



Sensitive method for glycosaminoglycan analysis of tissue sections

Lilla Turiák^{a,*}, Gábor Tóth^a, Oliver Ozohanics^b, Ágnes Révész^a, András Ács^{a,c},
Károly Vékey^a, Joseph Zaia^d, László Drahos^{a,b}

^a MS Proteomics Research Group, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Magyar Tudósok Körútja 2, H-1117, Budapest, Hungary

^b MTA TTK NAP B MS Neuroproteomics Group, Hungarian Academy of Sciences, Magyar Tudósok Körútja 2, H-1117, Budapest, Hungary

^c Semmelweis University, PhD School of Pharmaceutical Sciences, Üllői út 26, H-1085, Budapest, Hungary

^d Department of Biochemistry, Center for Biomedical Mass Spectrometry, Boston University, Boston, MA, 02215, United States

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ABSTRACT

A simple, isocratic HPLC method based on HILIC-WAX separation, has been developed for analyzing sulfated disaccharides of glycosaminoglycans (GAGs). To our best knowledge, this is the first successful attempt using this special phase in nano-HPLC–MS analysis. Mass spectrometry was based on negative ionization, improving both sensitivity and specificity. Detection limit for most sulfated disaccharides were approximately 1 fmol; quantitation limits 10 fmol. The method was applied for glycosaminoglycan profiling of tissue samples, using surface digestion protocols. This novel combination provides sufficient sensitivity for GAG disaccharide analysis, which was first performed using prostate cancer tissue microarrays. Preliminary results show that GAG analysis may be useful for identifying cancer related changes in small amounts of tissue samples (ca. 10 µg).

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1. Introduction

Nano-scale liquid chromatography (nano-LC) coupled to mass spectrometry (MS) is an excellent technique used in routine characterization of different types of molecules (e.g. peptides [1,2], glycans [3], etc.), with still growing application areas. The field of proteomics greatly benefited from its constant improvements; however, a similar breakthrough is still waiting to occur in case of glycomics and especially glycosaminoglycan (GAG) analysis. GAGs are an often overlooked class of compounds, characterized by highly sulfated, long, linear polysaccharide chains. Their characterization poses several challenges [4]. The saccharide units can be sulfated at various positions and epimerization may also occur along the chain. Most GAGs are covalently attached to core proteins and form proteoglycans (PGs). They are localized in the extracellular matrix, cell surfaces and intercellular granules and mediate various physiological and pathophysiological processes including coagulation, cancer metastasis, and inflammation [5–8]. Involvement of both GAGs and PGs in cancer progression has been reported [7,9,10]. GAG chains may interact with different effector proteins

(e.g. chemokines, cytokines and growth factors) and thus regulate processes such as tumor cell growth, metastasis, and angiogenesis [10]. Interaction between the GAG chains and the effector proteins strongly depends on sulfation motifs within the chain.

The building blocks of GAG chains are repeating disaccharide units of a uronic acid or galactose unit and an amino sugar (often abbreviated as HexA/Gal–HexNAc). GAGs are divided into four different classes: hyaluronan (HA), heparan sulfate (HS)/heparin, chondroitin sulfate/dermatan sulfate (CS/DS) and keratan sulfate (KS). Perhaps the most widely known group is HS, consisting of a hexuronic acid (HexA) and *N*-acetyl glucosamine (GlcNAc) disaccharide units.

Analytical characterization of GAGs is usually performed after hydrolysis of the polymeric chain into the constituent disaccharide units. Bacterial polysaccharide lyase enzymes can cleave the glycosidic bond between the HexA and HexNAc sugars via an eliminative mechanism and produce $\Delta^{4,5}$ -unsaturated disaccharides with varying degrees of sulfation; HS disaccharides may be sulfated by up to three sulfate groups. The structures, masses and nomenclature [11] of HS disaccharides are summarized in Table 1. Determining the ratio of these different structures is important in understanding mechanisms underlying diseases such as cancer.

Several different chromatographic techniques can be used to analyze $\Delta^{4,5}$ -unsaturated, and variously sulfated disaccharide

* Corresponding author.

E-mail address: turiak.lilla@ttk.mta.hu (L. Turiák).

Table 1
Structure and nomenclature of the most common unsaturated HS disaccharides obtained following heparin lyases digestion. Note, that D2A0/D0A6 and D2S0/D0S6 are positional isomers and are not distinguished in the present study.

HS disaccharide structure	Traditional name	Lawrence code [11]	<i>m/z</i> in negative 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

units of HS. These include derivatization followed by reversed-phase chromatography (RP) [12–17]; reversed-phase ion-pairing chromatography (RPIP) [18–20]; size exclusion chromatography (SEC) [21–23]; graphitized carbon [24,25]; amide-HILIC [26,27]; or HILIC-WAX [28] chromatography. These separation methods can be on-line coupled to mass spectrometry (MS) and yield useful information about the involvement of GAGs in various biochemical processes [29,30]. These analytical approaches have been reviewed recently [31]. The main disadvantage of the above mentioned methods is their relatively low sensitivity, which is generally in the low pmol range [23,28]. Perhaps the currently most sensitive method for GAG disaccharide profiling is fluorescent labelling and HPLC separation [15]. Limit of detection of this method for most HS disaccharides was ca. 0.3 pmol (~0.1 ng); for the trisulfated D2S6 it was ca. 2 pmol (~1 ng). This sensitivity is sufficient to study bulk tissues, but is insufficient to study tissue slices or tissue microarrays (TMAs). In fact, recent examples of GAG analysis as much as 1 g tissue amount were required [16].

The main reason for developing our method was the need to have a technology capable of GAG analysis of small size tissue slices. Our plan is utilizing tissue microarrays (set of 1.5 mm diameter, 5 μm thick tissue slices arranged in array format, ca. 10 μg tissue), which provide an opportunity to study relatively large number of well characterized, well matched human cancer tissue

samples. Target sensitivity is the low fmol range (for individual disaccharides), which is the amount foreseen to be present in tissue microarray digests. To achieve this goal, special, self-packed, commercially unavailable capillary columns were designed. 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 glycans based on charge, size and polarity [28,32]. Use of capillary columns allows the use of on-line nano-UHPLC–MS coupling; needed to achieve the necessary sensitivity. The packing material was obtained by opening a commercially available analytical (2.1 mm diameter) column, and repacked into 100 μm i.d. capillaries.

2. Materials & methods

2.1. Chemicals and reagents

The following standards and enzymes were purchased from Iduron (Cheshire, UK): unsaturated heparan sulfate disaccharides, heparinase I, II and III enzymes. LC–MS grade solvents, ammonium formate solution were purchased from Sigma-Aldrich (Sigma-Aldrich Kft., Budapest, Hungary).

2.2. Capillary column packing

A GlycanPac™ AXH-1 1.9 μm analytical column (2.1 $\times$ 100 mm, Thermo Fisher Scientific, Waltham, MA USA) was unpacked and repacked into 100 μm internal diameter capillaries using a pressure injection cell (Next Advance Inc., NY, USA). For this purpose, 20–30 cm 100 μm internal diameter capillaries were cut and fritted as previously reported [33]. Briefly, capillaries were dipped in a solution containing Kasil® 1624, Kasil 1 and formamide in a ratio of 3:1:1. The capillaries were then placed in an oven at 80 °C for 4 h. Capillaries were examined under a microscope and in case of fully porous frit the excess was trimmed to 0.5 cm in order to reduce dead volume. Capillary was then placed in the pressure injection cell and was washed with methanol. A 1 mg/mL suspension was prepared from the GlycanPac™ AXH-1 resin in 75% Acetonitrile – 25% Methanol. The slurry was continuously vortexed using a magnetic stir bar and the column was packed using nitrogen at 2000 psi. After reaching the desired length – generally between 10 and 11 cm – the pressure was carefully released overnight. Due to the pressure drop inhomogeneity may appear, therefore 30 min long compression step (at 5000 psi) was necessary following mounting the column on the nano-HPLC.

2.3. Liquid chromatography–mass spectrometry

The in-house packed capillary column was mounted on a Waters® nanoAcquity UPLC system (Waters, Milford, MA, USA) coupled to a high resolution Waters® Q-ToF Premier™ Mass Spectrometer (Waters, Milford, MA, USA) via nanoelectrospray ionization source. Precut silica tips (360 μm OD, 20 μm ID, 10 μm tip ID, DNU – MS GbR, Berlin, Germany) were used as emitters.

2.3.1. LC parameters

A flow rate of 0.6 $\mu\text{L}/\text{min}$ was found to give stable signal and gave reasonable back pressure (2000 psi). Solvent A was 50 mM ammonium formate pH 4.4 (pH adjusted with formic acid) and solvent B was 95% acetonitrile and 5% water, without any buffer. Method optimization was performed on the self-packed columns using commercially available $\Delta^{4,5}$ -unsaturated HS disaccharide standards. The following isocratic methods were tested: 90%B, 85%B, 80%B, 75%B and 70% B.

2.3.2. MS parameters

Negative ionization mode was used. Correct tuning of MS parameters is critical when analyzing labile molecules such as sulfated sugars. The instrument was tuned by direct infusing the most labile triply sulfated heparan sulfate disaccharide (D2S6). Decreasing the cone voltage to 15 eV from the generally used 35 eV resulted in minimal (<5%) sulfate loss. Extraction cone voltage was also lowered to 1 V. MS data was acquired in the m/z 180–800 mass range.

2.4. Enzymatic digestion of TMA cores

Prostate cancer tissue microarrays T191a and T196 were obtained from US Biomax, Inc. (Derwood, MD, USA). Tissue sections were processed (dewaxing and antigen retrieval) as previously described [34]. TMA cores corresponding to normal (healthy prostate tissue, male, 33 years), grade 1 (adenocarcinoma, male, 72 years) and grade 2–3 (adenocarcinoma, male, 60 years) cancer were chosen for the current analysis. In case of each patient the TMA contained 3 cores/case allowing to assess the reproducibility of the developed method. Heparinase I–III digestion was performed on the surface of the TMA cores as previously described [34]. Following 5 cycles of enzyme addition (40 min 37 °C incubation/cycle) two additional cycles were carried out by pipetting the enzyme solution without the enzymes to ensure completion of the enzymatic

digestion process. The HS disaccharides were extracted from the individual cores using 0.3% ammonium-hydroxide solution, dried down and re-suspended in the LC starting conditions. During the experiments a quality control sample followed by two blank injections was ran before the tissue microarray samples.

2.5. Data analysis

Data acquisition was controlled by MassLynx, quantitative results were evaluated by QuanLynx software (Waters, Milford, MA).

3. Results & discussion

Detection of both neutral and highly acidic disaccharides by mass spectrometry presents a challenge; initial trials showed that negative electrospray ionization provides the best sensitivity. High mass resolution was needed to improve selectivity of disaccharide analysis as extracts of tissue surface digestion contain a large amount of impurities, the target disaccharides being minor components only. Sulfated disaccharides fragment easily in the mass spectrometer ion source under conventional conditions, compromising analysis. In order to avoid this problem ion source conditions have to be specially tuned. The most critical parameter is the cone voltage, which, in our instrument, had to be reduced from the conventionally used 30–35 eV to as low as 15 eV.

Based on the literature [28] we have selected a mixed mode HILIC-WAX stationary phase, which was shown to be efficient for analysis of acidic oligosaccharides [32]. Capillary columns were packed in-house with this packing material. We have used medium ion strength ammonium formate buffer and acetonitrile solvents. Method development was carried out using commercially available standards with the goal of analyzing small amounts of tissue digests (tissue microarrays) containing disaccharides in the low fmol range.

Initial trials using gradient elution showed that mass spectrometry sensitivity significantly changes with solvent composition; as a result, the late-eluting doubly and triply sulfated disaccharides had the lowest sensitivity among those tested. For example, in equimolar mixtures the signal intensity for the trisulfated HS disaccharide was 30 times less than those of the neutral or the monosulfated disaccharides, due to changes in solvent composition. For this reason, we have decided to develop an isocratic elution method.

3.1. Method optimization

First, the required ion strength of the strong eluent was established. It was found that the method is very sensitive to the salt concentration: sulfated (especially highly sulfated) disaccharides do not elute from the column neither at lower (10–40 mM) nor at higher (80 mM) ammonium formate concentration. Medium ion strength (50 mM ammonium formate) resulted in a reasonable chromatogram; and this was used in the following. The next step was the adjustment of the pH of the buffer, using formic acid. We tested pH values of 4.0, 4.4 and 4.6, respectively. At pH 4.0 resolution of monosulfated, at pH 4.6 resolution of disulfated disaccharides were unsatisfactory. The best results were achieved at pH 4.4 for which it was possible to detect and resolve the HS disaccharides according to composition.

Subsequently we evaluated five different isocratic conditions (90% B, 85% B, 80%B, 75%B and 70% B, Fig. 1). Note, Fig. 1 shows the sum of the peak intensities (sum of selected ion chromatograms at high resolution) of the HS disaccharides. When the eluent contained a high percentage of acetonitrile (90 and 85% B) the corresponding peaks were broad and shallow (Fig. 1E and D) for the highly sulfated structures and the triply sulfated disaccharide did not elute within 15 min. In case of 75% and 70% B partial co-elution of various

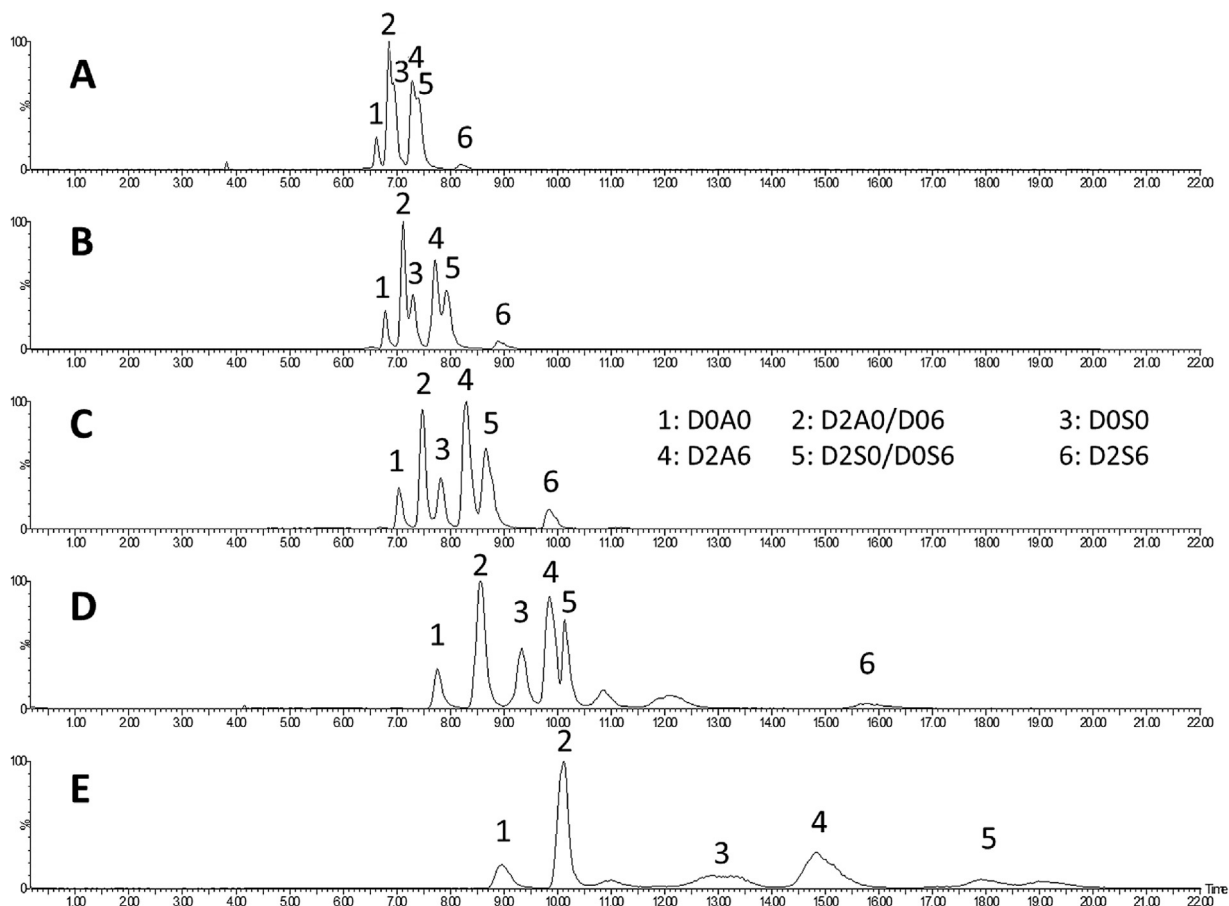


Fig. 1. Sum of selected ion chromatograms of 500 fmol heparan sulfate disaccharide standards (D0A0, D0S0, D0A6, D2A0, D2A6, D2S0, D0S6 and D2S6) on an in-house packed 100 μ m i.d. capillary column packed with 1.9 μ m particle size GlycanPac™ AXH-1 resin using the following isocratic methods: 70% B (A), 75% B (B), 80% B (C), 85% B (D), 90% B (E). Note, that D2A0/D0A6 and D2S0/D0S6 are positional isomers.

Table 2

Repeatability of analysis. Intra-Day and Inter-Day relative standard deviation of peak areas and retention times for the unsaturated HS disaccharides.

HS disaccharide	RSD Peak Area		RSD Retention time	
	Intra-Day	Inter-Day	Intra-Day	Inter-Day
D0A0	6.68%	7.91%	0.22%	0.45%
D0S0	5.15%	15.58%	0.27%	0.97%
D0A6/D2A0	3.11%	3.44%	0.26%	0.75%
D2S0/D0S6	3.67%	7.61%	0.48%	1.69%
D2A6	4.03%	7.37%	0.19%	1.35%
D2S6	10.18%	20.19%	0.35%	1.75%
Average	5.47%	10.35%	0.30%	1.16%

peaks was observed (Fig. 1A and B). The isocratic method at 80% B was optimal (Fig. 1C) because it resolved the HS disaccharides based on composition; peak shapes were symmetric, and all peaks eluted within 15 min. Individual selected ion chromatograms of the HS disaccharides are shown in Fig. 2. This isocratic method ameliorated the problem of low signal intensity for the triply sulfated HS. The main reason was that using gradient separation water and buffer concentration is increased at the elution of the triply sulfated disaccharide. Under such conditions the MS sensitivity decreases, resulting in low sensitivity for the highly sulfated derivatives. Note that D2S6 (the triply sulfated derivative) elutes in a fairly wide peak. This is due to the highly polar nature of this compound, which binds strongly to the HILIC-WAX resin.

We have determined the repeatability of analysis as well (Table 2). For the 500 fmol sample mixture intra-day repeatability of relative peak areas was 5% on average; inter-day repeatability

was 10% (relative standard deviation; 5 measurements in a day, measured on 3 different days). Repeatability (relative standard deviation) of retention times was, on average 0.3% within a day, and 1.2% between days.

3.2. Linearity studies

We also demonstrated the linearity (Fig. 3 and Supplementary material Fig. S1) of signal intensity with increasing sample amount, and the limits of quantitation. The following concentrations of HS disaccharide mixtures were tested with 80% B isocratic method: 10 fmol, 20 fmol, 50 fmol, 100 fmol, 200 fmol, 500 fmol and 1000 fmol. As 1 μ L sample volume was injected always, these sample amounts correspond to 10, 20, 50, 100 200, 500 and 1000 pmol/mL concentrations. Each concentration was measured in triplicate. For the mono and disulfated components at 10 fmol injection the signal-to-noise ratio was greater than 20:1 (indicating that the limit of quantitation (LOQ, defined as $S/N > 10$) is slightly better than 10 fmol). We estimate the limit of detection for these components ($S/N > 3$) to be approximately 1 fmol. For the non-sulfated D0A0 disaccharide, the detection limit was ca. 10 fmol (due to interference from an abundant background ion at a similar mass and retention time). The quantitation limit was ca. 20 fmol in this case. The trisulfated compound gave very low signal intensity at low concentration, detection limit was ca. 20 fmol, and quantitation limit was 50 fmol. We evaluated linearity of peak areas over a 10–1000 fmol range that is appropriate for biological samples. Excellent linearity was observed for four disaccharides in the 10–1000 fmol range (Fig. 3B–E), with an R^2 value > 0.99 for each. The

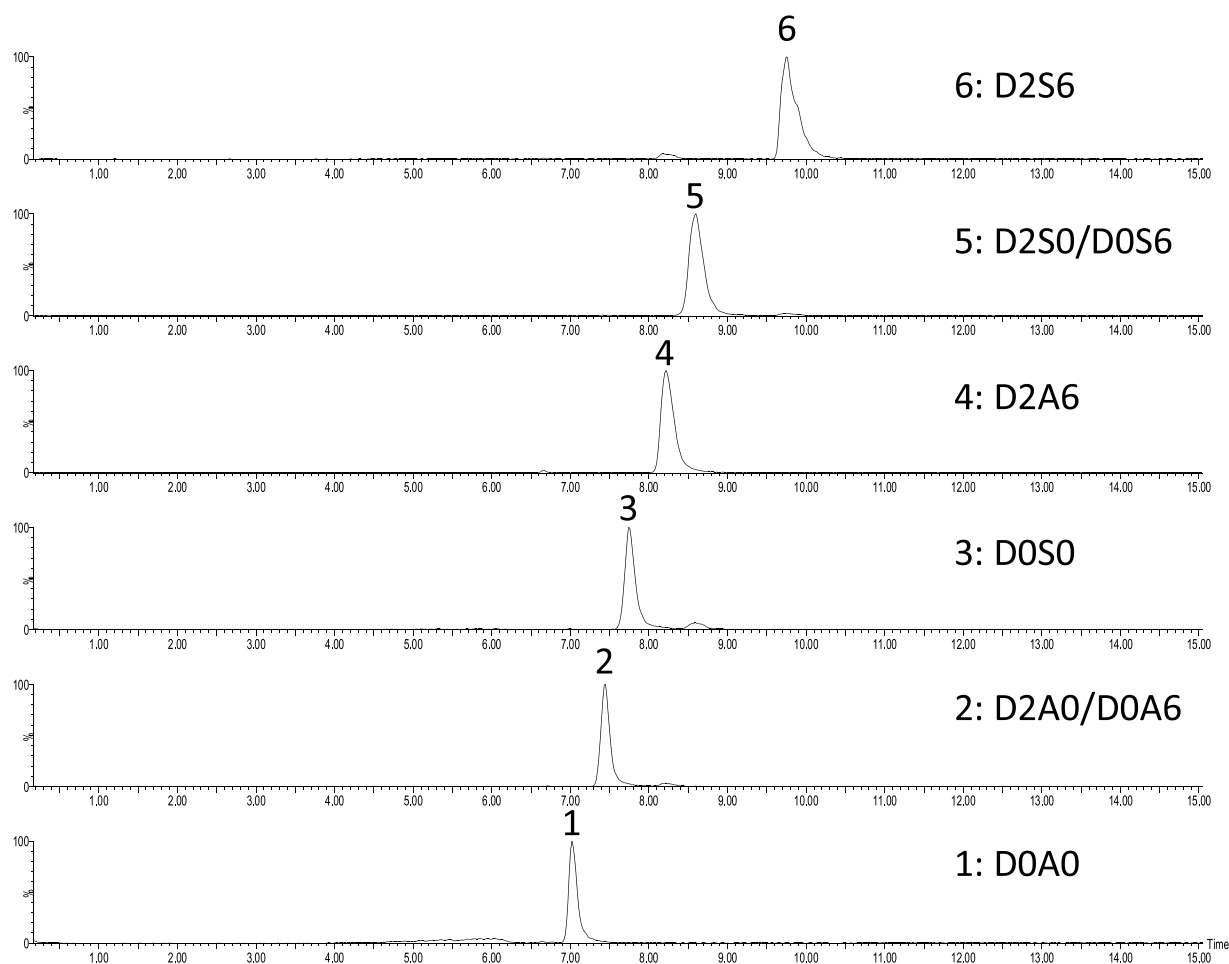


Fig. 2. Extracted ion chromatograms of 500 fmol heparan sulfate disaccharide standards (DOA0, D0S0, D0A6/D2A0, D2A6, D2S0/D0S6 and D2S6) on an in-house packed 100 μm i.d. capillary column packed with 1.9 μm particle size GlycanPac™ AXH-1 resin using isocratic separation (80% B).

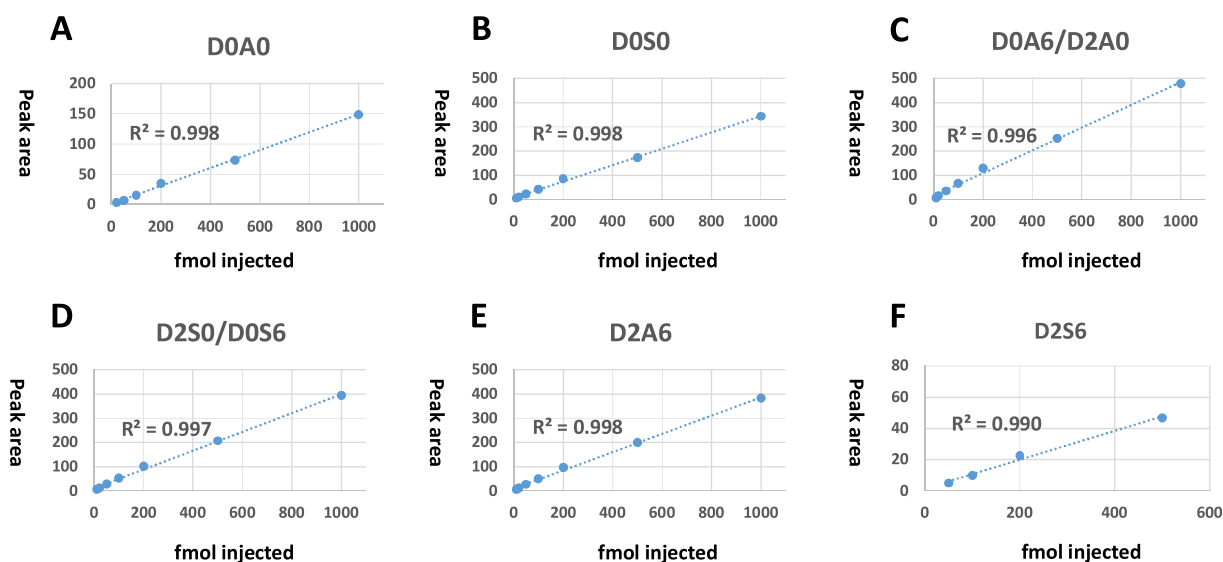


Fig. 3. Linearity of the method, calibration curves for the individual HS disaccharide standards: DOA0 (A), D0S0 (B), D0A6/D2A0 (C), D2S0/D0S6 (D), D2A6 (E), and D2S6 (F). Excellent linearity was observed for four disaccharides (B–E) in the 10–1000 fmol range with an R^2 value >0.99 for each. The non-sulfated disaccharide DOA0 (A) was measured in the 20–1000 fmol range, the trisulfated HS (F) in the 50–500 fmol range, both with $R^2 > 0.99$ linearity.

non-sulfated DOA0 disaccharide was measured in the 20–1000 fmol range (Fig. 3A), the trisulfated HS in the 50–500 fmol range (Fig. 3F), both with $R^2 > 0.99$ linearity. Average standard deviation of the HS

disaccharides, as expected, decreased with increasing concentration of the standard mixture (Table 3). Average standard deviations were found to be 4.2% for the 1000 fmol mixture and 14% for the

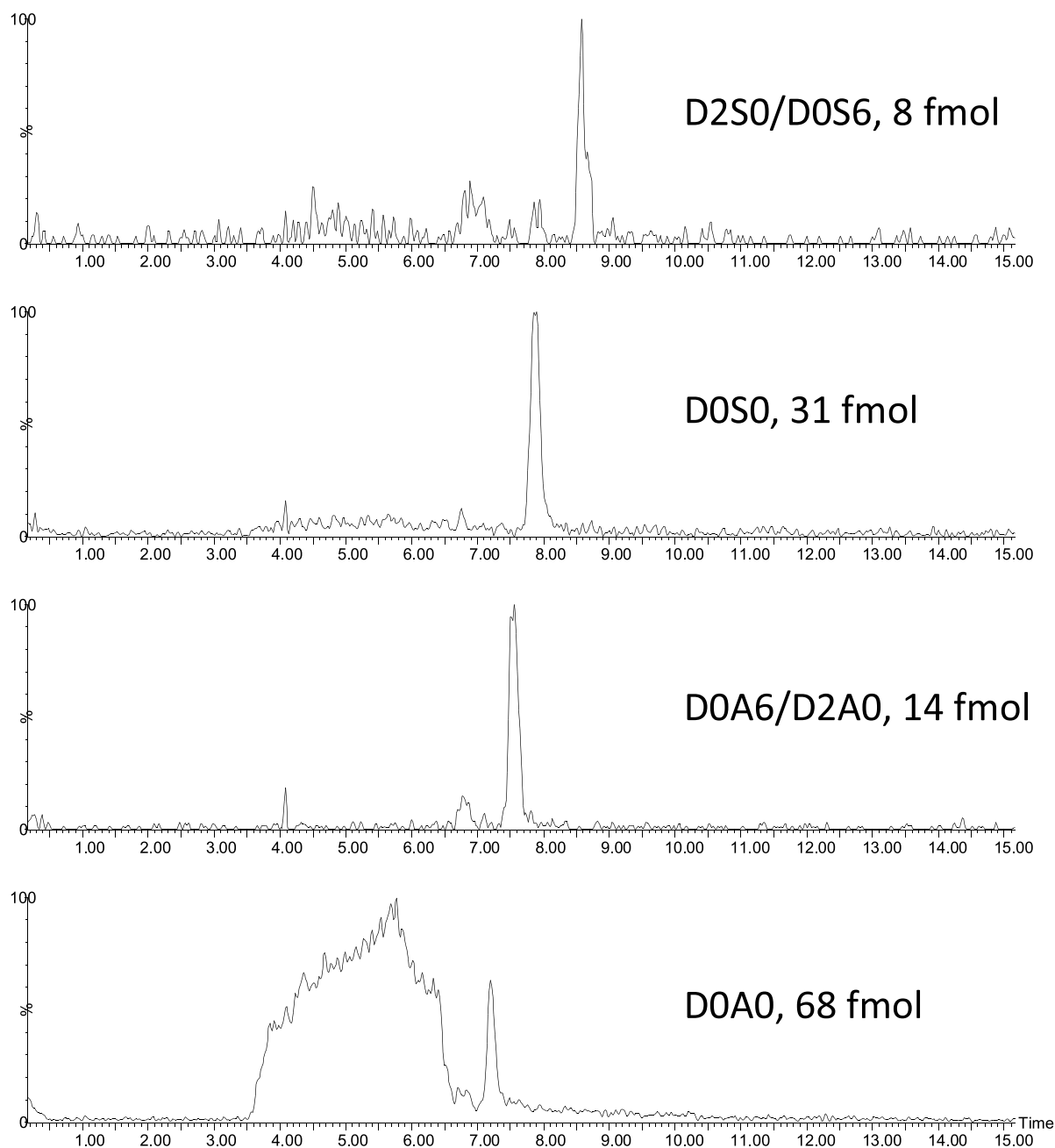


Fig. 4. Extracted ion chromatograms of heparan sulfate disaccharides extracted from the surface of a grade 2–3 prostate cancer tissue microarray core following on surface heparinase I–III digestion. The amount of the extracted disaccharides were estimated based on the calibration curve obtained using the standards.

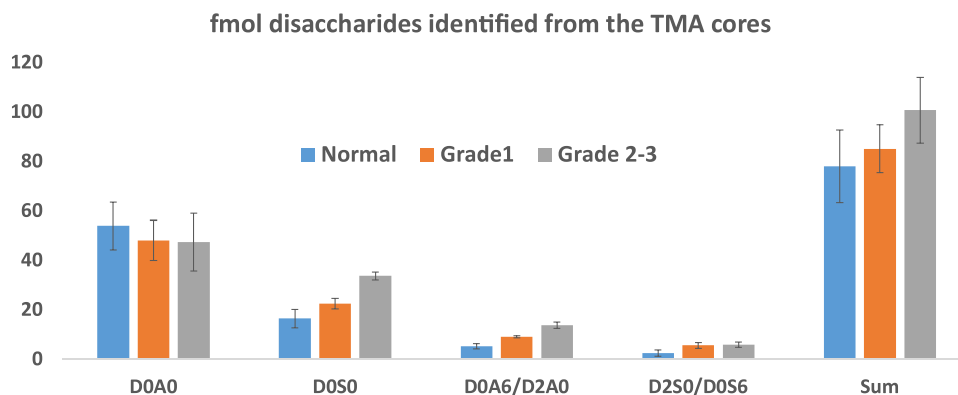


Fig. 5. Average amount of various GAG related HS disaccharides in normal, grade 1 and grade 2–3 prostate cancer TMA cores. The error bars represent standard deviation of the method.

Table 3

Relative standard deviation of peak areas for the unsaturated HS disaccharides at different concentrations measured in triplicates.

	10 fmol	20 fmol	50 fmol	100 fmol	200 fmol	500 fmol	1000 fmol
D0A0	ND	18.04%	10.98%	5.82%	4.57%	4.92%	6.31%
D0S0	18.79%	13.07%	11.43%	15.27%	10.28%	4.84%	1.45%
D0A6/D2A0	13.95%	18.21%	20.89%	14.86%	14.97%	10.81%	10.98%
D2S0/D0S6	15.84%	15.88%	17.11%	17.28%	9.94%	5.53%	2.86%
D2A6	8.53%	11.56%	12.40%	13.75%	7.50%	1.20%	1.96%
D2S6	ND	ND	ND	19.85%	7.58%	3.52%	1.73%

100 fmol mixture. The present experiments do not distinguish the positional isomers (D2A0/D0A6 and D2S0/D0S6). We have checked, that the ionization efficiencies of the two isomer pairs are within 15%; well within the intended accuracy of the present study. For this reason, quantitation will determine the sum of the two positional isomers. Relative sensitivity of the isomers is shown in Supplementary material Table S1.

3.3. Analysis of tissue microarrays

The next phase of method development was establishing, whether the method developed on standards would be suitable for analyzing small amounts of biological material. As test examples we have chosen tissue microarrays (TMA), which are gaining importance in biomedical workflows; the main challenge being the limited sample amount (1.5 mm diameter, 5 μ m thin cores). Sample amount in such cores is sufficient for proteomics workflows based on nanoLC–MS methodologies. TMA cores were digested using heparinase enzymes on their surface (see details in the Materials & Methods section), yielding $\Delta^{4,5}$ -unsaturated HS disaccharides. The digestion products were pipetted off the cores, and analyzed by the LC–MS method described above. Selected example chromatograms are shown in Fig. 4 for a grade 2–3 prostate cancer TMA cores. Based on the calibration curve obtained using the standards it was possible to estimate the amount of the extracted disaccharides (Fig. 4).

In this study TMA sections were analyzed corresponding to prostate tissue biopsy from one healthy individual, one grade 1 and one grade 2–3 patient, respectively. Three serial biopsies were analyzed for each TMA sample. We observed the 4 most abundant disaccharides in these samples. Among these, one doubly sulfated and the triply sulfated disaccharide were not detected. Approximate quantitation based on Fig. 3 indicated that the most abundant component is the non-sulfated D0A0 disaccharide being in the 20–80 fmol range in the various samples. The big peak in the D0A0 ion chromatogram, before the sample at $R_t = 3.5$ – 6.5 min is coming from the background. This does not interfere with detecting and quantifying D0A0. The two mono-sulfated components are present in comparable amounts; 10–40 fmol in the case of D0S0 and 5–20 fmol in the case of D0A6/D2A0. Among the higher sulfated components only the disulfated D2S0/D0S6 HS disaccharide was observed in each case, but only in low amounts (less than 10 fmol).

Three biological samples were analyzed in triplicates. The results showed an increased abundance in cancer of the mono-sulfated D0S0 and the D0A6/D2A0 components (Fig. 5).

4. Conclusion

We demonstrated that sulfated HS disaccharides can be advantageously studied using LC–MS with isocratic nano-chromatography conditions. Isocratic elution is advantageous for mass spectrometry analysis, as ion spray conditions and sensitivity issues do not complicate analysis. It is of particular importance in those cases, when the analyte has unfavorable MS characteristics. Using a combination of nano-HPLC and negative ionization, we detected HS disaccharides in the low fmol range.

We used the technique to analyze HS in tissue slices, using digestion directly on the tissue surface. Both sensitivity and specificity were sufficient to analyze small, ca. 2 mm² size areas on the tissue surface, opening up the possibility of studying tissue microarrays. We expect that this will allow systematic studies of human disease biospecimens. In the present paper, as an illustrative example, we studied TMA of healthy, grade 1 and grade 2–3 prostate tissue biopsies. These pilot studies demonstrated the ability to differentiate the biospecimens based on HS disaccharide abundances. This is consistent with known roles of HS proteoglycans in dysregulation of receptor tyrosine kinase signaling in cancer phenotypes [35–37]. In the future studies, we will extend to analyze GAGs from a large number of TMA cores, in order to build statistically relevant models correlating with disease progression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.chroma.2018.02.034>.

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