-made frits for nanoscale columns in*
575 *proteomics.* Proteomics, 2005. **5**(15): p. 3847-3850.
576 37. Xia, J. and P.J. Gilmer, *Organic modifiers in the anion-exchange chromatographic separation*
577 *of sialic acids.* Journal of Chromatography A, 1994. **676**(1): p. 203-208.
578 38. Espinosa, S., E. Bosch, and M. Rosés, *Retention of Ionizable Compounds on HPLC. 12. The*
579 *Properties of Liquid Chromatography Buffers in Acetonitrile−Water Mobile Phases That*
580 *Influence HPLC Retention.* Analytical Chemistry, 2002. **74**(15): p. 3809-3818.
581 39. Subirats, X., M. Rosés, and E. Bosch, *On the Effect of Organic Solvent Composition on the pH*
582 *of Buffered HPLC Mobile Phases and the pK a of Analytes—A Review.* Separation &
583 Purification Reviews, 2007. **36**(3): p. 231-255.
584 40. Alpert, A.J., et al., *Peptide Orientation Affects Selectivity in Ion-Exchange Chromatography.*
585 Analytical Chemistry, 2010. **82**(12): p. 5253-5259.
586 41. Food And Drug Administration, U.S., *Bioanalytical Method Validation. Guidance for Industry*.
587 2018.
588 42. Shao, C., et al., *Mass Spectral Profiling of Glycosaminoglycans from Histological Tissue*
589 *Surfaces.* Analytical Chemistry, 2013. **85**(22): p. 10984-10991.

Table 1. Structure, nomenclature, and m/z values of the HS disaccharides investigated. Note, that D2A0/D0A6 and D2S0/D0S6 are positional isomers and are not distinguished in the present study.

Chemical structure	Traditional name	Lawrence code	m/z (-) mode
	ΔHexA-GlcNAc	**D0A0**	378.1
	ΔHexA2S-GlcNAc	**D2A0**	458.1
	ΔHexA-GlcNAc6S	**D0A6**	458.1
	ΔHex-GlcNS	**D0S0**	416.1
	ΔHexA2S-GlcNAc6S	**D2A6**	538.1
	ΔHexA2S-GlcNS	**D2S0**	496.1
	ΔHex-GlcNS6S	**D0S6**	496.1
	ΔHexA2S-GlcNS6S	**D2S6**	576.1



596

597 **Fig. 1.**

598

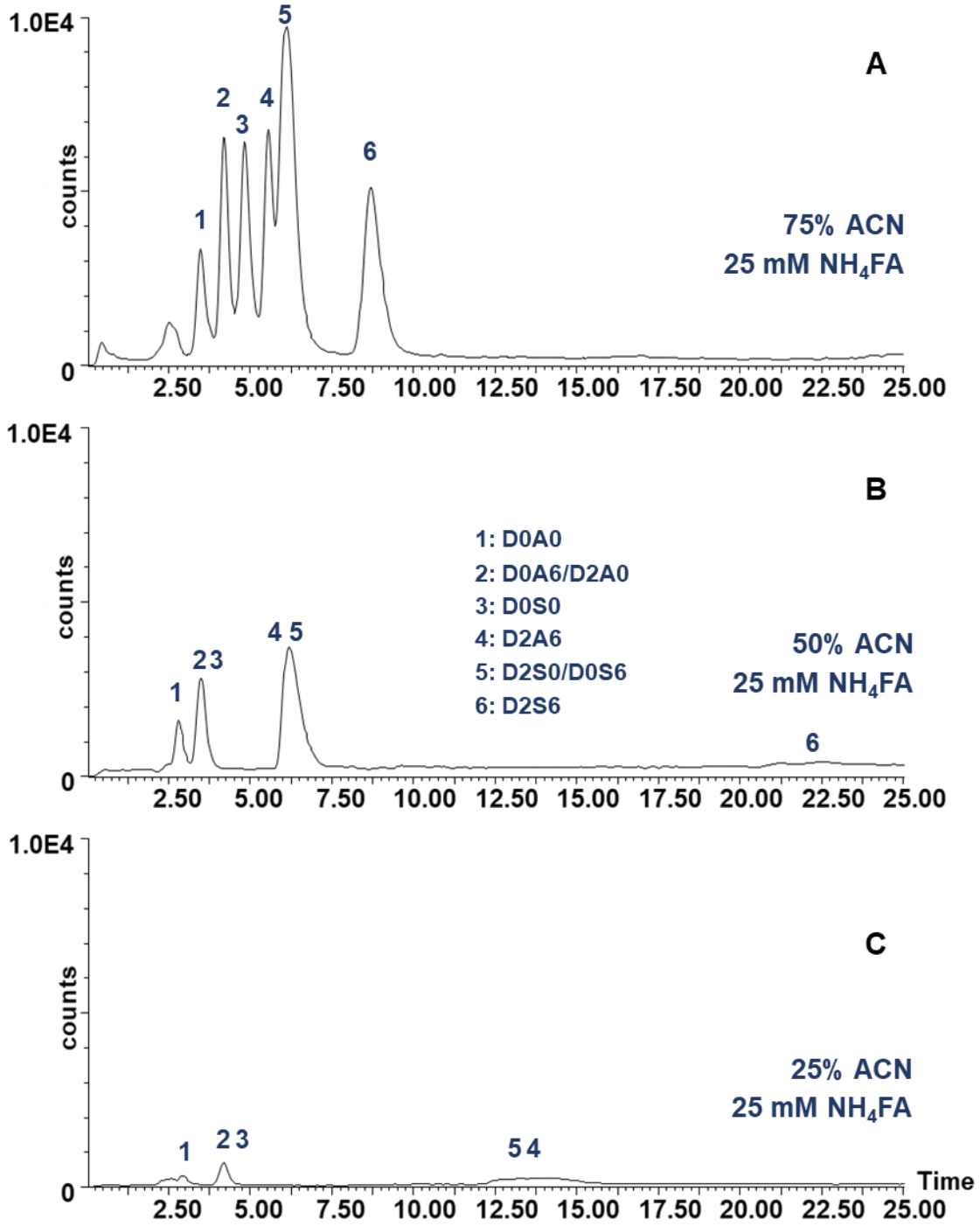

1.0E4
counts
0
A
1
2
3
4
5
6
75% ACN
25 mM NH4FA
2.50 5.00 7.50 10.00 12.50 15.00 17.50 20.00 22.50 25.00
B
1: D0A0
2: D0A6/D2A0
3: D0S0
4: D2A6
5: D2S0/D0S6
6: D2S6
50% ACN
25 mM NH4FA
C
25% ACN
25 mM NH4FA
Time

Fig. 2

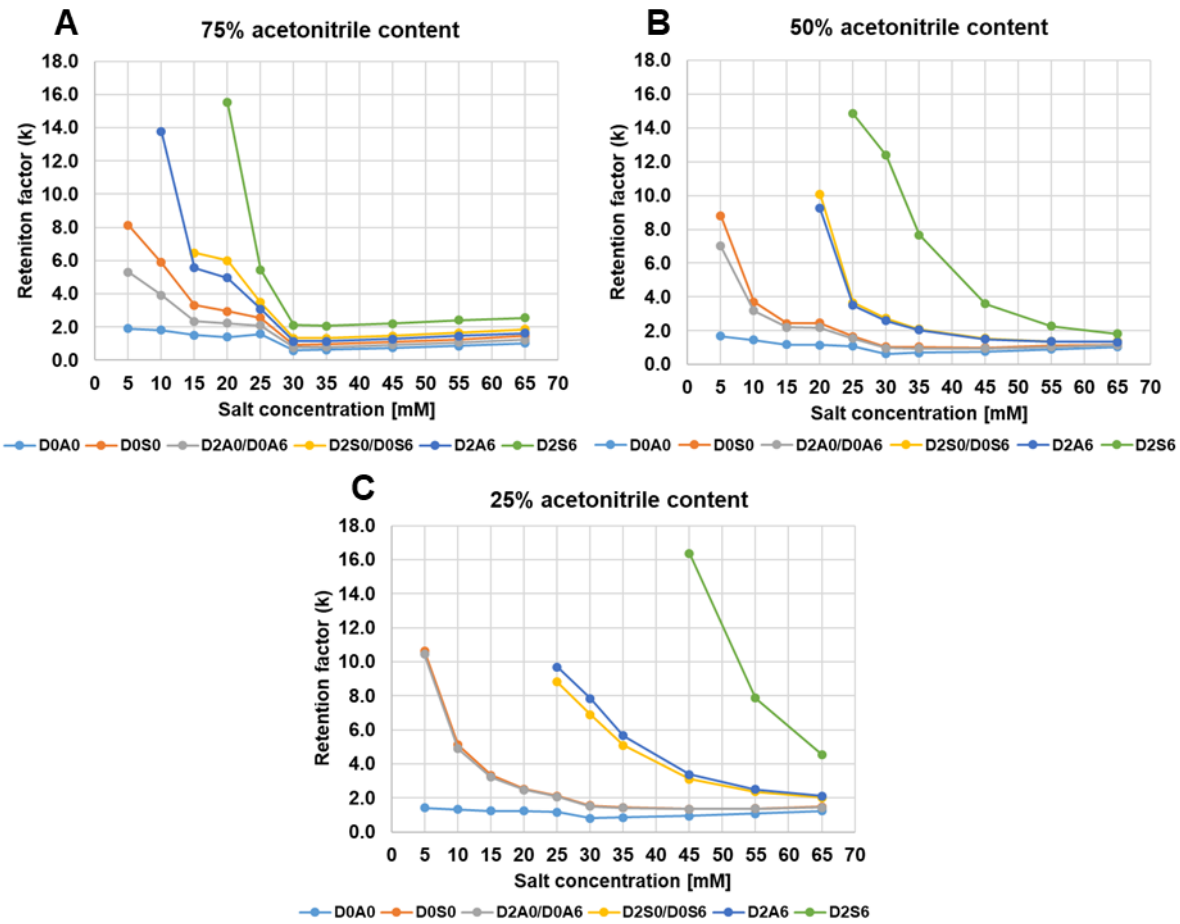

A
75% acetonitrile content
Reteniton factor (k)
Salt concentration [mM]
B
50% acetonitrile content
Retention factor (k)
Salt concentration [mM]
C
25% acetonitrile content
Retention factor (k)
Salt concentration [mM]
D0A0
D0S0
D2A0/D0A6
D2S0/D0S6
D2A6
D2S6

Fig.3

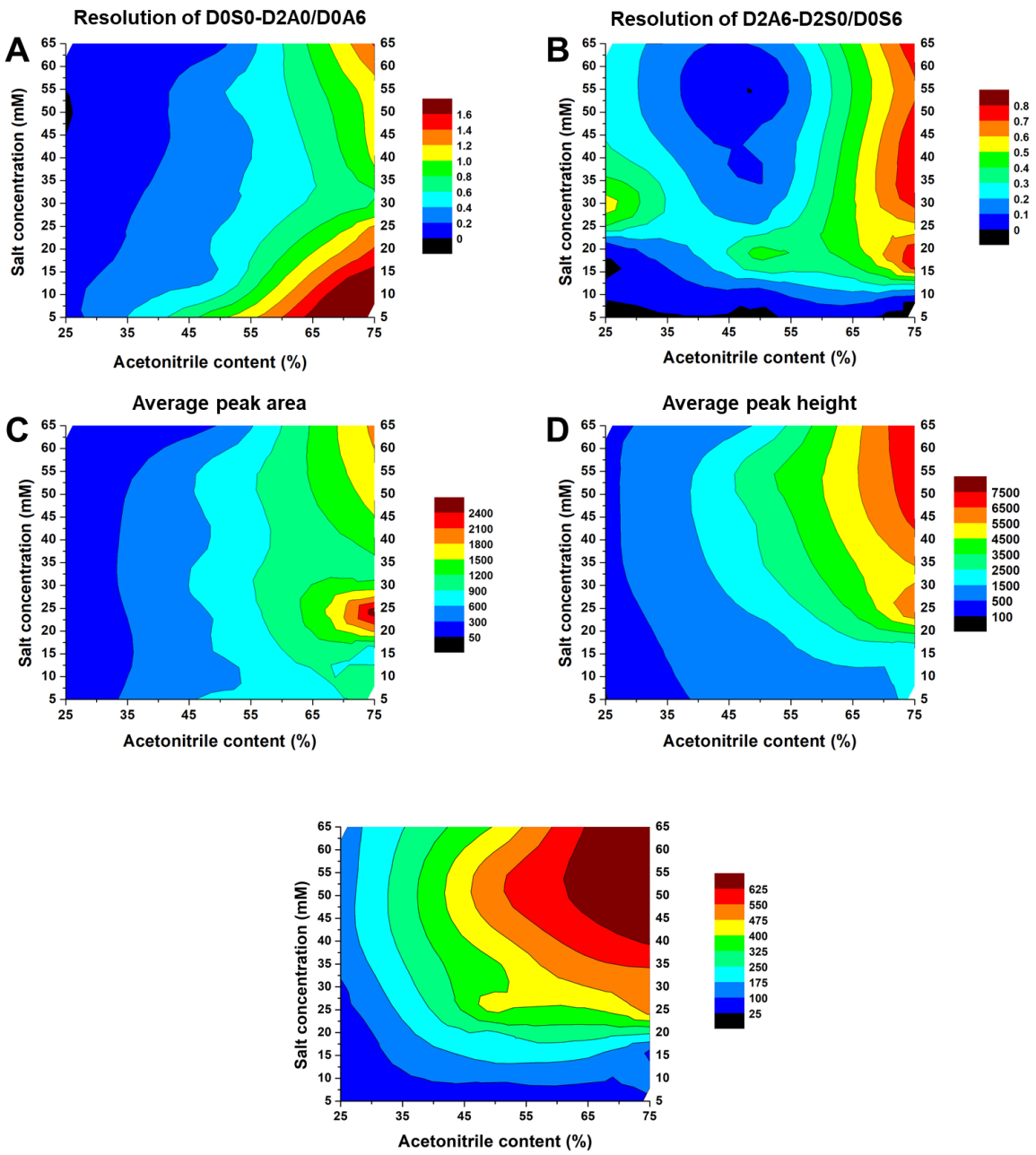

A
Resolution of D0S0-D2A0/D0A6
Salt concentration (mM)
Acetonitrile content (%)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
B
Resolution of D2A6-D2S0/D0S6
Salt concentration (mM)
Acetonitrile content (%)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
C
Average peak area
Salt concentration (mM)
Acetonitrile content (%)
2400
2100
1800
1500
1200
900
600
300
50
D
Average peak height
Salt concentration (mM)
Acetonitrile content (%)
7500
6500
5500
4500
3500
2500
1500
500
100
Salt concentration (mM)
Acetonitrile content (%)
625
550
475
400
325
250
175
100
25

Fig.4

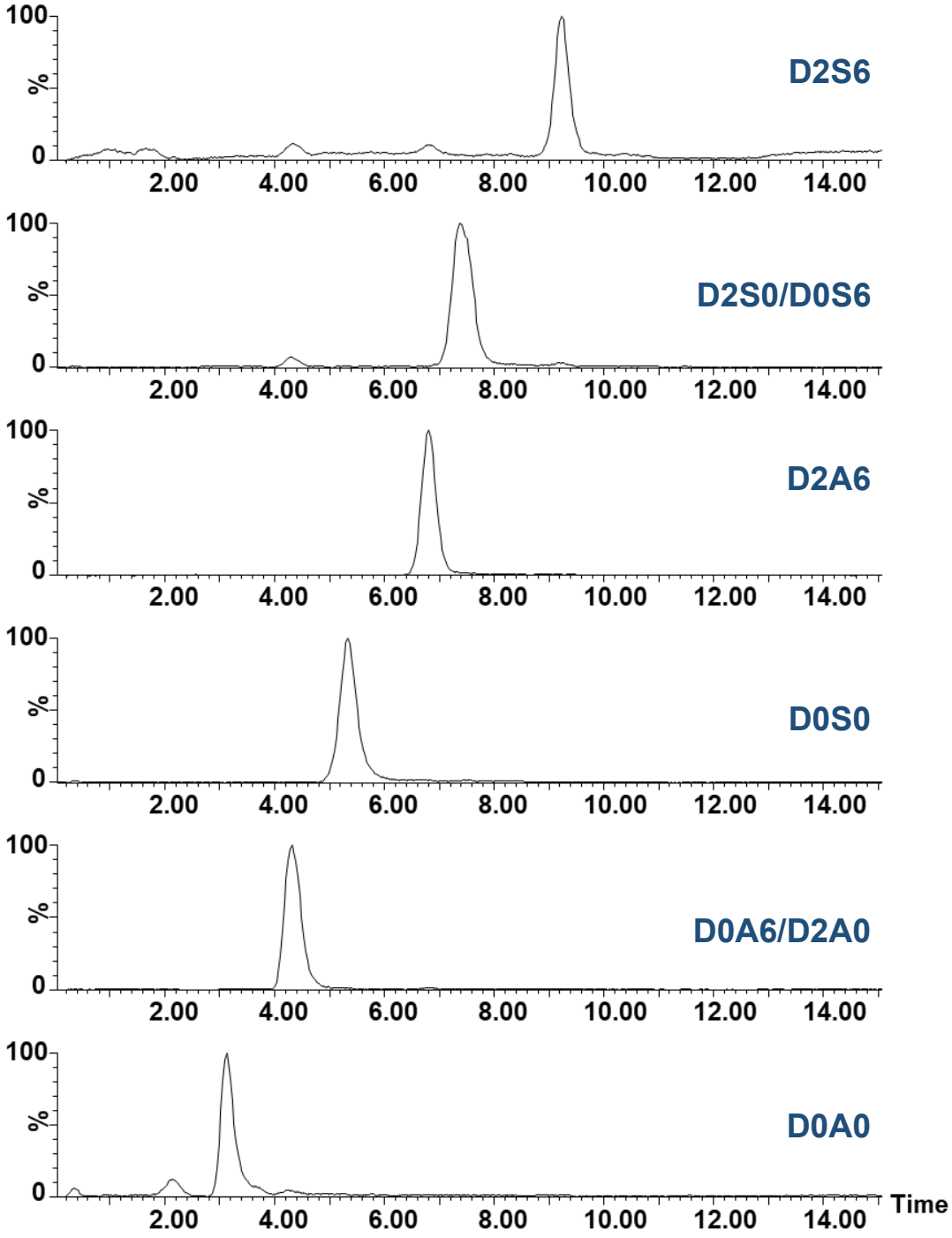

D2S6
D2S0/D0S6
D2A6
D0S0
D0A6/D2A0
D0A0
100
%
0
2.00
4.00
6.00
8.00
10.00
12.00
14.00
Time

Fig.5

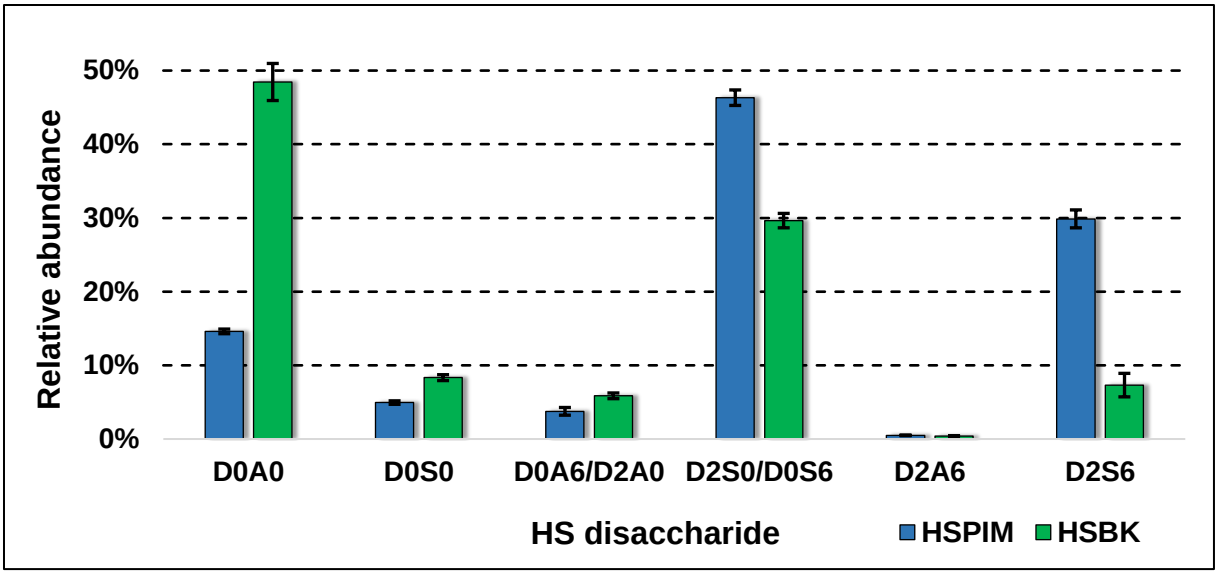

50%
40%
30%
20%
10%
0%
Relative abundance
D0A0
D0S0
D0A6/D2A0
D2S0/D0S6
D2A6
D2S6
HS disaccharide
HSPIM
HSBK

Fig.6