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# Discrimination of sulfated isomers of chondroitin sulfate disaccharides by HILIC chromatography and on-line coupled tandem mass spectrometry

Salomé Poyer,<sup>1,2\*</sup> Ilham Seffouh,<sup>1,2</sup> Chrystel Lopin-Bon,<sup>3</sup> Jean-Claude Jacquinet,<sup>3</sup> Jean-Yves Salpin,<sup>1,2</sup>  
Régis Daniel.<sup>1,2\*</sup>

<sup>1</sup> Université Paris-Saclay, CNRS, Univ Evry, LAMBE, Evry-Courcouronnes, 91025, France

<sup>2</sup> CY Cergy Paris Université, LAMBE, Evry-Courcouronnes, 91025, France

<sup>3</sup> ICOA, CNRS UMR 7311, Université d'Orléans, F-45067 Orléans

\* To whom correspondence should be addressed. E-mail: [salome.poyer@univ-evry.fr](mailto:salome.poyer@univ-evry.fr) Phone: +33 169 47 7XXX. Email: [regis.daniel@univ-evry.fr](mailto:regis.daniel@univ-evry.fr). Phone: +33 169 47 7641.

## Highlights

- HILIC optimized conditions for the separation of mono-, di- and tri-sulfated disaccharide isomers from chondroitin sulfate
- On-line detection of sulfated isomers of chondroitin sulfate disaccharides by HILIC-MS coupling
- Monitoring of a CS sulfatase and of a chondroitin lyase activity by HILIC-MS
- ~~— Differentiation of  $\alpha$  and  $\beta$  anomers~~
- ~~— Identification of disaccharides resulting from the depolymerization by the chondroitinase lyase ABC on endocan~~
- ~~— Determination of the kinetic performance and selectivity of CS 4-O-endosulfatase~~

## **Abstract**

Chondroitin sulfate (CS) are sulfated polysaccharides in particular present in the extracellular matrix of the central nervous system. In this study, we developed a HILIC-MS coupling enabling differentiation of both positional isomers and anomers of mono- di- and trisulfated CS disaccharides. The mobile phase composition in terms of pH and concentration showed high influence on chromatographic separation and was varied to allow the distinction of each CS without signal overlapping in 25 minutes total. This methodology was applied to identify the disaccharides isomers resulting from the depolymerization by the chondroitinase lyase ABC on endocan and the enzymatic kinetic of CS 4-*O*-endosulfatase. We have determined that the depolymerization of endocan yielded 99% of monosulfated disaccharides CS-A/CS-C in a 71/29 ratio, and trace amounts of disulfated CS-D, CS-E and unsulfated CS-0 disaccharides. In addition, the off-line desulfation kinetic performance of CS 4-*O*-endosulfatase on the monosulfated CS-A disaccharide allowed determining the half-time of the reaction at  $4.5 \pm 0.9$  min in the experimental setup.

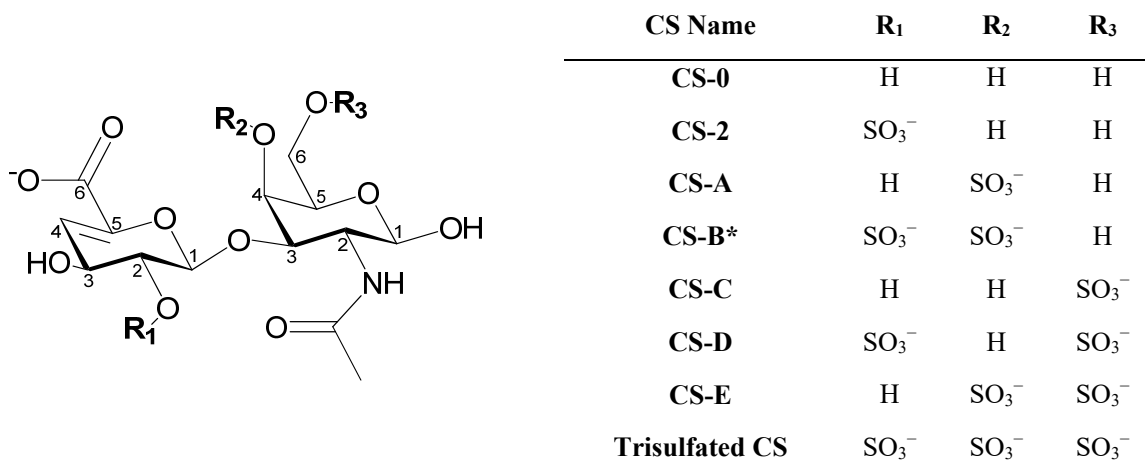
## **Keywords**

Chondroitin sulfate, glycosaminoglycans, hydrophilic interaction liquid chromatography, mass spectrometry, sulfatase, chondroitin lyase.

## 1. Introduction

Chondroitin sulfate (CS) are sulfated polysaccharides of the glycosaminoglycan (GAG) family, which are widely distributed in body compartments, with the notable feature representing the most abundant GAGs in brain. Being the prevalent carbohydrate component of the extracellular matrix of the central nervous system,[1, 2] CS are involved in neural development and regeneration, cell migration and axonal pathfinding, and recent findings highlight their critical roles in various physio-pathological processes such as in neuro-inflammation.[3-6] Structural analysis of CS faces to a high isomeric barrier due to the various sulfate distributions and presence of epimers along the polysaccharide chain. The repeating disaccharide units of CS-A, -C and -E are made up of glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc) sulfated on C4, C6 and both C4 and C6, respectively (Scheme 1). In CS-D, both GlcA and GalNAc are sulfated on C2 and C6, respectively. The C<sub>5</sub> epimerization of GlcUA into iduronic acid (IdoUA) in variable proportion lead to CS-B, also named dermatan sulfate.[7] From the diversity of sulfation pattern originates a variety of CS-protein binding partners.[8] To date, isolation and sequencing of bioactive CS sequences with specific sulfate pattern triggering the selective and high affinity protein binding remain challenging tasks, so that structure-activity relationship could not be easily established. In this context, development of efficient analytical methods for the sensitive detection and discrimination of various sulfation patterns within CS sequences is of high necessity to associate biological functions with well-defined sulfated structures. We have previously identified diagnostics ions for 4-*O* and 6-*O* sulfation, which have been used for the discrimination of sulfated isomers of CS oligosaccharides by electrospray-tandem mass spectrometry (ESI-MS/MS).[9] Real CS oligosaccharide samples from biological sources or formed by enzyme depolymerization, are complex mixtures which require their separation before MS analysis. This can be achieved through on-line liquid chromatography, generally using a reversed stationary phase, which is ~~offers the main advantage of volatile organic mobile phase compatible with ESI-MS.~~ However, given the polar nature of GAGs, such reversed phase separation requires their prior derivatization and/or ion-pairing.[10-15] Over the

last decade, hydrophilic interaction chromatography (HILIC) has emerged as an attractive alternative method for the separation of polar compounds.[16, 17] Indeed, HILIC is based on a polar stationary phase grafted with hydrophilic groups which is much more favorable for the separation of polar analytes. Furthermore, it uses organic polar solvents mixed with low proportion of water or aqueous buffer as eluents, which allows efficient electrospray ionization and is compatible with on-line MS coupling. HILIC-MS is widely expanding to the analysis of glycans, phosphorylated saccharides and glycopeptides.[18, 19] Comparatively, fewer reports describe applications of HILIC to GAG oligosaccharides, mainly for heparin oligosaccharides.[20-23] Recently, the HILIC-MS analysis of CS oligosaccharides has been described according to procedures requiring a preliminary derivatization of the reducing end by AMAC or 2-aminopyridine.[24, 25] In the present study, we have developed a HILIC separation method that does not require prior derivatization and allows the resolution of mono-, di- and trisulfated CS disaccharides (Scheme 1). Different parameters of separation conditions were optimized. We report the coupling of this HILIC method to ESI-MS in negative ion mode, and its application to the monitoring of the enzyme activity of a CS sulfatase and of a chondroitin lyase.



**Scheme 1.** Chemical structure of the chondroitin sulfate oligosaccharides studied. \*trisulfated, CS-0, CS-2, -A, -C, -D and -E, are composed of GlcA $\beta$ 1-3GalNAc repeating disaccharide units, contrary to CS-B which is composed of IdoA $\alpha$ 1-3GalNAc repeating units for natural disaccharides.

## 2. Materials and methods

### 2.1. Chemicals and standards

Synthetic disaccharides of pure CS presented in Scheme 1 were prepared as previously described.[26] CS disaccharides produced by digestion of CS by bacterial chondroitinase were purchased from Iduron (UK). HPLC-grade acetonitrile was supplied by VWR (France). Tris-HCl and phosphate buffers, ammonium formate and formic acid were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France), as well as Actinase E and chondroitinase ABC. Endocan was provided by LungInnov (Lille, France). CS 4-*O*-endosulfatase prepared as previously reported [27], was kindly provided by Dr. JL Neira (Miguel Hernandez Univ., Spain). Deionised water (18.2 M $\Omega$ ) was obtained from a Milli-Q (Millipore) purification system.

### 2.2. HILIC-MS analysis

HILIC-MS experiments were carried out using a quadrupole ion trap mass spectrometer (AmaZon Speed ETD, Bruker) equipped with an Apollo electrospray source operated in the negative ionization mode. MS and MS/MS experiments were performed in enhanced resolution mode in the 200 to 700 mass range. ESI source parameters were set as follows: capillary voltage 3.5 kV, end plate offset -140 V, nebulizer gas flow 20 psi, dry gas flow 10 L min<sup>-1</sup> and dry gas temperature 250 °C. Data analysis v4.0 software was used to process data. HILIC separation of CS analogues was achieved using a ZIC-cHILIC column (SeQuant® ZIC®-cHILIC 3  $\mu$ m, 100 Å 150 x 2,1 mm, VWR, France). Optimized chromatographic conditions were: solvent A 7.5 mM ammonium formate adjusted at pH 4.0 using formic acid, solvent B acetonitrile; flow rate: 300  $\mu$ L/min; injected volume of CS mixtures: 2.5 $\mu$ L; column temperature: 20°C, and autosampler temperature was set at 10°C; linear gradient: 0 min (26%A), 4 min (26%A), 6 min (35%A), 8 min (35%A), 9 min (85%A), 12 min (85%A), 13 min (10%A), 16 min (10%A), 17 min (26%A), followed by 8 min of reconditioning for a total analysis time

of 25 min. A volume split of 1/3 was used at the end of the column to get 100  $\mu\text{L}/\text{min}$  flow rate at the entrance of the mass spectrometer.

### 2.3. *Chondroitin Sulfate 4-O-endosulfatase assay*

The desulfation reaction catalyzed by the bacterial CS 4-*O*-endosulfatase was carried out as previously described.[28] Briefly, the enzyme reaction was performed at 37 °C in 800  $\mu\text{L}$  50 mM phosphate buffer, pH 7, containing 50  $\mu\text{M}$  of unsaturated chondroitin 4-*O*-sulfate disaccharide  $\Delta 4,5\text{HexUA}1-3\text{GalNAc}(4\text{S})$  as substrate. The reaction was initiated by the addition of 40  $\mu\text{L}$  of 0.6 mg/mL of CS 4-*O*-endosulfatase. 100  $\mu\text{L}$  samples were withdrawn from the reaction mixture upon reaction course, and then two-fold diluted in water. Resulting diluted samples were filtered on a 3 kDa centrifugal filter (Ultra, Amicon, USA). The filtered sample solutions were stored at 4 °C for subsequent HILIC-MS analysis.

### 2.4. *Essay of chondroitin disaccharides from endocan*

Endocan (8  $\mu\text{g}$ ) was incubated overnight with Actinase E (2.7  $\mu\text{g}$ ) in 100  $\mu\text{l}$  20 mM Tris-HCl buffer, pH 7.2 at 37°C to release the CS chain. The resulting peptides were removed by dialysis overnight in water using a 3.5 kDa cut-off dialysis cassette (Slide-A-Lyzer, ThermoFisher). The dialyzed solution was evaporated to dryness, resuspended in 10  $\mu\text{l}$  20 mM Tris-HCl buffer, pH 7.2, and heated at 95°C for 15 min to inactivate remaining active Actinase E. The CS chain in the dialyzed solution was depolymerized into disaccharides by incubation overnight with 20 mU (5 $\mu\text{l}$ ) of chondroitinase ABC at 37°C. The resulting CS disaccharides were recovered through filtration on a 3 kDa centrifugal filter (Ultra, Amicon, USA). The filtered solution was stored at 4 °C for subsequent HILIC-MS analysis.

## 3. Results and discussion

The development and optimization of the separation method by HILIC were performed using mono-, di- and trisulfated CS disaccharides, including the monosulfated CS-2 carrying a sulfate group on the

C<sub>2</sub> of the GlcA unit (Scheme 1). The detection was performed by on-line coupled mass spectrometry in negative-ion mode.

[29]

### 3.1. Optimization of HILIC-MS conditions

The eluent system chosen for both the HILIC separation and the on-line coupling to MS, was made of ammonium formate for the aqueous eluent A, and acetonitrile (MeCN) for the eluent B. It has been optimized as regards the concentration and pH of the aqueous eluent A, the A/B ratio in the mobile phase, and the gradient slope. The sample injection volume and the column temperature were also examined.

#### 3.1.1. Gradient optimization

In the first step, isocratic elution was tested at different percentages of eluent A 25 mM ammonium formate ranging from 25 to 30% mixed with eluent B MeCN to achieve the separation of the two monosulfated disaccharide isomers CS-A and CS-C. The isocratic elution at 26, 28 or 30% solvent A led to the separation of the two monosulfated disaccharide isomers CS-A and CS-C in less than 7 minutes (Figure 1). The retention time increased with lowering solvent A proportion, in agreement with the expected stronger interaction with the HILIC phase in low aqueous mobile phase. Accordingly, a satisfying baseline separation was obtained at 26% solvent A, even allowing the differentiation of each  $\alpha/\beta$  anomer couples (Figure 1b). A broad peak of low intensity could be observed before the elution of CS disaccharides and was identified to CS-C and CS-A isomers in mixture by MS/MS analysis (Figure S2). The presence of this unwanted peak may result from column overloading as it disappears for injection at lower concentration (see sections from 3.1.2).

A more complex sample was then tested by adding the disulfated isomers CS-D and CS-E disaccharides to the monosulfated isomers mixture. Isocratic elution at 26% solvent A did not permit the disulfated CS isomers to be distinguished and led to the co-elution of  $\beta$ -CS-A with  $\alpha$ -CS-D between 7 and 7.5



min. Therefore, different gradients were tested to improve the separation of disulfated CS disaccharides. A direct gradient from 25 to 30% of eluent A in 10 min led to a poor separation of the two monosulfated disaccharide CS-A and CS-C, which were eluted in less than 7.5 minutes (Figure S1). An isocratic step was then introduced before launching the gradient. A 4- or a 8-min isocratic step of 26 % eluent A followed by a 2-min gradient increasing the aqueous mobile phase from 26 to 35 % was tested. While the 8-minute isocratic step followed by the 2-minute gradient did not significantly improve the separation between mono and disulfated CS isomers (Figure S2a), the 4-minute isocratic step followed by the 2-minute gradient results in a clear separation of the disulfated CS anomers and the differentiation of their corresponding anomers (Figure S2b). The aqueous eluent A was increased to 85% after the 2-minute gradient to ensure the total elution of injected sulfated disaccharides, then it was decreased to 10% for allowing column reactivation. In sections 3.1.2. to 3.1.4, the following elution system will be considered: 0 min (26%A), 4 min (26%A), 6 min (35%A), 8 min (35%A), 9 min (85%A), 12 min (85%A), 13 min (10%A), 16 min (10%A), 17 min (26%A) followed by 8 min of reconditioning, for a total analysis running time of 25 min.

### 3.1.2. Mobile phase composition

The influence of pH of the aqueous solvent A on the HILIC separation was studied by varying the pH of the ammonium formate solution from 3.5 to 6 (in steps of 0.5 pH unit by adding formic acid), and by using the gradient system determined above. The pH effect was studied on the HILIC separation of two different disaccharide mixtures, one containing CS-0, CS-A, CS-C, CS-E and CS-D, and the second containing CS-2, CS-B and the trisulfated CS, in low enough concentration range (2 to 50  $\mu$ M) to prevent trail of peak as observed in Figure 1.

We observed that CS disaccharides are more retained in acidic conditions (Figure 2 and Figure S4), as illustrated by the elution of all the disaccharides between 5 and 13.5 minutes at pH 3.5 compared to their elution in 3 - 10 minutes at pH >5. Moreover, the elution order at pH 3.5 followed the disaccharide polarity, as 0-, mono- and di- and finally trisulfated species were successively separated, while at higher

pH values, some mixing occurred between non- and monosulfated disaccharides (from pH 4) and between mono- and disulfated disaccharides (from pH 5). Trisulfated CS eluted last whatever the pH value. EIC shows some co-elution of anomers as observed for the  $\alpha$ -CS-C and  $\alpha$ -CS-A monosulfated disaccharides ( $m/z$  458.1) and  $\alpha$ -CS-B and  $\beta$ -CS-D disulfated disaccharides ( $m/z$  538.1) at pH 3.5, and for the  $\beta$ -CS-2 and  $\alpha$ -CS-A monosulfated disaccharides ( $m/z$  458.1) between pH 4.5 and 5 (Figure 2b and 2c). At pH 5,  $\beta$ -CS-A and the disulfated disaccharide  $\beta$ -CS-D (detected either as singly deprotonated,  $m/z$  538.1, or doubly deprotonated,  $m/z$  268.7) co-elute at  $t_R=4.1$  min. Similarly, co-elution is also observed at  $t_R=3.6$  min for  $\beta$ -CS-C and  $\alpha$ -CS-D (Fig. 2d).

From these results, we can notice the importance of the sulfate group position of CS analogues on HILIC elution order. Indeed, for monosulfated species, elution order is as follows: CS-2 (sulfate location C<sub>2</sub> of GlcA) elutes first, then CS-C (sulfate location C<sub>6</sub> of GalNAc) and CS-A (sulfate location C<sub>4</sub> of GalNAc). For disulfated species, order elution is similar with first eluted CS-D (sulfate location on C<sub>2</sub> of GlcA and C<sub>6</sub> of GalNAc) then CS-B (sulfate location on C<sub>2</sub> of GlcA and C<sub>4</sub> of GalNAc) and finally CS-E (sulfate location C<sub>6</sub> and C<sub>4</sub> of GalNAc). Expectingly, CS analogues sulfated on C<sub>2</sub> of GlcA are eluted first as they are less polar than CS analogues sulfated on GalNAc saccharide. It is interesting to note that sulfate location on C<sub>6</sub> of GalNAc yields less polar compounds than corresponding CS analogues sulfated on the C<sub>4</sub> of the same saccharide.

The pH of eluent A also affected both the intensity and the charge state of the disaccharide species detected by ESI MS in negative ionization mode. The most intense MS signal was observed either at pH 4.0 or 4.5 depending on the disaccharide species. For the sake of sensitive analysis, the detection of a given disaccharide dispatched under different charge states is not desirable as it results in a signal dilution. From that perspective, di- and trisulfated disaccharides were preferentially detected as doubly charged species  $[\text{CS}-2\text{H}]^{2-}$  at  $m/z$  268.7 and 308.6, respectively. Accordingly, the disulfated CS-D and trisulfated disaccharides, poorly observed at pH 3.5 and pH 4, were mainly observed as  $[\text{CS}-2\text{H}]^{2-}$  from pH 4.5 (Figure 2c-d). By contrast, disulfated disaccharides CS-B and CS-E were exclusively observed as singly charged ions below pH 4.5 (Figure 2a-c). In addition to multiple charge states, H/Na exchange likely due to residual sodium present in the sample resulted in sodium in adducts likely on

the sulfate moieties, which can further complicate the mass spectra. The abundance of  $[\text{CS}+\text{Na}-2\text{H}]^-$  ions are constant below pH 4.5, while it tends to disappear from pH 5 (Figure S5). Indeed, the Na/H back exchange can occur above pH 5 as the relative proportion of  $\text{Na}^+$  decreases to the benefit of  $\text{H}^+$  when pH increases (Figures S6 and S7).

Intact sulfated disaccharides were mostly detected. However, we noticed some losses of  $\text{SO}_3$  in the case of di- and trisulfated disaccharides, likely due to in-source fragmentation. It leads to the formation of the partially desulfated ions at  $m/z$  458.1  $[\text{disulfated CS}-\text{SO}_3-\text{H}]^-$  from the disulfated disaccharide, and at  $m/z$  538.1  $[\text{trisulfated-CS}-\text{SO}_3-\text{H}]^-$  from the trisulfated disaccharide, which both are indistinguishable from the intact deprotonated mono- and disulfated CS, respectively. It should be pointed out that the in-source desulfation is observed whatever the pH studied (Figure S6).

Overall, the separation of the CS disaccharide isomers is fully observed at pH 4 and pH 4.5. As the separation is more resolved and the signal is less diluted at pH 4 thanks to the prominent detection of singly charged species, ammonium formate solvent A in eluent system was then adjusted to pH 4 for the subsequent optimization steps.

The influence of ionic strength of the eluent A ammonium formate, pH 4, was studied by varying its concentration from 2 to 20 mM (Figure 3). Short retention times and consequently a poor separation of CS disaccharides were observed at the lowest ammonium formate concentration (Figure 3a). By contrast, retention times significantly increased as the ionic strength increased, and a complete separation of CS isomers was achieved from 5 to 10 mM ammonium formate. At the highest concentration (20 mM), broadening of chromatographic peaks occurred and disulfated CS isomers were no more differentiated. Variation in buffer concentration also affected the charge state of both di- and trisulfated disaccharides. The doubly-charged CS ions were very intense at 2 and 3.5 mM, and they largely decreased at highest concentrations compared to singly charged species. Despite the sensitive detection of multiple charge state ions at low concentration, the quasi absence of H/Na exchange above 5mM resulted in more sensitive MS analysis (Figure S7). Based on this optimization of pH and ionic

strength, 7.5 mM ammonium formate, pH 4, was selected as eluent A, leading to suitable MS peak intensities and resolution of CS isomers.

### 3.1.3. Column temperature

The influence of the temperature on the HILIC separation of CS oligosaccharides was studied by varying the column temperature from 10 to 50 °C with 10 °C steps. The increase in temperature improved the HILIC separation of sulfated disaccharides (Figure 4): at 10 °C,  $\beta$ -CS-0 is eluted after  $\alpha$ -CS-2 and a slight peak overlap is observed between  $\beta$ -CS-A and  $\alpha$ -CS-D, while from 20 °C  $\beta$ -CS-A and  $\alpha$ -CS-D were baseline resolved, and from 30 °C, CS-0 anomers were eluted before any sulfated CS disaccharides. The improved separation observed when increasing the temperature resulted from the weaker retention of the non-sulfated disaccharide CS-0 and the concomitant stronger retention of the mono- and disulfated disaccharides. The temperature effect depended on sulfate content: the first eluted monosulfated  $\alpha$ -CS-2 exhibited a  $t_R$  shift of less than 1 min, whereas the first eluted disulfated  $\alpha$ -CS-D showed a  $t_R$  shift of almost 2 min upon temperature increase. Note that the trisulfated CS retention time was almost not affected by temperature variation as its elution depends mainly on the polarity gradient. Separation improvement at increased temperature was however counterbalanced by peak tailoring at 30°C and above, as shown for example at 50°C by  $\beta$ -CS-C being almost not differentiated from CS-A peak tail observed between  $\alpha$ - and  $\beta$ -CS-A (Figure 4e). Being more important as temperature increased, we assume that it is due to *in-situ* anomerization (Figure S10). The temperature had low impact on both the charge state of the detected species and the extent of the in-source desulfation process (Figures S11 and S12). Indeed, between 10 and 50 °C, a small decrease in the signal intensity of doubly charged ions for di- and trisulfated disaccharides is observed. A slight increase in H/Na exchange on sulfate moieties was observed only for disulfated disaccharides (Figure S12) when temperature increased, while  $[M-H]^-$  and  $[M+Na-2H]^-$  ratios remained stable with temperature for monosulfated CS (Figure S11). Based on these results, 20°C was finally chosen as the optimal temperature. It is worth noting that the column pressure at this temperature does not allow the

flow rate to be increased, so that this parameter was not varied and was kept at 300  $\mu\text{L}/\text{min}$  during analysis.

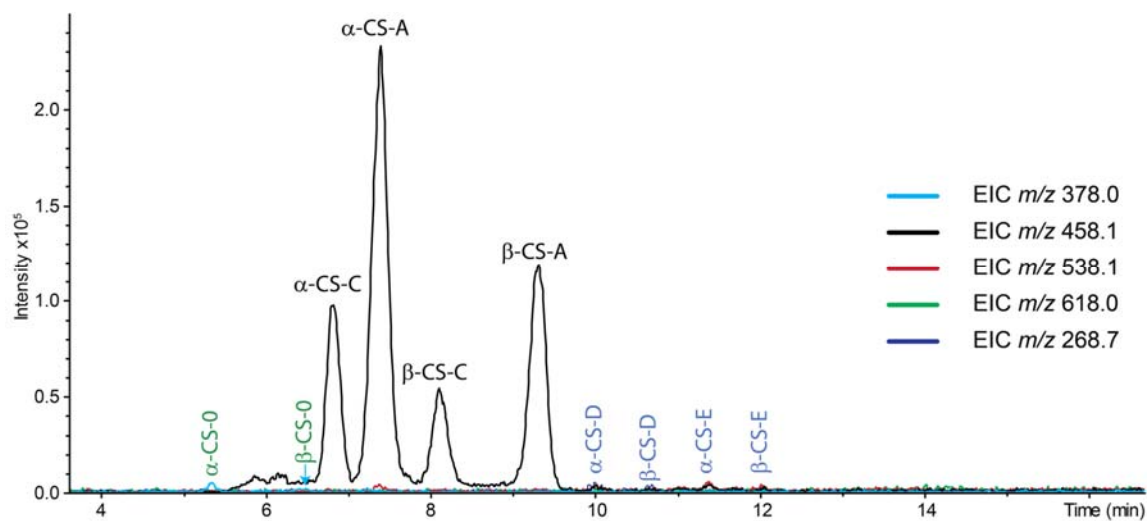
Taken together, these data lead to the following optimized HILIC method comprising a first isocratic step and then an increasing gradient of the MS compatible buffer 7.5 mM ammonium formate, pH 4, as polar eluent A, and run at 300  $\mu\text{L}/\text{min}$ , 20  $^{\circ}\text{C}$ . The injected volume, which is usually a key parameter for HILIC analysis, was varied from 0.5 to 2.5  $\mu\text{L}$  without showing any chromatographic differences (Figure S9) and was then set a 2.5  $\mu\text{L}$ .

### 3.2. *Application to enzyme activity kinetic*

The optimized HILIC separation of sulfated disaccharides was applied to the monitoring of two CS-specific enzymatic reactions: the depolymerization of CS by the lyase chondroitinase ABC, and the regio-selective desulfation of CS by the bacterial CS 4-*O*-endosulfatase. Both enzymes are very useful tools for exploring the structure-function relationships of CS since they can be used for disaccharide compositional analysis and to probe the role of sulfate groups in biological activities.

The depolymerization reaction has been carried out on endocan, a circulating proteoglycan secreted by endothelial cells and to which a single CS chain is attached. [REF] After its release from the core protein by enzyme proteolysis, the GAG chain was depolymerized by the chondroitinase lyase ABC and the resulting disaccharides were analyzed by HILIC-MS. Monosulfated disaccharides accounted for 99% of the MS signal on the obtained HILIC chromatogram, while disulfated and unsulfated disaccharides were detected as trace amounts and no signal was observed for trisulfated disaccharides (Figure 5). Based on EIC, the monosulfated disaccharides formed were 4-*O*-sulfated (CS-A) and 6-*O*-sulfated (CS-C) in a  $71/29 \pm 5$  ratio. Note that the quantitation was performed from UV as different ionization efficiencies in MS can be observed depending on the sulfate location, and influence this ratio. Thus, the enzyme depolymerization mainly yielded monosulfated disaccharides in agreement with the literature data indicating 1 sulfate/disaccharide on average in endocan and 65% of 4-*O*-sulfated

disaccharides (JBC 2001), therefore validating this LC-MS method for the identification of CS disaccharides.

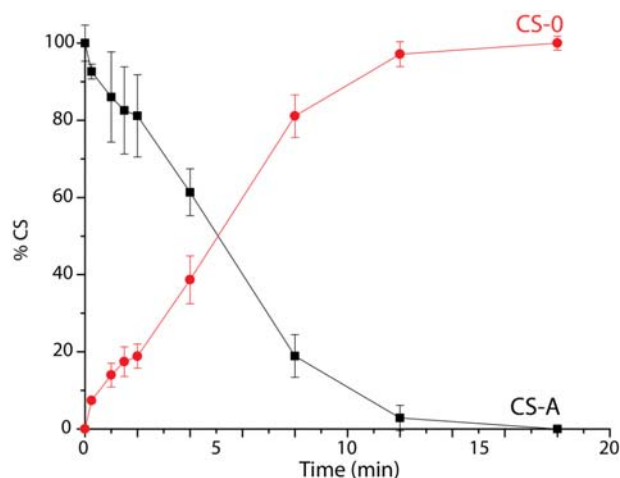


**Figure 5.** LC-MS experiments performed after the depolymerization of endocan with EIC of CS-0 in light blue at  $m/z$  378.0, EIC to mono sulfated CS at  $m/z$  458.1 in black, EIC of disulfated CS at  $m/z$  538.1 and 268.7 in red and dark blue respectively and EIC of trisulfated CS at  $m/z$  618.0 in green.

The enzymatic desulfation of natural sulfated carbohydrate substrate is an important post-editing and catabolic reaction, the time-course of which could not be easily followed. Here, the kinetics of desulfation of the disaccharide substrate  $\Delta$ UA $\beta$ -(1,3)-GalNAc4S (CS-A) catalyzed by the bacterial CS 4-*O*-endosulfatase and the concomitant formation of the desulfated product  $\Delta$ UA $\beta$ -(1,3)-GalNAc (CS-0), was recorded by HILIC-MS. The enzyme kinetics was recorded by sampling 100  $\mu$ L of the reaction mixture at regular time intervals (from 0.25 min to 18 min, and before the addition of the CS 4-*O*-endosulfatase). The time course of CS-A substrate consumption showed a complete desulfation in less than 20 min in these conditions (Figure 6), following a first order kinetic (Figure S13). plots of the CS dp2 percentages in function of the time are represented in. These results show a first order kinetic (in accordance with the literature ?) which can be expressed as:

$$[CS - A] = [CS - A_0] \exp(-kt)$$

where  $[CS-A]$  is the concentration of CS-A at time  $t$ ,  $[CS-A_0]$  is the initial concentration and  $k$  is the reaction rate coefficient. The plot  $\ln(CS-A) = f(t)$  has a slope equal to  $-k$  and gives us access to the half-time of the reaction  $\ln 2 / k = 4.5 \pm 0.9$  min (Figure S13).



**Figure 6.** Plots of %CS =  $f(t)$  of the kinetic of enzyme desulfation with CS-A isomer represented in squares and CS-0 in circles. The kinetic was performed two times and analyzed in triplicate.

The kinetic performed by LC-MS shows that the desulfation of CS-A by the bacterial CS 4-*O*-endosulfatase is complete in less than 20 min in these conditions. We could then study the 4-*O* desulfation specificity by reacting the CS 4-*O*-endosulfatase in presence of disaccharides CS-A and CS-C. For this experiment, CS 4-*O*-endosulfatase was incubated in presence of 100  $\mu$ M of CS-A and 0, 0.5, 1 or 2 equivalents of CS-C. While the sample containing only CS-A disaccharides showed a complete desulfation, the mixtures containing CS-C isomer are observed intact (Figure S14), confirming the CS 4-*O* desulfation specificity ~~even in presence of its substrate~~. [30]

#### 4. Conclusions

The HILIC-MS methodology developed allows a fast and unambiguous identification of mono- di- and trisulfated chondroitin sulfates, including anomers in the case of reducing sugars. We could highlight the importance of the mobile phase composition essentially in terms of pH and buffer concentration. Indeed, for reproducibility, these parameters have to be strictly identical as minimal variations result

in shifts of retention times of several minutes and can lead to misattribution. The temperature of the column should also be limited to avoid *in-situ* anomerization.

However, taking into account the optimized chromatographic parameters, we were able to use this HILIC-MS method to identify disaccharides isomers resulting from the depolymerization by the chondroitinase lyase ABC of endocan and determine the kinetic performance and selectivity of CS 4-*O*-endosulfatase successfully. Work is in progress in our laboratory to distinguish higher polymerization degree CS using a similar methodology. Given the analytical challenge represented by the analysis of saccharides and more particularly reducing sugars, the development of such a method can already be used for many applications in the field of GAGs.

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**Figure 1.** Isocratic elution of CS-A (4-*O*-sulfated) and CS-C (6-*O*-sulfated) disaccharides upon HILIC-MS separation. **A.** 28% of solvent A: 5mM ammonium formate, pH 3.75, solvent B: MeCN. **B.** 26% of solvent A: 5mM ammonium formate, pH 3.75, solvent B: MeCN. Extracted ion chromatograms of deprotonated [M-H]<sup>-</sup> ion at *m/z* 458.06. Injection volume: 2μL of 10 μM CS-A and CS-C disaccharides in H<sub>2</sub>O/MeCN 25:75; flow rate: 300μL/min; column temperature: 30°C.

**Figure 2.** Effect of pH of the 5mM ammonium formate solvent A on the HILIC-MS separation of mono-, di- and trisulfated CS disaccharides. pH: 3.5 (**A**), 4.0 (**B**), 4.5 (**C**) and 5.0 (**D**). Solvent B: MeCN, gradient elution as described in the section 2.2 of Materials and methods. Extracted ion chromatograms of the following deprotonated CS disaccharides in H<sub>2</sub>O/MeCN 25:75: CS-0 (10μM), CS-A (4μM), CS-C (2μM), CS-E (20μM), CS-D (20μM), CS-2 (2μM), CS-B (20μM) and the trisulfated CS (50μM). Injection volume: 2μL; flow rate: 300μL/min; column temperature: 30°C.

**Figure 3.** Effect of the ionic strength of the pH 4.0 ammonium formate solvent A on the HILIC-MS separation of mono-, di- and trisulfated CS disaccharides. Concentration of solvent A: 2 mM (**A**), 3.5 mM (**B**), 5mM (**C**), 7.5 mM (**D**), 10 mM (**E**) and 20 mM (**F**). Solvent B: MeCN, gradient elution as described in the section 2.2 of Materials and methods. Conditions and extracted ion chromatograms of deprotonated CS disaccharides as in Fig. 2.

**Figure 4.** Effect of the column temperature on the HILIC-MS separation of mono-, di- and trisulfated CS disaccharides. Column temperature: 10°C (**A**), 20 °C (**B**), 30 °C (**C**), 40 °C (**D**)

and 50 °C (E). Solvent A: 7.5mM ammonium formate pH 4.0, solvent B: MeCN. Gradient elution as described in the section 2.2 of Materials and methods. Conditions and extracted ion chromatograms of deprotonated CS disaccharides as in Fig. 2.

**Figure 5.** LC-MS experiments performed after the depolymerization of endocan with EIC of CS-0 in light blue at  $m/z$  378.0, EIC to mono sulfated CS at  $m/z$  458.1 in black, EIC of disulfated CS at  $m/z$  538.1 and 268.7 in red and dark blue respectively and EIC of trisulfated CS at  $m/z$  618.0 in green.

**Figure 6.** Plots of %CS =  $f(t)$  of the kinetic of enzyme desulfation with CS-A isomer represented in squares and CS-0 in circles. The kinetic was performed two times and analyzed in triplicate.

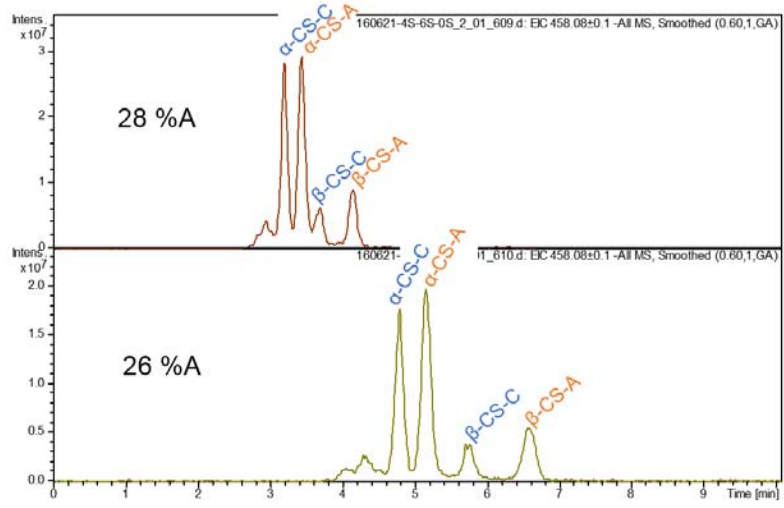
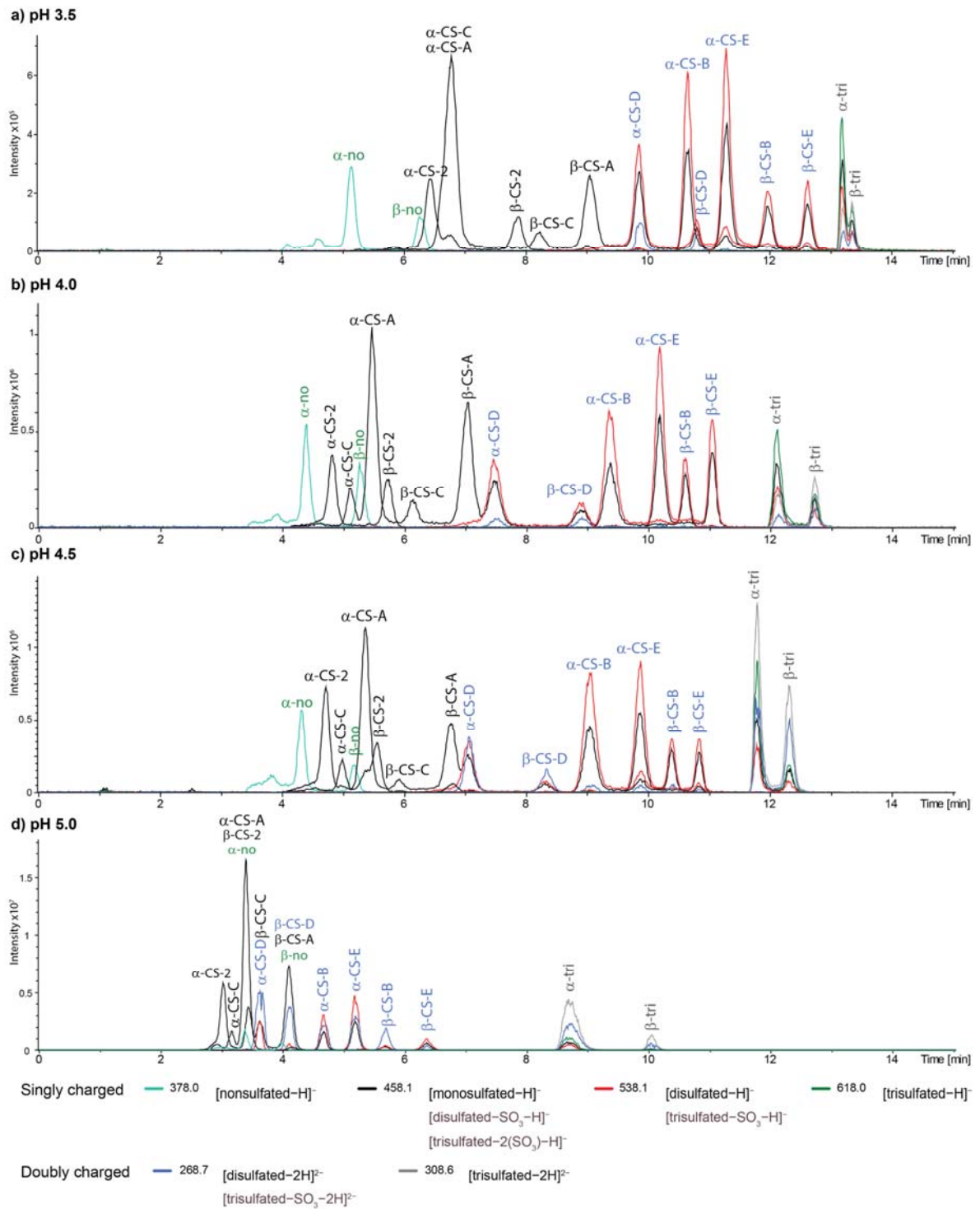
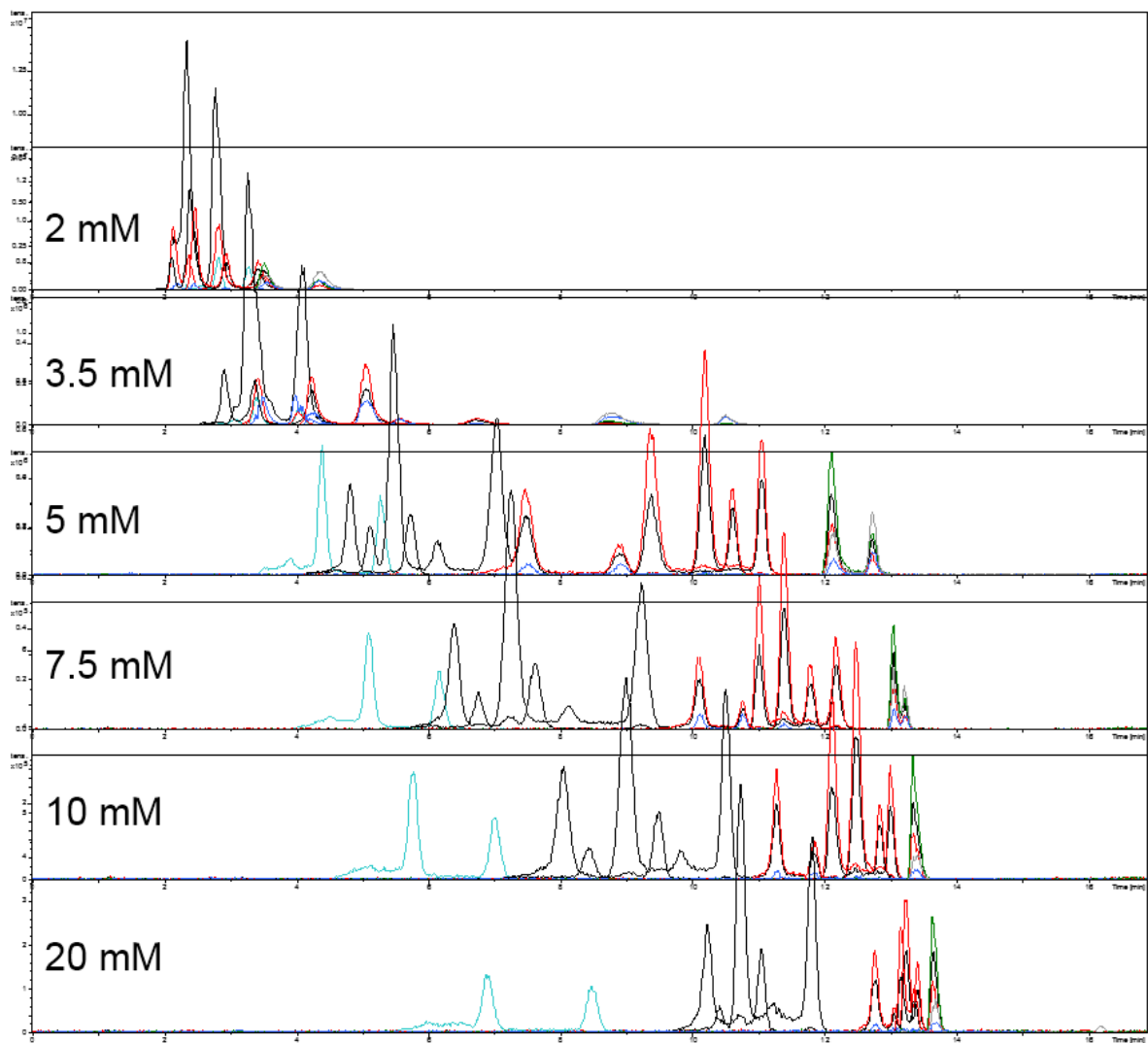


Figure 1



**Figure 2**



**Figure 3**

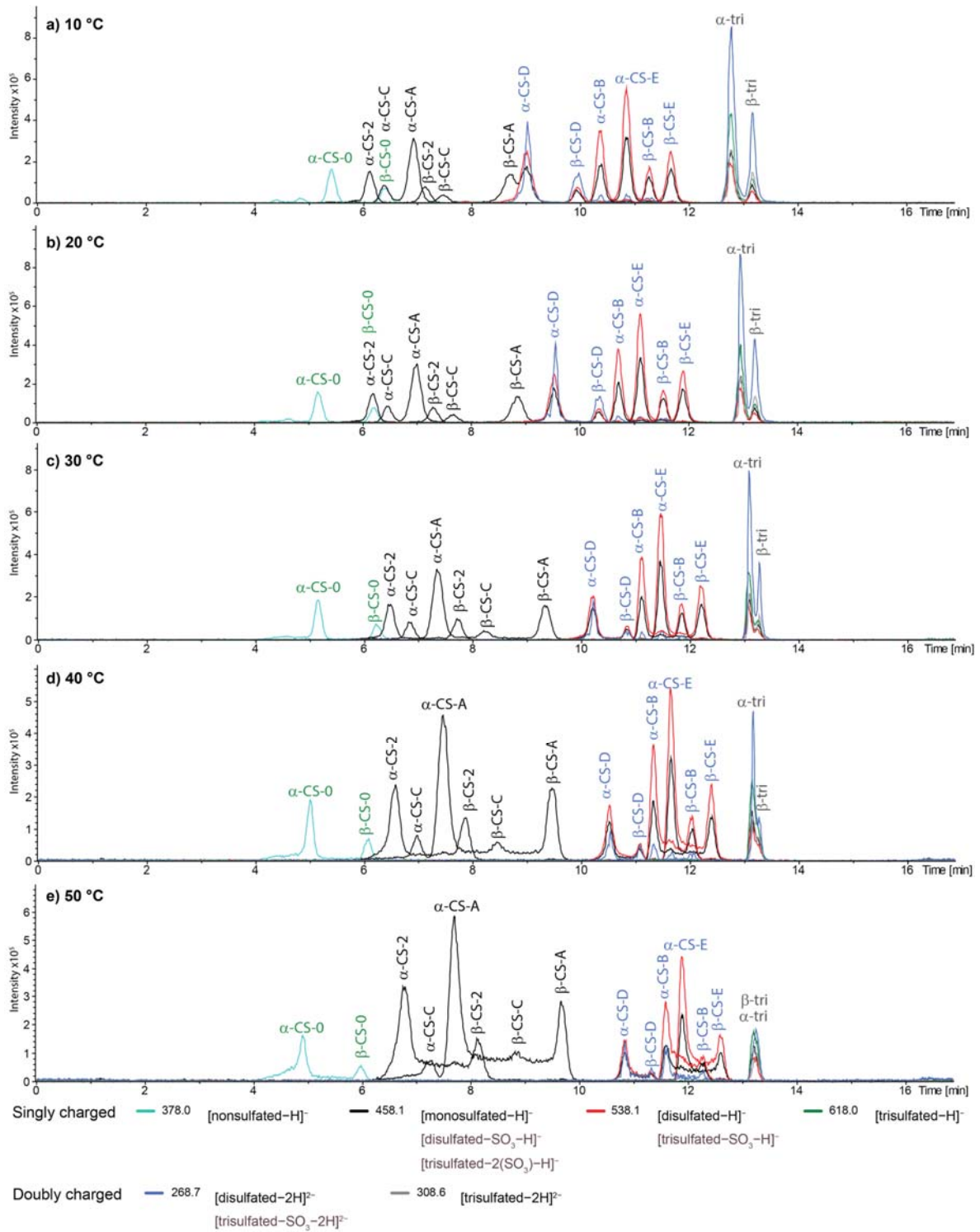


Figure 4



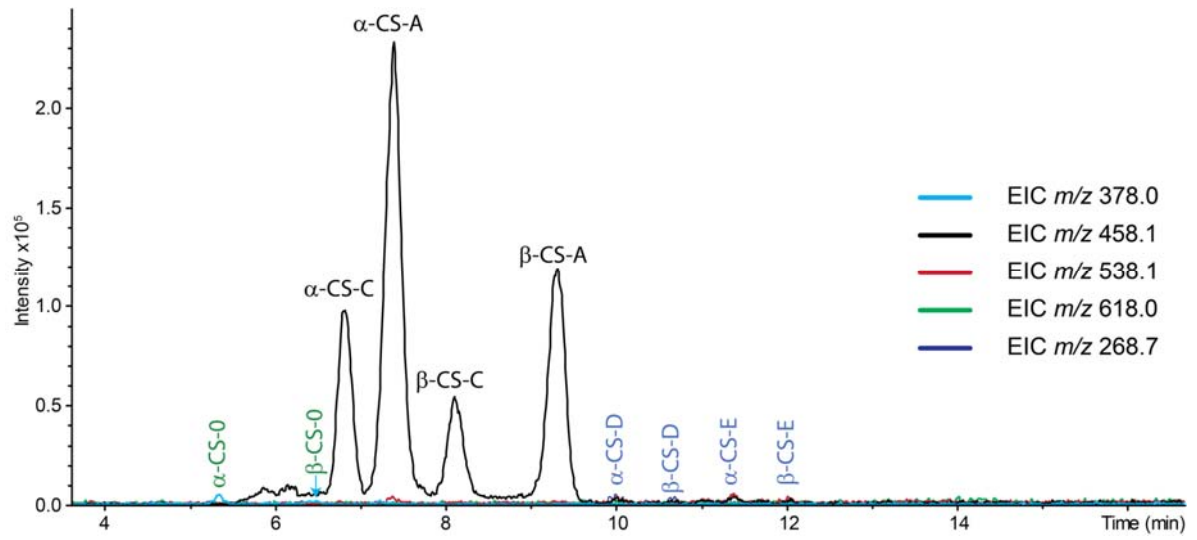


Figure 5

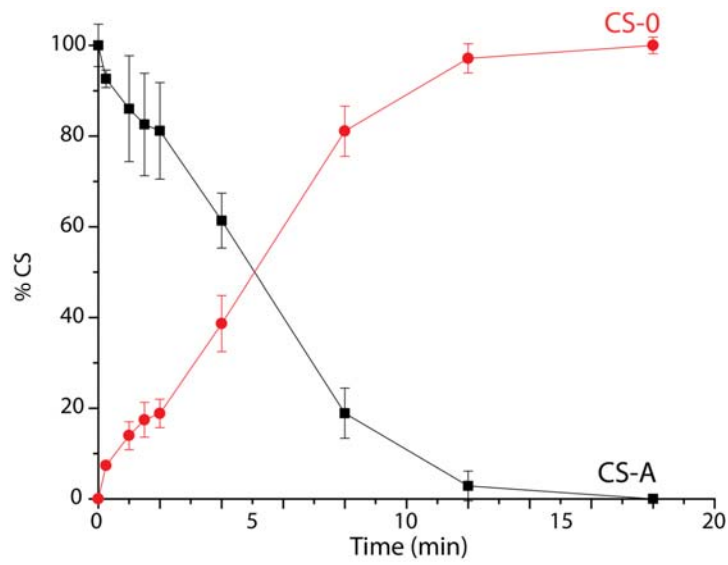


Figure 6