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Short communication

Salt gradient chromatographic separation of chondroitin sulfate disaccharides

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ABSTRACT

In the present study, we describe the development of a fast, 2-step salt gradient for analysis of chondroitin sulfate disaccharides. Using salt gradients, which is somewhat unusual in HILIC-based separations, provides relatively fast chromatography with excellent sensitivity (15 min cycle time, 10–20 fmol/μL detection, 30–50 fmol/μL quantitation limit), and good linearity. The efficiency of the new method is demonstrated by measuring human tissue slices of healthy, cirrhotic, and cancerous liver samples. Preliminary results show major differences among the quantity and sulfation pattern of the various sample types.

1. Introduction

Chondroitin sulfate (CS) is a class of long linear polysaccharides (glycosaminoglycans, GAGs), composed of alternating saccharide units of N-acetylgalactosamine (GalNAc) and glucuronic acid. The synthesis of the polysaccharide backbone is carried out in the Golgi apparatus. A very important step of CS chain formation is sulfation, carried out by sulfotransferases [1]. The GalNAc residues may be sulfated at the 4-OH and/or 6-OH positions, while glucuronic acid may be sulfated at the 2-OH position [2]. The sulfated CS chains are attached to a core protein via a tetrasaccharide linkage, forming proteoglycans (PGs). PGs are localized in the extracellular matrix and on cell surfaces controlling various biochemical functions [3–5]. The GAG chains are responsible for cellular signaling and recognition, governed by the size and the sulfation pattern of the respective chains [6–8]. Alterations in the ratio of the differentially sulfated disaccharide building blocks may be descriptive of various diseases, e.g. sulfation pattern changes have been observed between healthy and cancerous tissue [9,10]. Hepatic cirrhosis is accompanied by increased synthesis of connective tissue, with the predominant increase of CS GAGs. Hepatocellular carcinoma also exhibits a selective increase in CS levels [10,11]. Thus, these malfunctions provide a good basis for CS compositional studies.

Structural characterization of CS is usually performed by analyzing their disaccharide and/or oligosaccharide building blocks after enzymatic digestion (e.g. Chondroitinase ABC) or chemical degradation (deaminative cleavage with nitrous acid) [12,13]. The resulting disaccharides have a characteristic sulfation pattern, which is descriptive of sulfation motifs of the original molecule. Structure and description of $\Delta^{4,5}$-unsaturated CS disaccharides originating from Chondroitinase ABC digestion are summarized in Table 1.

Various chromatographic methods have been reported to analyze the sulfation of CS-derived disaccharides. These include reversed-phase chromatography with derivatization or ion-pairing [14,15], size exclusion (SEC) [16], graphitized carbon [17], HILIC [18,19], or HILIC-WAX [9,20] chromatography. Most of these separation methods can be (and often are) coupled on-line to mass spectrometry (MS), providing reliable structural information [21,22]. Diversity of the reported methods suggests that they have major drawbacks, mainly due to the diverse polarity of CS disaccharides and their unfavorable ionization characteristics in ESI-MS. The main disadvantages of reported methods include limited sensitivity (LODs in the picomole - high femtomole range [21,23]), long analysis time, and limited reproducibility. The current state of the art on GAG disaccharide analysis has been reviewed recently [2].

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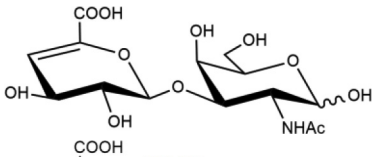
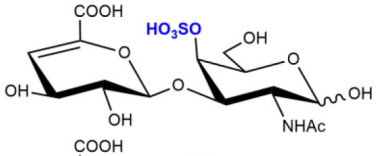
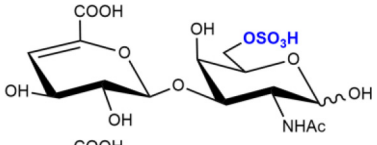
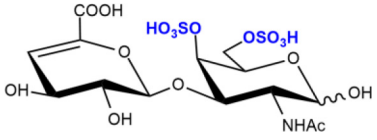
Table 1
Structure, nomenclature, and *m/z* values of the CS disaccharides investigated. Note, that D0a4 and D0a6 are positional isomers and are distinguished via MS/MS measurements in the present study.

Chemical structure	Traditional name	Lawrence code	*m/z* (-) mode	MS/MS transition
	ΔHexA-GalNAc	**D0a0**	378.1	-
	ΔHexA-GalNAc4S	**D0a4**	458.1	300.1
	ΔHexA-GalNAc6S	**D0a6**	458.1	282.1
	ΔHexA-GalNAc4S6S	**D0a10**	538.1	-

We have recently reported a promising salt-gradient μHPLC-MS method [20] for heparan sulfate (HS) analysis, with sensitivity down to 1 fmol/μL. Our present aim is to extend the applicability of this method to CS disaccharides: this is a group of GAGs more challenging to analyze, due to their 5–10-times lower sensitivity and even less ideal chromatographic behavior experienced in our setup. The aim of pushing down the limits of quantitation is to have a method capable of quantitative analysis from small tissue slices or biopsies. These sample types pose a real challenge to instrumental analytical techniques, necessitating utmost sensitivity and repeatability. The applicability of our method will be shown in the case of limited sample size human liver tissue slides, describing the changes occurring in cirrhosis and hepatocellular carcinoma in comparison to healthy species.

2. Materials and methods

2.1. Chemicals and reagents

The $\Delta^{4,5}$-unsaturated chondroitin sulfate disaccharide standards (listed in Table 1, 'CS disaccharides' hereinafter) were purchased from Iduron (Cheshire, UK). Crystalline ammonium formate, ammonium bicarbonate, Chondroitinase ABC, and formic acid (FA) were purchased from Merck (Budapest, Hungary). Glygen graphite+C_{18} TopTips were purchased from Sunchrom GmbH (Friedrichsdorf Germany). LC-MS grade water, acetonitrile, xylene, and ethanol were purchased from VWR International Ltd. (Debrecen, Hungary).

2.2. Column packing

A GlycanPac^{TM} AXH-1 1.9 μm analytical HPLC column (2.1 × 100 mm, Thermo Fisher Scientific, Waltham, MA USA) was unpacked and repacked into 250 μm i.d. capillaries based on a method published recently [9]. Briefly, a 1 mg/mL resin suspension (75% acetonitrile - 25% water) was driven through a fritted capillary until reaching the 13 cm bed-length. After careful pressure release, and UPLC compression procedure, the column was ready to use.

2.3. Liquid chromatography-mass spectrometry

For microscale chromatography, a Waters® nanoAcquity UPLC system (Waters, Milford, MA, USA) was coupled to a high-resolution Waters® QTOF Premier^{TM} Mass Spectrometer (Waters, Milford, MA, USA) via normal electrospray ionization source.

2.3.1. MS parameters

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. For the MS/MS measurements of monosulfated CS disaccharides, the collision energy was set to 22 eV, following detailed optimization (details not shown).

2.3.2. UHPLC parameters

For the investigation of the chromatographic behavior, we injected a mixture of the CS standards: 1 pmol/μL of D0a0, D0a10; 0.5 pmol/μL of the D0a4 and D0a6 standards (positional isomers, thus resulting in a total of 1 pmol/μL D0a4/D0a6 content). Throughout the whole study, 1 μL volumes were injected.

A self-packed GlycanPac AXH-1 capillary column (250 μm i.d.) was used, the column temperature was adjusted to 45 °C using an AgileSleeve capillary heater with a MonoSleeve column heater controller (Analytical Sales and Services Inc, Flanders, NJ USA), and the flow rate was adjusted to 8 μL/min.

2.4. Salt gradient HILIC-WAX separation of CS disaccharides

The finally developed gradient program was the following. Eluent A was 10 mM ammonium formate in 75:25 v/v ACN:water (pH 4.4); Eluent B was 65 mM ammonium formate in 75:25 v/v ACN:water (pH 4.4). Starting from 6% B, the eluent ratio changed in 0.5 min to 12% B, and then in 4.5 min to 60% B. As a washing step, the composition was elevated to 100% B and held for 4 min and it was followed by a 5-minute-long equilibration at the initial composition.

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2.5. Preparation of FFPE tissue slices

Surgically removed control, cirrhotic, and cancer-bearing liver specimens were sent from the Transplantation and Surgical Clinic of Semmelweis University to the 1st Department of Pathology and Experimental Cancer Research for diagnostic purpose. Samples were fixed in 10% buffered formaldehyde and embedded into paraffin. 3-µm-thick sections were cut and stained with Hematoxylin-eosin for diagnostic evaluation. Subsequently, 7-µm-thick paraffin-embedded sections were prepared from four of each control, cirrhotic and hepatocellular cancer containing paraffin-embedded blocks for chondroitin sulfate analysis. The work was approved by Semmelweis University Regional and Institutional Committee of Science and Research Ethics (TUKEB permit number: 95/1999).

2.6. On-tissue chondroitin sulfate sample preparation

De-paraffinization and antigen retrieval of FFPE liver tissue slides were performed as described recently [9]. After that, enzymatic digestion was performed based on a previously developed methodology [24]. Briefly, an aqueous digestion solution with the following composition was prepared: 25 mM ammonium bicarbonate, 2.5 mM ammonium acetate, 1 mU/µL Chondroitinase ABC. The used buffer ensures the selectivity of Chondroitinase ABC towards only CS. The enzyme solution was added in five cycles as follows: 5 µL droplets of digestion solution were pipetted onto the surface, and the samples were incubated in a humidified box for 1 h at 37 °C. The resulting disaccharides were extracted from the surface with 25 µL 0.3% ammonium hydroxide solution via 5 cycles of repeated pipetting. The samples were then dried down and resuspended in 15 µL of injection solvent (10 mM ammonium formate in 75:25 v/v ACN:water (pH 4.4)), from which 1 µL was injected.

2.7. Data evaluation and interpretation

Peaks were integrated manually and with the QuanLynx add-in of Waters MassLynx 4.1 software. Data visualization was done using Microsoft Excel.

3. Results and discussion

3.1. Method development

Building on the previously mapped behavior of HS disaccharides on the GlycanPac AXH-1 column [20], we used 75:25 v/v ACN:water solvent composition and ammonium formate salt in the present study. The method development focused on determining the best salt gradient in the 10 mM – 80 mM range in order to achieve fast baseline separation. We have tested several linear gradients with various length and steepness, a few examples are shown in Fig. 1. This shows the effect of starting and ending conditions of the gradient, using identical-length linear gradients. The peak before 'peak 1' is a buffer contaminant, not affecting proper CS disaccharide detection.

The 15 mM → 30 mM gradient (Fig. 1A) shows the partial separation of the D0a4/D0a6 isomer pair. Using lower salt concentrations and shallower gradients this resolution can be increased but the resulting peaks are broad and shallow. This increases the detection limits unacceptably (at least while working with biological samples). For this reason, we decided to go in another direction; developing a method with which the D0a4/D0a6 isomer pair co-elutes, but subsequently distinguish these two isomers using tandem mass spectrometry. By running a 20 mM → 55 mM salt gradient (Fig. 1B), sharp and high-intensity peaks were observed. Operating at a higher salt concentration (30 mM → 80 mM gradient) caused a minor decrease both in retention times and intensities (Fig. 1C).

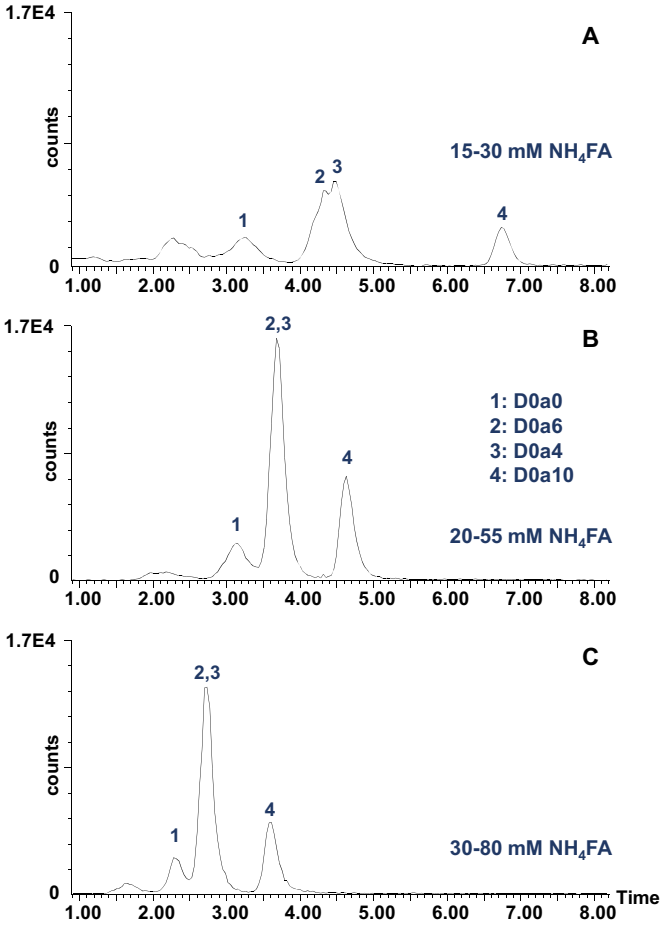

Fig. 1. Effect of different starting and ending salt concentrations using 75% acetonitrile – 25% water solvent composition and identical-length linear gradient ramps. Sums of extracted ion chromatograms (EICs) are shown in the diagram for A: 15 mM → 30 mM; B: 20 mM → 55 mM; and C: 30 mM → 80 mM ammonium formate salt concentrations.

We concluded that, as a linear gradient, the 20 mM → 55 mM range was close to optimal. Next, we improved performance using a 2-step gradient. The key points were to lower the starting salt concentration, start with a shallow, and follow it with a steeper gradient. The ending salt concentration was also lowered, in order to decrease the probability of clogging. Therefore, we designed a method that used a 2-step salt gradient starting from 14 mM to 43 mM ammonium formate concentration. This way we obtained a chromatogram (Fig. 2) with close to ideal peak shapes, good resolution, selectivity, and sensitivity. Note that the salt concentration has a major effect on peak retention and peak shape, but contrary to usual mass spectrometry experience, the ion suppression was negligible. The resolution for the peak pair 1–2,3 was 1.3, and for peak pair 2,3–4, it was 2.2. The positional isomers D0a4 and D0a6 were distinguished using MS/MS measurements: The predominant daughter ion from D0a4 is *m/z* 300.1, and that from D0a6 is *m/z* 282.1 [25,26].

3.2. Method characterization

The limit of quantitation (LOQ, FDA Bioanalytical Method Validation guidelines [27]) was below 50 fmol/µL for the D0a0 and the D0a10 components and 30 fmol/µL for the D0a4/D0a6 isomer

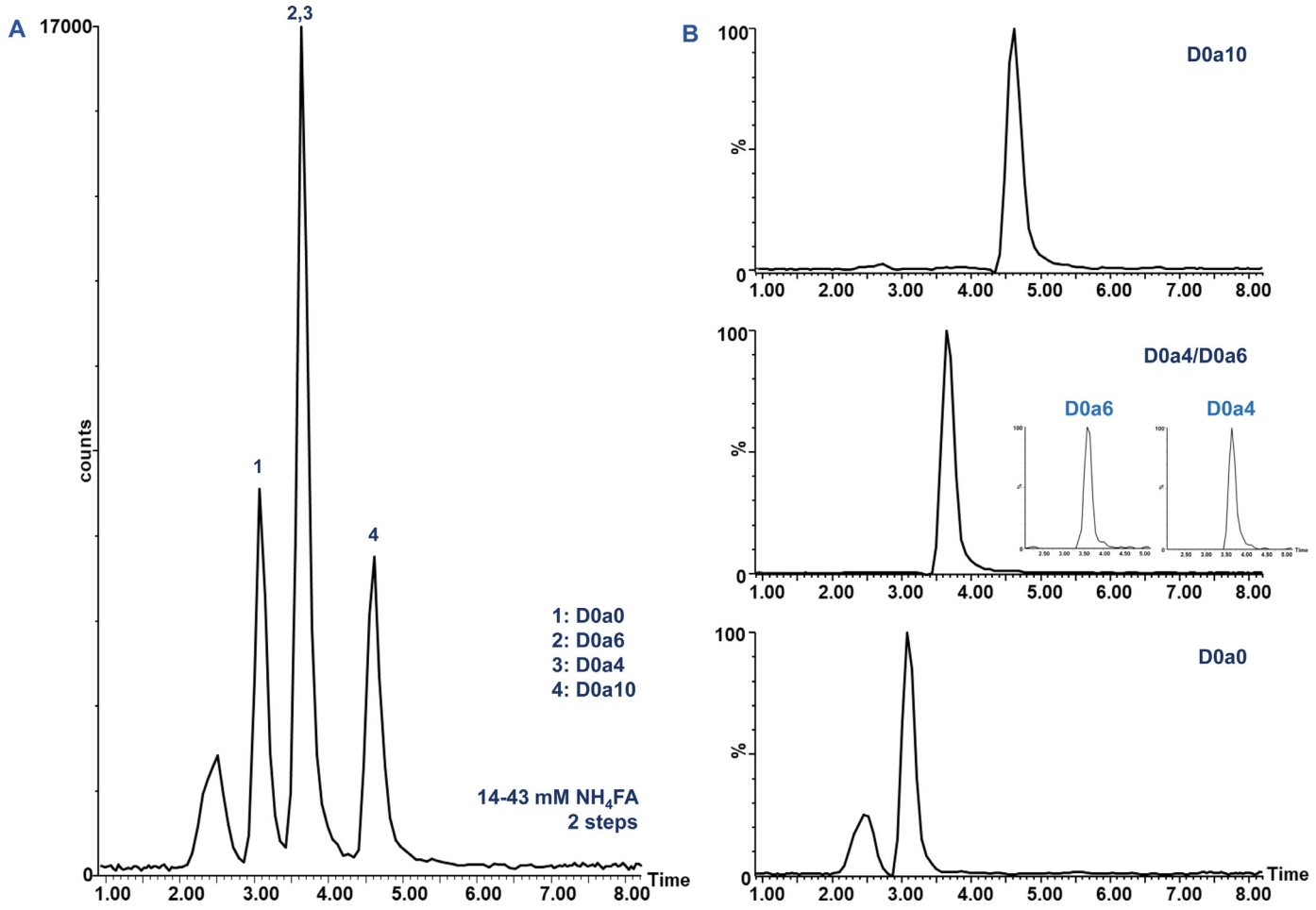

Fig. 2. Chromatograms using the optimized 2-step gradient. A: Sum of extracted ion chromatograms; B: Individual extracted ion chromatograms for the respective compounds.

pair, while the limit of detection (LOD, estimated as 3-times S/N) was approximately 20 fmol/µL for the D0a0 and the D0a10 components and 10 fmol/µL for the D0a4/D0a6 isomer pair. These values show that our method is approximately 5-times more sensitive than what was reported in a recent paper [28]. Improved sensitivity may be attributed to gradient focusing and low chemical noise. Note, our gradient is also relatively fast (5 min gradient and 15 min overall cycle time), which is about 2–3 times shorter than the generally used methods for GAG separations [2].

The linearity of the method was characterized in a wide range from 50 fmol/µL to 5 pmol/µL, each concentration was measured in triplicate. The R^2 values covering the whole range were all above 0.99. Calibration curves are shown in Fig. 3, along with the calibration for the intensities of MS/MS transitions of the positional isomers.

The repeatability of the method was analyzed in 5 consecutive runs (intra-day repeatability) and on 3 different days of the week (inter-day repeatability) using a 500 fmol/µL CS disaccharide standard mixture. Intra-day variability (relative standard deviation of peak areas) was between 1.90 and 4.49%, while inter-day variability was between 10.56% and 15.62% (see Table S-1). The latter can be easily compensated using before-batch calibrations. The retention time variability was between 0.14% and 0.95% intra-day, and between 0.20% and 1.12% inter-day (RSD values). Working with relatively short gradients, these numbers suggest very stable chromatography. The variability of peak areas is mainly due to changes in ion-source conditions since the stability of the CS disaccharides in the applied injection solvent (75% ACN, 25% H2O, 10 mM NH_4FA) proved to be satisfactory under autosampler conditions in the timeframe of a usual batch (4 h). However, one should be careful, when storing the samples for a longer period of time in this solution, since the disaccharides showed a major degradation at 4 °C when stored for 12–24 h. For details on sample stability, see Table S-2.

Spike/recovery test was carried out to determine the accuracy of the method: measurements were performed at low (100 fmol/µL), medium (500 fmol/µL), and high (2000 fmol/µL) spike concentrations on three parallel samples each. The recovery values were in the range of 87.0% - 114.9% at all concentrations for each disaccharide. For detailed values, see Table S-3 in the supplementary material.

Long-term robustness of the system was outstanding: over 3 months of problem-free operation was observed, with no carry-over, even after injecting as much as 10 pmol/µL samples.

3.3. Chondroitin sulfate composition in cirrhosis and liver cancer

Using the developed method, we aimed to determine key changes of CS composition with respect to cirrhotic and cancerous malfunctions of the human liver. The samples were enzymatically degraded into unsaturated CS disaccharides using Chondroitinase ABC. Tissue slices from three individuals in each group were digested, and all the samples were injected in triplicates and separated using the developed salt gradient µHPLC-MS method.

As liver cancer is often induced by cirrhotic malfunction, we expected similar changes in CS content and sulfation pattern.

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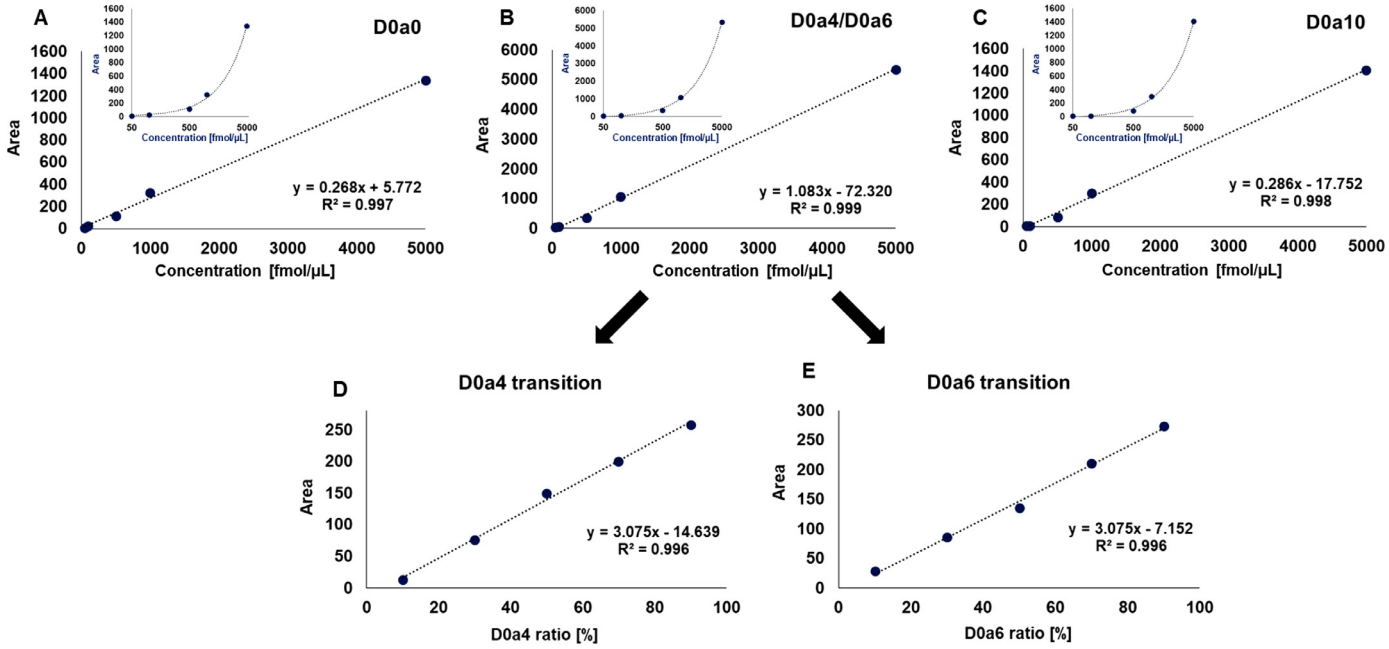

Fig. 3. Calibration curves (linear and logarithmic plotting) for the investigated compounds' MS1 signals in the 50 fmol/µL – 5 pmol/µL range and the MS/MS transitions using 1 pmol/µL mixture of the isomers. A: D0a0; B: D0a4/D0a6; C: D0a10; D: D0a4 transition (458.1 – 300.1); E: D0a6 transition (458.1 – 282.1).

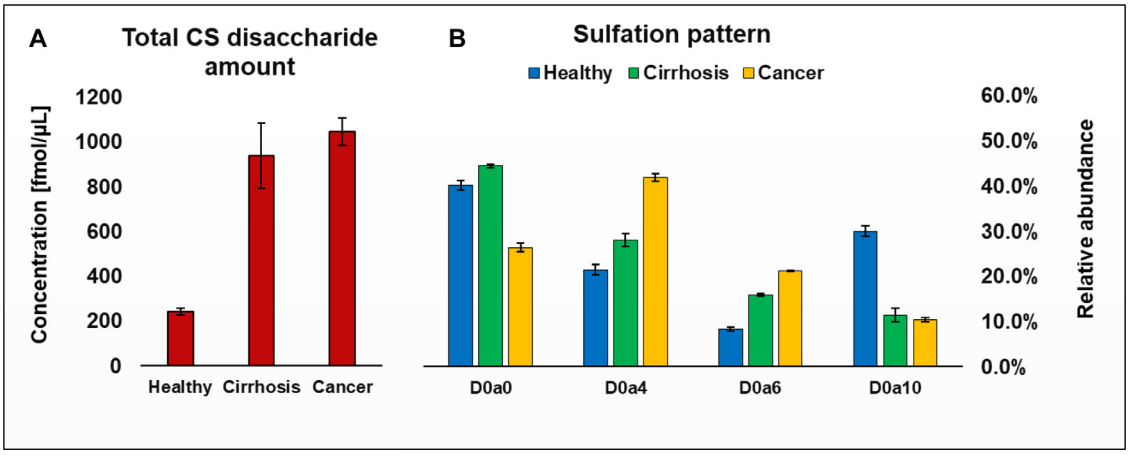

Fig. 4. Comparison of healthy, cirrhotic and cancerous liver samples. A: Sum of absolute concentrations of all CS disaccharides (total chondroitin sulfate content). B: Sulfation pattern changes with respect to individual disaccharide ratios.

Evidence of elevated CS content has already been reported in the literature [10,11], therefore it was used to demonstrate the applicability of the method from limited-size tissue surface samples. The total CS amount showed an over 4-fold increase in both cirrhosis and cancer, to the same extent within statistical significance borders. As is seen in Fig. 4, the relative standard deviations remained under 10% (often around 1–2%) for individual disaccharides and were between 10% and 22% for cumulative absolute amounts. This is well acceptable for measurements from biological material, especially when taking into account that sub-picomole quantities were measured. Therefore it is concluded that the developed method can be reliably used.

The changes in the sulfation pattern showed interesting results. The average numbers of sulfate groups per disaccharide are similar to one another: it is 0.90 in healthy, 0.63 in cirrhotic and 0.84 in cancerous samples. In contrary to the relatively similar average sulfation, there are substantial changes in the ratio of the individual disaccharides (Fig. 4B). The nonsulfated disaccharide (D0a0) shows a small increase in cirrhotic state and a large decrease in cancer. The monosulfated components (D0a4 and D0a6) both show a medium increase in cirrhosis and a much larger increase in cancer. Their ratio relative to each other showed a small but significant change: in healthy tissue, the D0a4/D0a6 ratio was 2.61, and it decreased to 1.75 in cirrhosis and to 1.97 in cancer. The ratio of the doubly sulfated component (D0a10) showed a major decrease both in cirrhotic and cancerous states. These data are in correlation with others reported in the literature, performed by other techniques, eg. SAX chromatography or gel-electrophoresis [10,11].

The changes discussed above indicate that CS may play a role in the malfunctions investigated, thus proposing the basis of a future large-scale study focusing on the regulatory mechanisms of GAGs in connection with liver cancer and cirrhosis.

4. Conclusions

The diverse polarity and unfavorable ionization characteristics of CS disaccharides necessitate the continuous development of techniques used for their analysis. In this short communication,

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we described a salt gradient method for their analysis, which enables fast and reliable quantitation of small amounts of CS disaccharides. The developed method shows faster chromatography, improved sensitivity, and good repeatability compared to previous methods. Using a short, 2-step salt gradient (5 min gradient, 15 min cycle time) we were able to separate and identify CS disaccharides down to 10–20 fmol/μL and quantify them down to 30–50 fmol/μL from biological matrices. The applicability of the developed method for limited-size samples was demonstrated by measuring various liver tissue slices. The observed changes propose the necessity of a future large-scale study.

Author contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Gábor Tóth: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft. **Károly Vékey:** Conceptualization, Data curation, Writing - original draft, Funding acquisition. **Simon Sugár:** Data curation, Writing - original draft. **Ilona Kovalszky:** Resources, Writing - original draft. **László Drahos:** Data curation, Writing - original draft, Funding acquisition. **Lilla Turiák:** Conceptualization, Data curation, Writing - original draft, Supervision, Funding acquisition, Project administration.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.460979.

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