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1 **Discrimination of sulfated isomers of chondroitin sulfate disaccharides**
2 **by HILIC-MS**

3

4 Salomé Poyer,^{1,2*} Ilham Seffouh,^{1,2} Chrystel Lopin-Bon,³ Jean-Claude Jacquet,³ José L. Neira,⁴
5 Jean-Yves Salpin,^{1,2} Régis Daniel.^{1,2*}

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7 ¹ Université Paris-Saclay, CNRS, Univ Evry, LAMBE, Evry-Courcouronnes, 91025, France

8 ² CY Cergy Paris Université, LAMBE, Evry-Courcouronnes, 91025, France

9 ³ ICOA, CNRS UMR 7311, Université d'Orléans, F-45067 Orléans

10 ⁴ Instituto de Biología Molecular y Celular, Universidad Miguel Hernandez, Elche, Alicante, Spain

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12 * To whom correspondence should be addressed. E-mail: salome.poyer@univ-evry.fr Phone: +33 169 47 77
13 38. Email: regis.daniel@univ-evry.fr. Phone: +33 169 47 7641.

14

15

16 **Abstract**

17 Chondroitin sulfate (CS) glycosaminoglycans are biologically active sulfated polysaccharides that
18 pose an analytical challenge for their structural analysis and functional evaluation. In this study, we
19 developed a HILIC separation method and its online coupling to mass spectrometry (MS) allowing
20 efficient differentiation and sensitive detection of mono-, di- and trisulfated CS disaccharides and
21 their positional isomers, without requiring prior derivatization. The composition of the mobile phase
22 in terms of pH and concentration showed great influence on the chromatographic separation and was
23 varied to allow the distinction of each CS without signal overlap in a total analysis a 25 min. This
24 methodology was applied to determine the disaccharide composition of biological reaction media
25 resulting from various enzymatic transformations of CS, such as enzymatic desulfation of CS
26 disaccharides by a CS 4-*O*-endosulfatase, and depolymerization of the CS endocan by chondroitinase
27 lyase ABC.

28

29 **Keywords**

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

32 1. Introduction

33 Chondroitin sulfate (CS) are sulfated polysaccharides of the glycosaminoglycan (GAG) family,
34 which are widely distributed in body compartments, with the notable feature representing the most
35 abundant GAGs in brain. Being the prevalent carbohydrate component of the extracellular matrix of the
36 central nervous system,(1, 2) CS are involved in neural development and regeneration, cell migration
37 and axonal pathfinding, and recent findings highlight their critical roles in various physio-
38 pathological processes such as in neuro-inflammation.(3-6) Structural analysis of CS faces to a high
39 isomeric barrier due to the various sulfate distributions and presence of epimers along the
40 polysaccharide chain. The repeating disaccharide units of CS-A, -C and -E are made up of glucuronic
41 acid (GlcA) and *N*-acetylgalactosamine (GalNAc) sulfated on C4, C6 and both C4 and C6,
42 respectively (Table 1). In CS-D, both GlcA and GalNAc are sulfated on C2 and C6, respectively. The
43 C₅ epimerization of GlcA into iduronic acid (IdoA) in variable proportion lead to CS-B, also named
44 dermatan sulfate.(7) From the diversity of sulfation pattern originates a variety of CS-protein binding
45 partners.(8) To date, isolation and sequencing of bioactive CS sequences with specific sulfate pattern
46 triggering the selective and high affinity protein binding remain challenging tasks, so that structure-
47 activity relationship could not be easily established. In this context, development of efficient
48 analytical methods for the sensitive detection and discrimination of various sulfation patterns within
49 CS sequences is of high necessity to associate biological functions with well-defined sulfated
50 structures. We have previously identified diagnostics ions for 4-*O* and 6-*O* sulfation, which have
51 been used for the discrimination of sulfated isomers of CS oligosaccharides by electrospray-tandem
52 mass spectrometry (ESI-MS/MS).(9) Real CS oligosaccharide samples from biological sources or
53 formed by enzyme depolymerization are complex mixtures requiring their separation before MS
54 analysis. This can be achieved through on-line liquid chromatography, generally using a reversed
55 stationary phase, which is compatible with ESI-MS. However, given the polar nature of GAGs, such
56 reversed phase separation requires their prior derivatization and/or ion-pairing.(10-15) Over the last

57 decade, hydrophilic interaction liquid chromatography (HILIC) has emerged as an attractive
58 alternative method for the separation of polar compounds.(16, 17) Indeed, HILIC is based on a polar
59 stationary phase grafted with hydrophilic groups, making it much more favorable to the separation of
60 polar analytes. Furthermore, it uses polar solvents mixed with low proportion of water or aqueous
61 buffer as eluents, which allows efficient electrospray ionization and is compatible with on-line MS
62 coupling. HILIC-MS is widely expanding to the analysis of glycans, phosphorylated saccharides and
63 glycopeptides.(18, 19) In comparison, fewer applications of the HILIC method to the separation of
64 GAG oligosaccharides have been reported, most of which focus primarily on heparin
65 oligosaccharides.(20-26) Recently, the HILIC-MS analysis of CS oligosaccharides has been
66 described according to procedures requiring a preliminary derivatization of the reducing end by
67 AMAC or 2-aminopyridine (27, 28) or using a salt gradient (29). In the present study, we have
68 developed a HILIC separation method that does not require prior derivatization and allows the
69 resolution of mono-, di- and trisulfated CS disaccharides (Table 1). Different parameters of
70 separation conditions were optimized. We report the coupling of this HILIC method to ESI-MS in
71 negative ion mode, and its application to the monitoring of the enzyme activity of a CS sulfatase and
72 of a chondroitin lyase.

73

74 **2. Materials and methods**

75 *2.1. Chemicals and standards*

76 CS disaccharides produced by digestion of CS by bacterial chondroitinase were purchased from
77 Iduron (UK). HPLC-grade acetonitrile was supplied by VWR (France). Tris-HCl and phosphate
78 buffers, ammonium formate and formic acid were obtained from Sigma-Aldrich (Saint-Quentin
79 Fallavier, France), as well as Actinase E and chondroitinase ABC. Endocan was provided by
80 LungInnov (Lille, France). CS 4-*O*-endosulfatase prepared as previously reported (30), was kindly

81 provided by Dr. JL Neira (Miguel Hernandez Univ., Spain). Deionised water (18.2 MΩ) was
82 obtained from a Milli-Q (Millipore) purification system.

83 2.2. *HILIC-MS analysis*

84 HILIC-MS experiments were carried out using a quadrupole ion trap mass spectrometer (AmaZon
85 Speed ETD, Bruker) equipped with an Apollo electrospray source operated in the negative ionization
86 mode. MS and MS/MS experiments were performed in enhanced resolution mode in the 200 to 700
87 mass range. ESI source parameters were set as follows: capillary voltage 3.5 kV, end plate offset
88 -140 V, nebulizer gas flow 20 psi, dry gas flow 10 L/min and dry gas temperature 250 °C. Data
89 analysis v4.0 software was used to process data. HILIC separation of CS analogues was achieved
90 using a ZIC-cHILIC column (SeQuant® ZIC®-cHILIC 3 μm, 100 Å 150 x 2,1 mm, VWR, France).
91 Optimized chromatographic conditions were: solvent A 7.5 mM ammonium formate adjusted at pH
92 4.0 using formic acid, solvent B acetonitrile; flow rate: 300 μL/min; injected volume of CS mixtures:
93 2.5 μL; column temperature: 20 °C, and autosampler temperature was set at 10 °C; linear gradient:
94 0 min (24%A), 4 min (24%A), 6 min (35%A), 8 min (35%A), 9 min (85%A), 12 min (85%A),
95 13 min (10%A), 16 min (10%A), 17 min (24%A), followed by 8 min of reconditioning for a total
96 analysis time of 25 min. A volume split of 1/3 was used at the end of the column to get 100 μL/min
97 flow rate at the entrance of the mass spectrometer.

98 2.3. *Chondroitin sulfate 4-O-endosulfatase assay*

99 The desulfation reaction catalyzed by the bacterial CS 4-O-endosulfatase was carried out as
100 previously described.(31) Briefly, the enzyme reaction was performed at 37 °C in 800 μL 50 mM
101 phosphate buffer, pH 7, containing 50 μM of unsaturated chondroitin 4-O-sulfate disaccharide
102 Δ4,5HexUA1-3GalNAc(4S) as substrate. The reaction was initiated by the addition of 40 μL of
103 0.6 mg/mL of CS 4-O-endosulfatase. 100 μL samples were withdrawn from the reaction mixture
104 upon reaction course, and then two-fold diluted in water. Resulting diluted samples were filtered on a

105 3 kDa centrifugal filter (Ultra, Amicon, USA). The filtered sample solutions were stored at 4 °C for
106 subsequent HILIC-MS analysis.

107 2.4. *Essay of chondroitin disaccharides from endocan*

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

117

118 3. Results and discussion

119 The development and optimization of the separation method by HILIC were performed using mono-,
120 di- and trisulfated CS disaccharides, including the monosulfated CS-2 carrying a sulfate group on the
121 C₂ of the GlcA unit (Table 1). The detection was performed by on-line coupled mass spectrometry in
122 negative-ion mode.

123 3.1. *Optimization of HILIC-MS conditions*

124 The eluent system chosen for both the HILIC separation and the on-line coupling to MS, was made
125 of ammonium formate for the aqueous eluent A, and acetonitrile (MeCN) for the eluent B. It has been
126 optimized as regards the concentration and pH of the aqueous eluent A, the A/B ratio in the mobile

127 phase, and the gradient slope. The sample injection volume and the column temperature were also
128 examined.

129 *3.1.1. Gradient optimization*

130 In the first step, isocratic elution was tested at different percentages of eluent A 5 mM ammonium
131 formate ranging from 25 to 30% mixed with eluent B MeCN to achieve the separation of the two
132 monosulfated disaccharide isomers CS-A and CS-C. The isocratic elution at 24 or 28 % solvent A led
133 to the separation of the two monosulfated disaccharide isomers CS-A and CS-C in less than 7 min
134 (Figure 1). The retention time increased with lowering solvent A proportion, in agreement with the
135 expected stronger interaction with the HILIC phase in low aqueous mobile phase. Accordingly, a
136 satisfying baseline separation was obtained at 26% solvent A, even allowing the differentiation of
137 each α/β anomer couples as previously observed for neutral carbohydrates (Figure 1b). (32)A broad
138 peak of low intensity could be observed before the elution of CS disaccharides and was identified as
139 a CS-C analogue by MS/MS analysis (Figure S1). The presence of this unwanted peak may result
140 from column overloading as it disappears for injection at lower concentration (see sections from
141 3.1.2).

142 A more complex sample was then tested by adding the disulfated isomers CS-D and CS-E
143 disaccharides to the monosulfated isomers mixture. Isocratic elution at 26% solvent A did not permit
144 the disulfated CS isomers to be eluted in less than 15 min (data not shown). Therefore, different
145 gradients were tested to allow the elution and separation of disulfated CS disaccharides. A direct
146 gradient from 25 to 30% of eluent A in 10 min led to a poor separation of the two monosulfated
147 disaccharide CS-A and CS-C, which were eluted in less than 7.5 min (Figure S2). An isocratic step
148 was then introduced before launching the gradient. A 4- or a 8-min isocratic step of 24 % eluent A
149 followed by a 2-min gradient increasing the aqueous mobile phase from 24 to 35 % was tested. While
150 the 8-minute isocratic step followed by the 2-minute gradient did not significantly improve the

151 separation of disulfated CS isomers (Figure S3a), the 4-minute isocratic step followed by the 2-
152 minute gradient results in a clear separation of the disulfated CS anomers and the differentiation of
153 their corresponding anomers in 11 min (Figure S3b). The aqueous eluent A was increased to 85%
154 after the 2-minute gradient to ensure the total elution of injected sulfated disaccharides, then it was
155 decreased to 10% for allowing column reactivation. In sections 3.1.2. to 3.1.4, the following elution
156 system will be considered: 0 min (24%A), 4 min (24%A), 6 min (35%A), 8 min (35%A), 9 min
157 (85%A), 12 min (85%A), 13 min (10%A), 16 min (10%A), 17 min (24%A) followed by 8 min of
158 reconditioning, for a total analysis running time of 25 min.

159 3.1.2. Mobile phase composition

160 The influence of the pH of the aqueous solvent A on the HILIC separation was studied by varying the
161 pH of the ammonium formate solution from 3.5 to 6 (in steps of 0.5 pH unit by adding formic acid),
162 and by using the gradient system determined above. The pH effect was studied on the HILIC
163 separation of two different disaccharide mixtures, one containing CS-0, CS-A, CS-C, CS-E and CS-
164 D, and the second containing CS-2, CS-2,4 and the trisulfated CS, in low enough concentration range
165 (2 to 50 μ M) to prevent trail of peak as observed in Figure 1.

166 We observed that CS disaccharides are more retained in acidic conditions (Figure 2 and Figure S4),
167 as illustrated by the elution of all the disaccharides between 5 and 13.5 min at pH 3.5 compared to
168 their elution in 3 - 10 min at pH \geq 5. Moreover, the elution order at pH 3.5 followed the disaccharide
169 polarity, as non-, mono- and di- and finally trisulfated species were successively separated, while at
170 higher pH values, some mixing occurred between non- and monosulfated disaccharides (from pH 4)
171 and between mono- and disulfated disaccharides (from pH 5). Trisulfated CS eluted last whatever the
172 pH value. EIC shows some co-elution of anomers as observed for the α -CS-C and α -CS-A
173 monosulfated disaccharides (m/z 458.1) and α -CS-2,4 and β -CS-D disulfated disaccharides (m/z
174 538.1) at pH 3.5, and for the β -CS-2 and α -CS-A monosulfated disaccharides (m/z 458.1) between

175 pH 5 and 6 (Figure 2d and Figure S4). At pH 5, β -CS-A and the disulfated disaccharide β -CS-D
176 (detected either as singly deprotonated, m/z 538.1, or doubly deprotonated, m/z 268.7) co-elute at
177 $t_R=4.1$ min. Similarly, co-elution is also observed at $t_R=3.6$ min for β -CS-C and α -CS-D (Fig. 2d).

178 From these results, we can notice the importance of the sulfate group position of CS analogues on
179 HILIC elution order. Indeed, for monosulfated species, elution order is the following: CS-2 (sulfate
180 location C₂ of GlcA) elutes first, then CS-C (sulfate location C₆ of GalNAc) and CS-A (sulfate
181 location C₄ of GalNAc). For disulfated species, order elution is similar with first eluted CS-D (sulfate
182 location on C₂ of GlcA and C₆ of GalNAc) then CS-2,4 (sulfate location on C₂ of GlcA and C₄ of
183 GalNAc) and finally CS-E (sulfate location C₆ and C₄ of GalNAc). Expectingly, CS analogues
184 sulfated on C₂ of GlcA are eluted first as they are less polar than CS analogues sulfated on GalNAc
185 saccharide. It is interesting to note that sulfate location on C₆ of GalNAc yields less polar compounds
186 than corresponding CS analogues sulfated on the C₄ of the same saccharide.

187 The pH of eluent A also affected both the intensity and the charge state of the disaccharide species
188 detected by ESI MS in negative ionization mode. The most intense MS signal was observed either at
189 pH 4.0 or 4.5, depending on the disaccharide species. For the sake of sensitivity, the detection of a
190 given disaccharide dispatched under different charge states is not desirable as it results in a signal
191 dilution. From that perspective, di- and trisulfated disaccharides were preferentially detected as
192 doubly charged species $[\text{CS}-2\text{H}]^{2-}$ at m/z 268.7 and 308.6, respectively. Accordingly, the disulfated
193 CS-D and trisulfated disaccharides, poorly observed at pH 3.5 and pH 4, were mainly observed as
194 $[\text{CS}-2\text{H}]^{2-}$ from pH 4.5 (Figure 2c-d). By contrast, disulfated disaccharides CS-2,4 and CS-E were
195 exclusively observed as singly charged ions below pH 4.5 (Figure 2a-c). In addition to multiple
196 charge states, H/Na exchange likely due to residual sodium present in the sample resulted in sodium
197 adducts probably on the sulfate moieties, which can further complicate the mass spectra. The
198 abundance of $[\text{CS}+\text{Na}-2\text{H}]^-$ ions are relatively constant regardless of the pH (Figure S5 and S6)

199 meaning that the pH of the mobile phase do not influence the H/Na exchange, while it strongly
200 decreases as the proportion of MeCN increases.

201 Intact sulfated disaccharides were mostly detected. However, we noticed some losses of SO₃ in the
202 case of di- and trisulfated disaccharides, likely due to in-source fragmentation. It leads to the
203 formation of the partially desulfated ions at m/z 458.1 [disulfated-CS-SO₃-H]⁻ from the disulfated
204 disaccharide, and at m/z 538.1 [trisulfated-CS-SO₃-H]⁻ from the trisulfated disaccharide, which both
205 are indistinguishable from the intact deprotonated mono- and disulfated CS, respectively. It should be
206 pointed out that the in-source desulfation is observed whatever the pH studied (Figure S6).

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

211 The influence of ionic strength of the eluent A ammonium formate, pH 4, was studied by varying its
212 concentration from 2 to 20 mM (Figure 3). Short retention times and consequently a poor separation
213 of CS disaccharides were observed at the lowest ammonium formate concentration (Figure 3a). By
214 contrast, retention times significantly increased as the ionic strength increased, and a complete
215 separation of CS isomers was achieved from 5 to 10 mM ammonium formate. At the highest
216 concentration (20 mM), broadening of chromatographic peaks occurred and disulfated CS isomers
217 were no more differentiated. Variation in buffer concentration also affected the charge state of both
218 di- and trisulfated disaccharides. The doubly-charged CS ions were mostly observed for buffer
219 concentrations comprised between 2 and 5 mM. Despite the sensitive detection of multiple charge
220 state ions at low concentration and the quasi absence of sodium adducts below concentration of
221 3.5mM, which resulted in more sensitive MS analysis (Figure S7), the isomer separation were not
222 fully achieved at these low buffer salt concentrations,. Based on these optimization of pH and ionic

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

225 3.1.3. Column temperature

226 The influence of the temperature on the HILIC separation of CS oligosaccharides was studied by
227 varying the column temperature from 10 to 50 °C with 10 °C steps. The increase in temperature
228 improved the HILIC separation of sulfated disaccharides (Figure 4): at 10 °C, β -CS-0 is eluted after
229 α -CS-2 and a slight peak overlap is observed between β -CS-A and α -CS-D, while from 20 °C β -CS-
230 A and α -CS-D were baseline resolved, and from 30 °C, CS-0 anomers were eluted before any
231 sulfated CS disaccharides. The improved separation observed when increasing the temperature
232 resulted from the weaker retention of the non-sulfated disaccharide CS-0 and the concomitant
233 stronger retention of the mono- and disulfated disaccharides. The temperature effect depended on
234 sulfate content: the first eluted monosulfated α -CS-2 exhibited a t_R shift of less than 1 min, whereas
235 the first eluted disulfated α -CS-D showed a t_R shift of almost 2 min upon temperature increase. Note
236 that the trisulfated CS retention time was almost not affected by temperature variation as its elution
237 depends mainly on the polarity gradient. Separation improvement at increased temperature was
238 however counterbalanced by peak tailoring at 30 °C and above, as shown for example at 50 °C by β -
239 CS-C being almost not differentiated from CS-A peak tail observed between α - and β -CS-A (Figure
240 4e). Being more important as temperature increased, we assume that it is due to *in-situ* anomerization
241 (Figure S9). The temperature had low impact on both the charge state of the detected species and the
242 extent of the in-source desulfation process (Figures S10 and S11). A slight increase in H/Na
243 exchange on sulfate moieties was observed only for disulfated disaccharides (Figure S11) when
244 temperature increased, while $[M-H]^-$ and $[M+Na-2H]^-$ ratios remained stable with temperature for
245 monosulfated CS (Figure S10). Based on these results, 20 °C was finally chosen as the optimal
246 temperature. It is worth noting that the column pressure at this temperature does not allow the flow

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

249 Taken together, these data lead to the following optimized HILIC method comprising a first isocratic
250 step and then an increasing gradient of the MS compatible buffer 7.5 mM ammonium formate, pH 4,
251 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
252 parameter for HILIC analysis, was varied from 0.5 to 2.5 μL without showing any chromatographic
253 differences (Figure S12) and was then set at 2.5 μL . This methodology was finally applied to all CS
254 isomers present in a single mixture in order to verify that no peak overlap could bias the isomer
255 assignment. The results obtained (Figure S13) confirm that the separation by HILIC-MS of the
256 totality of CS disaccharides can be obtained in less than 15 minutes for a total analysis (including the
257 elution of possible more polar compounds and a reactivation) of 25 minutes. Even if the selectivity
258 factors remain less good for some CS isomers than with RP-HPLC using derivatizations (mainly due
259 to the differentiation of anomers in HILIC), this method allows to identify each species
260 unambiguously for a 2 times shorter analysis time and from intact disaccharides.(11, 33, 34)
261 Moreover, the presence of salts in small quantities in the mobile phase does not clog the ESI source
262 nor the optics of the mass spectrometer (27) and allows us to obtain detection limits (Table S1) of the
263 same order of magnitude as previous methods of analysis of GAGS disaccharides by RP-HPLC with
264 derivatization or by HILIC-MS (Table S2).(26, 35).

265 3.2. *Application to the monitoring activity of GAG transforming enzymes*

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

271 The depolymerization reaction has been carried out on endocan, a circulating proteoglycan secreted
272 by endothelial cells and to which a single CS chain is attached.(36) After its release from the core
273 protein by Actinase E catalysed proteolysis, the GAG chain was depolymerized by the chondroitinase
274 lyase ABC and the resulting disaccharides were analyzed by HILIC-MS. Monosulfated disaccharides
275 accounted for 99% of the MS signal on the obtained HILIC chromatogram, while disulfated and
276 unsulfated disaccharides were detected as trace amounts and no signal was observed for trisulfated
277 disaccharides (Figure 5). Based on EIC, the monosulfated disaccharides formed were 4-*O*-sulfated
278 (CS-A) and 6-*O*-sulfated (CS-C) in a $71\pm 5/29\pm 5$ ratio. Note that the quantitation was performed from
279 UV as different ionization efficiencies in MS can be observed depending on the sulfate location, and
280 influence this ratio.(27) Moreover, although quantification can be performed from the peak of only
281 one of the anomers when calibration curves are performed in MS according to literature data (26), it
282 is however noted that in some cases, the α/β ratios can be different between standards and biological
283 samples, as observed here in the case of sulfated disaccharides (Table S3 and Figure S15). It would
284 therefore be preferable to perform the quantification on the sum of the anomers. Note also that MS
285 signals from different charge states and in-source dissociation as for sulfated disaccharides should be
286 taken into account. By taking all these precautions, it is possible to approach a reliable MS quantification by
287 the HILIC-MS method although this was not undertaken here. Thus, the enzyme depolymerization
288 mainly yielded monosulfated disaccharides in agreement with the literature data indicating one
289 sulfate per disaccharide unit on average in endocan and 65% of 4-*O*-sulfated disaccharides (37),
290 therefore validating this LC-MS method for the identification of CS disaccharides.

291 The enzymatic desulfation of natural sulfated carbohydrate substrate is an important reaction in post-
292 editing and catabolic processes, the time-course of which could not be easily followed. Here, the
293 kinetics of desulfation of the disaccharide substrate $\Delta\text{UA}\beta\text{-(1,3)-GalNAc}_4\text{S}$ (CS-A) catalyzed by the
294 bacterial CS 4-*O*-endosulfatase and the concomitant formation of the desulfated product $\Delta\text{UA}\beta\text{-(1,3)-}$
295 GalNAc (CS-0), was recorded by HILIC-MS. The enzyme kinetics was recorded by sampling 100 μL

296 of the reaction mixture at regular time intervals (from 0.25 min to 18 min, and before the addition of
297 the CS 4-*O*-endosulfatase). The time course of CS-A substrate consumption showed a complete
298 desulfation in less than 20 min in these conditions (Figure 6), following a first order kinetic (Figure
299 S14) which can be expressed as:

$$300 \quad [CS - A] = [CS - A_0] \exp(-kt)$$

301 where [CS-A] is the concentration of CS-A at time t, [CS-A₀] is the initial concentration and k is the
302 reaction rate coefficient. The plot Ln(CS-A) = f(t) has a slope equal to -k and gives us access to the
303 half-time of the reaction $\ln 2 / k = 4.5 \pm 0.9$ min (Figure S14).

304 The kinetic performed by LC-MS shows that the desulfation of CS-A by the bacterial CS 4-*O*-
305 endosulfatase is complete in less than 20 min in these conditions. HILIC-MS analysis could also be
306 conducted on a mixture of the two isomers disaccharides CS-A and CS-C. CS 4-*O*-endosulfatase was
307 incubated in presence of 100 μM of CS-A and 0, 0.5, 1 or 2 equivalents of CS-C. The CS-C
308 disaccharide was virtually unaffected, confirming the selectivity of the sulfatase for the 4-*O*- sulfate
309 group (Figure S15), confirming the CS 4-*O* desulfation specificity.(38)

310

311 4. Conclusions

312 The HILIC-MS methodology developed allows a fast and unambiguous separation and identification
313 of mono- di- and trisulfated chondroitin sulfate, including α/β anomers. As expected, the elution
314 order follows the increase in sulfate content, while the sulfate position also influences the retention
315 time as observed with the distinct elution of the different monosulfated disaccharides. With respect to
316 the separation of highly charged polar analytes, the study of the chromatographic parameters
317 underlines the critical importance of the characteristic of the mobile phase, and notably the pH value
318 and the buffer concentration. For reproducibility and robustness of the method, these parameters have
319 to be strictly maintained, as small variations result in retention time shifts of several minutes and can

320 lead to attribution errors. The temperature of the column should also be limited to avoid *in-situ*
321 anomerization.

322 Following these optimized chromatographic parameters, the HILIC-MS method can be used to
323 identify disaccharides isomers in biological reaction media. The disaccharide products from the
324 enzyme depolymerization of a CS polysaccharide can be quantitatively identified as shown by the
325 endocan degradation analysis. The change in sulfate content upon enzyme desulfation of a sulfated
326 substrate can be also monitored to determine the kinetic performance and selectivity of a sulfatase, as
327 successfully illustrated here for the CS 4-*O*-endosulfatase. Given the analytical challenge represented
328 by the analysis of sulfated saccharides, such a method can already be used for many applications in
329 the field of GAGs. This method has the potential to be extended to longer oligosaccharides, and work
330 is underway in our laboratory to evolve the chromatographic parameters to distinguish CS of higher
331 degree of polymerization.

332 **Conflicts of interest**

333 The authors declare that there is no conflict of interest concerning this manuscript

334 **Acknowledgment**

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339

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438

439 **Figure 1.** Extracted ion chromatograms of deprotonated $[M-H]^-$ ion at m/z 458.06 from isocratic
440 elution of CS-A (4-*O*-sulfated) and CS-C (6-*O*-sulfated) disaccharides upon HILIC-MS separation. **A.**
441 28% of solvent A: 5mM ammonium formate, pH 3.75, solvent B: MeCN. **B.** 26% of solvent A: 5mM
442 ammonium formate, pH 3.75, solvent B: MeCN. Injection volume: 2 μ L of 10 μ M CS-A and CS-C
443 disaccharides in H₂O/MeCN 25:75; flow rate: 300 μ L/min; column temperature: 30°C.

444

445 **Figure 2.** Effect of pH of the 5mM ammonium formate solvent A on the HILIC-MS separation of
446 mono-, di- and trisulfated CS disaccharides. pH: 3.5 (**A**), 4.0 (**B**), 4.5 (**C**) and 5.0 (**D**). Solvent B:
447 MeCN, gradient elution as described in the section 2.2 of Materials and methods. Extracted ion
448 chromatograms of the following deprotonated CS disaccharides in H₂O/MeCN 25:75: CS-0 (10 μ M),
449 CS-A (4 μ M), CS-C (2 μ M), CS-E (20 μ M), CS-D (20 μ M), CS-2 (2 μ M), CS-2,4 (20 μ M) and the
450 trisulfated CS (50 μ M). Injection volume: 2 μ L; flow rate: 300 μ L/min; column temperature: 30°C.

451

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

457

458 **Figure 4.** Effect of the column temperature on the HILIC-MS separation of mono-, di- and trisulfated
459 CS disaccharides. Column temperature: 10°C (**A**), 20 °C (**B**), 30 °C (**C**), 40 °C (**D**) and 50 °C (**E**).
460 Solvent A: 7.5mM ammonium formate pH 4.0, solvent B: MeCN. Gradient elution as described in
461 the section 2.2 of Materials and methods. Conditions and extracted ion chromatograms of
462 deprotonated CS disaccharides as in Fig. 2.

463

464 **Figure 5.** LC-MS experiments performed after the depolymerization of endocan with EIC of CS-0 in
465 light blue at m/z 378.0, EIC of mono sulfated CS at m/z 458.1 in black, EIC of disulfated CS at m/z
466 538.1 and 268.7 in red and dark blue respectively and EIC of trisulfated CS at m/z 618.0 in green.

467

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

470

Discrimination of sulfated isomers of chondroitin sulfate disaccharides by HILIC-MS

Supporting information

Salomé Poyer,^{1,2*} Ilham Seffouh,^{1,2} Chrystel Lopin-Bon,³ Jean-Claude Jacquinet,³ José L. Neira,⁴
Jean-Yves Salpin,^{1,2} Régis Daniel.^{1,2*}

¹ Université Paris-Saclay, CNRS, Univ Evry, LAMBE, Evry-Courcouronnes, 91025, France

² CY Cergy Paris Université, LAMBE, Evry-Courcouronnes, 91025, France

³ ICOA, CNRS UMR 7311, Université d'Orléans, F-45067 Orléans

⁴ Instituto de Biología Molecular y Celular, Universidad Miguel Hernandez, Elche, Alicante, Spain

* To whom correspondence should be addressed:

Salomé Poyer <https://orcid.org/0000-0003-0628-7383> E-mail: salome.poyer@univ-evry.fr Phone: +33 169 47 7738. Régis Daniel <https://orcid.org/0000-0003-0725-5439>, E-mail: regis.daniel@univ-evry.fr. Phone: +33 169 47 7641.

Figure S1. a) HILIC-MS/MS analysis of deprotonated monosulfated CS-A and CS-C using an isocratic elution of 26 % of solvent A using the following chromatographic conditions: Solvent A: 5 mM of ammonium formate adjusted at pH 3.75 with formic acid, Solvent B: MeCN, injection volume: 2 μ L of CS-A and CS-C at 10 μ M in H₂O/MeCN 25:75 flow rate: 300 μ L/min and column temperature: 30 °C. MS/MS spectra of the ion [CS-H]⁻ at m/z 458.1 are extracted from b) the peak tail at t_R 4.3 min, c) α -CS-C at t_R 4.8 min and d) α -CS-A at t_R 5.1 min..... 5

Figure S2. HILIC-MS/MS analysis of deprotonated monosulfated CS-A and CS-C using a linear gradient elution from 25 % to 30 % of solvent A in 10 minutes using the following chromatographic conditions: Solvent A: H₂O, Solvent B: MeCN, injection volume: 2 μ L of CS-A and CS-C at 10 μ M in H₂O/MeCN 25:75 flow rate: 300 μ L/min and column temperature: 30 °C..... 5

Figure S3. EIC of deprotonated CS from HILIC-MS separation using the following chromatographic conditions: Solvent A: 5 mM of ammonium formate adjusted at pH 3.75 with formic acid, Solvent B: MeCN, injection volume: 2 μ L, flow rate: 300 μ L/min and column temperature: 30 °C. An isocratic elution at 24% of A was performed for a) 8min or b) 4 min followed by a 2 min gradient to reach 35% of A for a total analysis time of 15 min. The injected mixture contains CS-0 at 10 μ M, CS-A at 4 μ M, CS-C at 2 μ M, CS-E and CS-D at 20 μ M in H₂O/MeCN 25:75. The color code of m/z values for each EIC is explain at the bottom of the Figure..... 6

Figure S4. EIC of deprotonated CS from HILIC-MS separation using the following chromatographic conditions: Solvent A: 5 mM of ammonium formate adjusted at pH a) 3.5, b) 4.0, c) 4.5, d) 5.0 e) 5.5 and f) 6.0 with formic acid, Solvent B: MeCN, injection volume: 2 μ L, flow rate: 300 μ L/min and column temperature: 30 °C. The gradient is: 0 min (24%A), 4 min (24%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 (24%A) followed by 8 min of reconditioning for a total analysis time of 25 minutes. Each chromatogram is an overlay of two analysis containing different injected CS as followed: the first mixture contains CS-0 at 10 μ M, CS-A at 4 μ M, CS-C at 2 μ M, CS-E and CS-D at 20 μ M, and the second one CS-2 at 2 μ M, CS-2,4 at 20 μ M and the trisulfated CS at 50 μ M in H₂O/MeCN 25:75. The color code of m/z values for each EIC is explain at the bottom of the Figure with intact ions annotated in black and in-source MS desulfated species annotated in dark red. 7

Figure S5. Extracted MS spectra of α -CS-A from HILIC-MS experiments presented in Figure 2 at pH a) 3.5 t_R 6.4-7.1 min, b) 4.0 t_R 5.4-5.7 min, c) 4.5 t_R 5.3-5.6 min and d) 5.0 t_R 3.3-3.5 min. 8

Figure S6. Extracted MS spectra of α -CS-E from HILIC-MS experiments presented in Figure 2 at pH a) 3.5 t_R 11.0-11.5 min, b) 4.0 t_R 10.0-10.4 min, c) 4.5 t_R 9.6-10.1 min and d) 5.0 t_R 5.0-5.4 min. 8

Figure S7. Extracted MS spectra of α -CS-A from HILIC-MS experiments presented in Figure 3 at ammonium formate concentrations of a) 2 mM t_R 2.2-2.5 min, b) 3.5 mM t_R 3.2-3.4 min, c) 5 mM t_R 5.3-5.6 min, d) 7.5 mM t_R 7.0-7.5 min, e) 10 mM t_R 8.8-9.2 min and f) 20 mM t_R 10.6-10.9 min. Note that in

the case of a) the MS signal at m/z 458.0 is the sum of the contributions from α -CS-A, α -CS-C and β -CS-D and for b) the contributions from α -CS-A and α -CS-C..... 9

Figure S8. Extracted MS spectra of α -CS-E from HILIC-MS experiments presented in Figure 3 at ammonium formate concentrations of a) 2 mM t_R 2.7-2.9 min, b) 3.5 mM t_R 4.9-5.3 min, c) 5 mM t_R 10.0-10.4 min, d) 7.5 mM t_R 11.2-11.5 min, e) 10 mM t_R 12.3-12.6 min and f) 20 mM t_R 13.1-13.3 min. Note that in the case of a) the MS signal at m/z 458.0 is the sum of the contributions from α -CS-E, β -CS-A, and β -CS-C. 9

Figure S9. Intensity zooms from 0 to 150000 counts of EIC of deprotonated CS from HILIC-MS separation using the following chromatographic conditions: Solvent A: 7.5 mM of ammonium formate adjusted at pH 4.0, Solvent B: MeCN, injection volume: 2.5 μ L, flow rate: 300 μ L/min and column temperature: a) 10 $^{\circ}$ C, b) 20 $^{\circ}$ C, c) 30 $^{\circ}$ C, d) 40 $^{\circ}$ C and e) 50 $^{\circ}$ C. The gradient is: 0 min (24%A), 4 min (24%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 (24%A) followed by 8 min of reconditioning for a total analysis time of 25 minutes. Each chromatogram contains CS-0 at 10 μ M, CS-A at 4 μ M, CS-C at 2 μ M, CS-E and CS-D at 20 μ M in H₂O/MeCN 25:75. The color code of m/z values for each EIC is explain at the bottom of the Figure with intact ions annotated in black and in-source MS desulfated species annotated in dark red..... 10

Figure S10. Extracted MS spectra of α -CS-A from HILIC-MS experiments presented in Figure 4 at a) 10 $^{\circ}$ C t_R 6.7-7.2 min, b) 20 $^{\circ}$ C t_R 6.8-7.2 min, c) 30 $^{\circ}$ C t_R 7.1-7.6 min d) 40 $^{\circ}$ C t_R 7.3-7.7 min and e) 50 $^{\circ}$ C t_R 7.5-8.0 min. 11

Figure S11. Extracted MS spectra of α -CS-E from HILIC-MS experiments presented in Figure 4 at a) 10 $^{\circ}$ C t_R 10.5-11.2 min, b) 20 $^{\circ}$ C t_R 10.9-11.3 min, c) 30 $^{\circ}$ C t_R 11.3-11.7 min d) 40 $^{\circ}$ C t_R 11.5-11.9 min and e) 50 $^{\circ}$ C t_R 11.8-12.1 min..... 11

Figure S12. EIC of deprotonated CS from HILIC-MS separation using the following chromatographic conditions: Solvent A: 7.5 mM of ammonium formate adjusted at pH 4.0, Solvent B: MeCN, injection volume: a) 0.5 μ L, b) 1.0 μ L, c) 1.5 μ L, d) 2.0 μ L and d) 2.5 μ L, flow rate: 300 μ L/min and column temperature: 20 $^{\circ}$ C. The gradient is: 0 min (24%A), 4 min (24%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 (24%A) followed by 8 min of reconditioning for a total analysis time of 25 minutes. Each chromatogram is an overlay of two analysis containing different injected CS as followed: the first mixture contains CS-0 at 10 μ M, CS-A at 4 μ M, CS-C at 2 μ M, CS-E and CS-D at 20 μ M, and the second one CS-2 at 2 μ M, CS-2,4 at 20 μ M and the trisulfated CS at 50 μ M in H₂O/MeCN 25:75. The color code of m/z values for each EIC is explain at the bottom of the Figure with intact ions annotated in black and in-source MS desulfated species annotated in dark red. 12

Figure S13. EIC of deprotonated CS from HILIC-MS separation using the following chromatographic conditions: Solvent A: 7.5 mM of ammonium formate adjusted at pH 4.0, Solvent B: MeCN, injection

volume: 2.0 μL , flow rate: 300 $\mu\text{L}/\text{min}$ and column temperature: 20 $^{\circ}\text{C}$. The gradient is: 0 min (24%A), 4 min (24%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 (24%A) followed by 8 min of reconditioning for a total analysis time of 25 minutes. The CS sample contains CS-0 at 65 μM , CS-A at 2.6 μM , CS-C at 0.65 μM , CS-E at 20 μM , CS-D at 0.5 μM , CS-2 at 0.35 μM , CS-2,4 at 0.4 μM and the trisulfated CS at 750 μM in $\text{H}_2\text{O}/\text{MeCN}$ 25:75. The color code of m/z values for each EIC is explain at the right of the Figure..... 13

Table S1. Limit of detection observed for HILIC-MS analysis with the injection of 2 μL of isomer mixtures in $\text{H}_2\text{O}/\text{MeCN}$ 25:75 13

Table S2. Comparison with other methods reported in the literature related to the separation of CS disaccharides. The list of publications is sorted by year..... 14

Table S3. Retention times, peak widths and peak area for singly- and doubly charged CS analogues obtained from HILIC-MS analysis presented on Figure 2. t_{R} and peak widths are in minutes and peak area are divided by 1.10^5 to simplify values comparison. 15

Figure S14. $\text{Ln}([\text{CS-A}]) = f(t)$ plots with the experimental errors to determine the rate coefficient of the desulfation reaction for CS-A. 16

Figure S15. EIC of deprotonated CS from HILIC-MS separation of CS-0 in green (m/z 378.0) and monosulfated CS in black (m/z 458.0) from a) CS standards, and after the reaction of 100 μM of CS-A with the CS 4-O-endosulfatase in presence of b) 0, c) 0.5, d) 1 and e) 2 equivalents of CS-C. 17

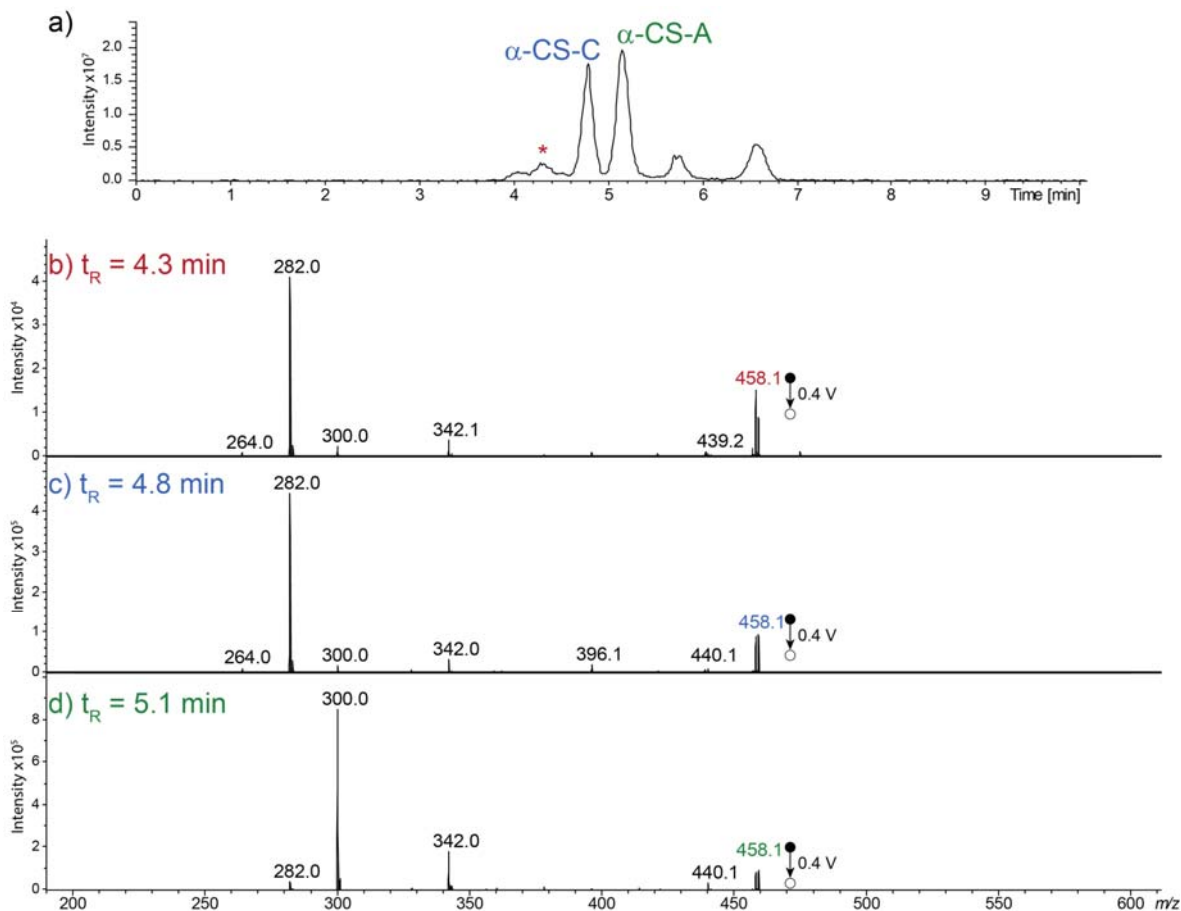


Figure S1. a) HILIC-MS/MS analysis of deprotonated monosulfated CS-A and CS-C using an isocratic elution of 26 % of solvent A using the following chromatographic conditions: Solvent A: 5 mM of ammonium formate adjusted at pH 3.75 with formic acid, Solvent B: MeCN, injection volume: 2 μ L of CS-A and CS-C at 10 μ M in H₂O/MeCN 25:75 flow rate: 300 μ L/min and column temperature: 30 $^{\circ}$ C. MS/MS spectra of the ion [CS-H]⁻ at m/z 458.1 are extracted from b) the peak tail at t_R 4.3 min, c) α -CS-C at t_R 4.8 min and d) α -CS-A at t_R 5.1 min.

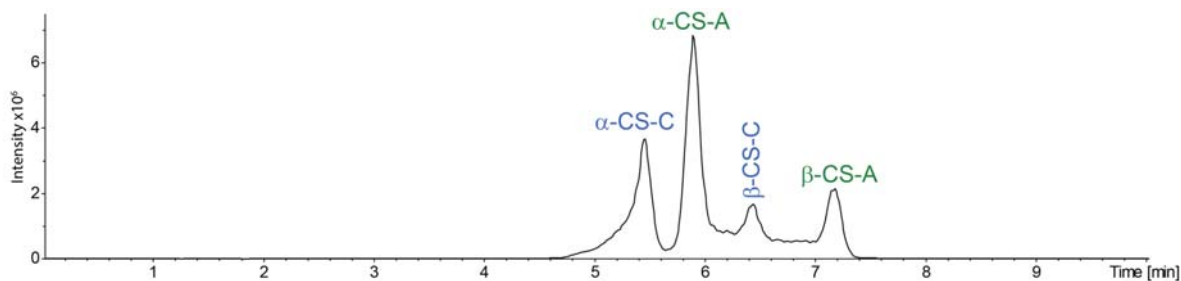


Figure S2. HILIC-MS/MS analysis of deprotonated monosulfated CS-A and CS-C using a linear gradient elution from 25 % to 30 % of solvent A in 10 minutes using the following chromatographic conditions: Solvent A: H₂O, Solvent B: MeCN, injection volume: 2 μ L of CS-A and CS-C at 10 μ M in H₂O/MeCN 25:75 flow rate: 300 μ L/min and column temperature: 30 $^{\circ}$ C.

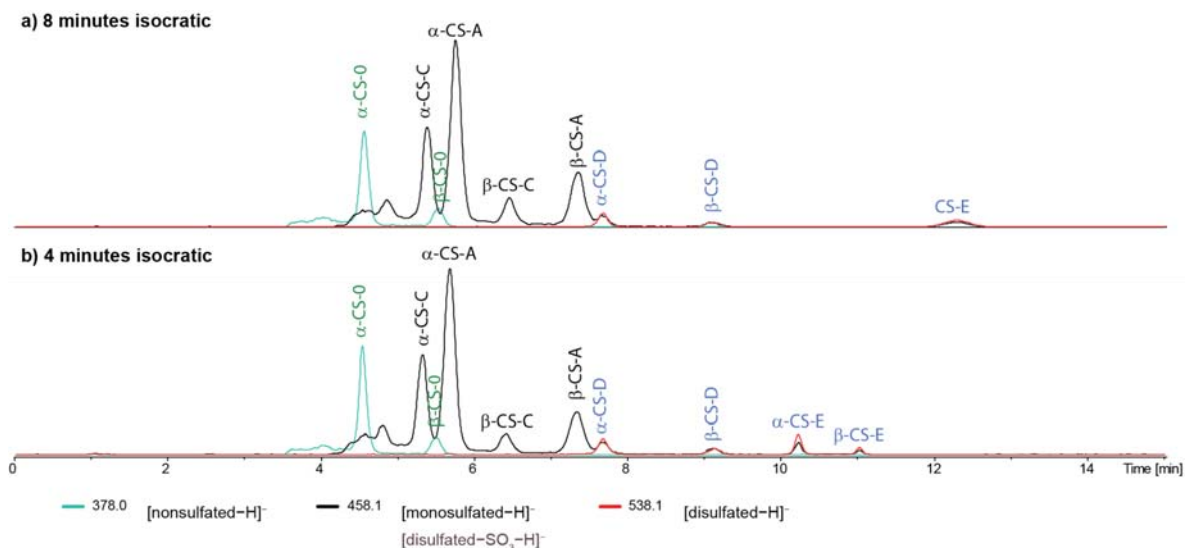


Figure S3. EIC of deprotonated CS from HILIC-MS separation using the following chromatographic conditions: Solvent A: 5 mM of ammonium formate adjusted at pH 3.75 with formic acid, Solvent B: MeCN, injection volume: 2 μ L, flow rate: 300 μ L/min and column temperature: 30 $^{\circ}$ C. An isocratic elution at 24% of A was performed for a) 8min or b) 4 min followed by a 2 min gradient to reach 35% of A for a total analysis time of 15 min. The injected mixture contains CS-0 at 10 μ M, CS-A at 4 μ M, CS-C at 2 μ M, CS-E and CS-D at 20 μ M in H₂O/MeCN 25:75. The color code of m/z values for each EIC is explain at the bottom of the Figure.

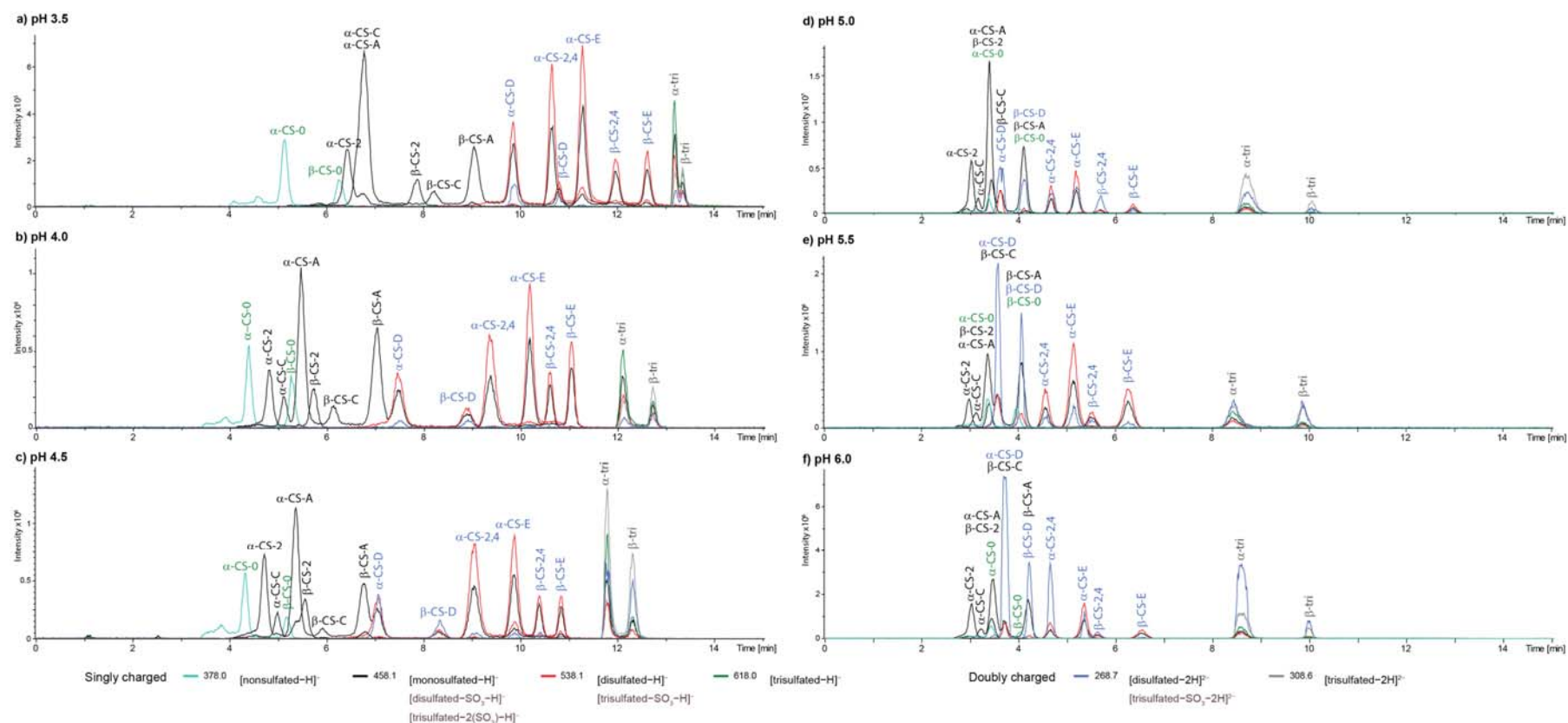


Figure S4. EIC of deprotonated CS from HILIC-MS separation using the following chromatographic conditions: Solvent A: 5 mM of ammonium formate adjusted at pH a) 3.5, b) 4.0, c) 4.5, d) 5.0 e) 5.5 and f) 6.0 with formic acid, Solvent B: MeCN, injection volume: 2 μ L, flow rate: 300 μ L/min and column temperature: 30 $^{\circ}$ C. The gradient is: 0 min (24%A), 4 min (24%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 (24%A) followed by 8 min of reconditioning for a total analysis time of 25 minutes. Each chromatogram is an overlay of two analysis containing different injected CS as followed: the first mixture contains CS-0 at 10 μ M, CS-A at 4 μ M, CS-C at 2 μ M, CS-E and CS-D at 20 μ M, and the second one CS-2 at 2 μ M, CS-2,4 at 20 μ M and the trisulfated CS at 50 μ M in H₂O/MeCN 25:75. The color code of m/z values for each EIC is explain at the bottom of the Figure with intact ions annotated in black and in-source MS desulfated species annotated in dark red.

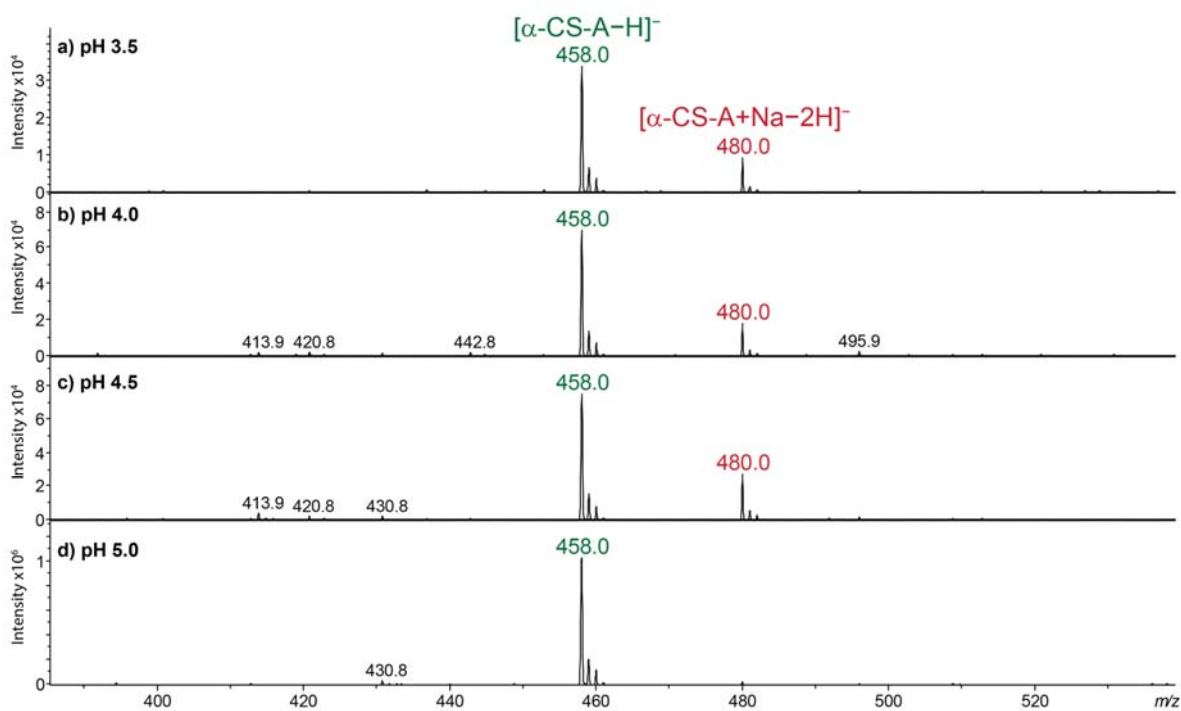


Figure S5. Extracted MS spectra of α -CS-A from HILIC-MS experiments presented in Figure 2 at pH a) 3.5 t_R 6.4-7.1 min, b) 4.0 t_R 5.4-5.7 min, c) 4.5 t_R 5.3-5.6 min and d) 5.0 t_R 3.3-3.5 min.

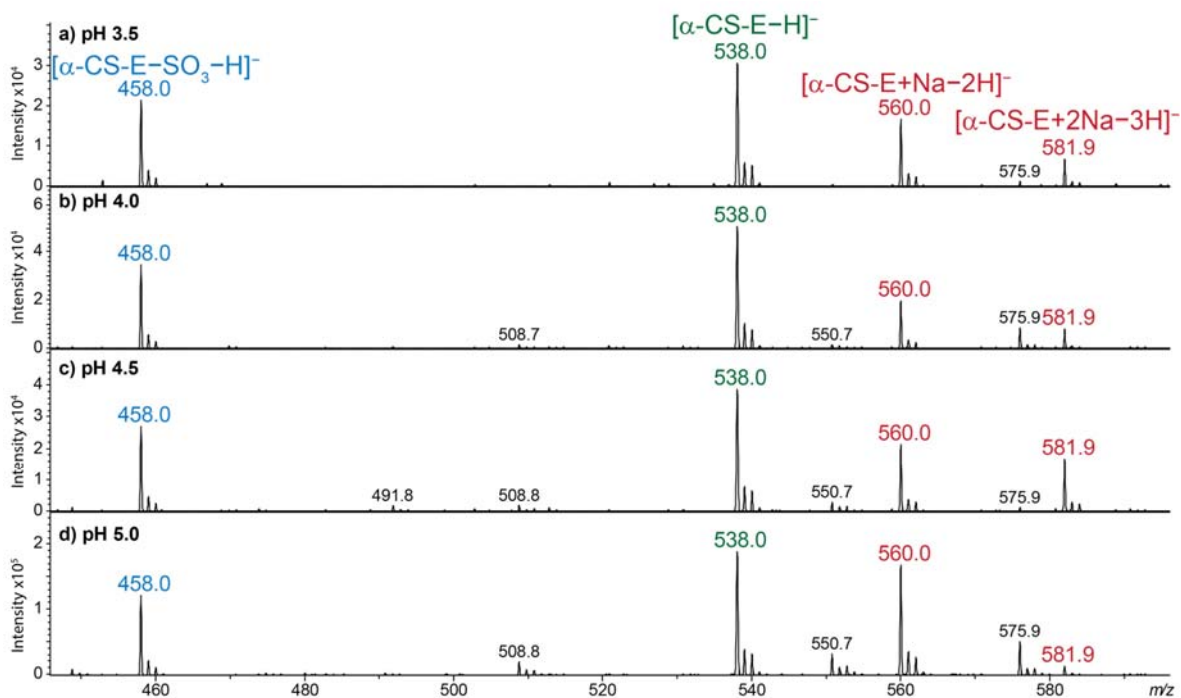


Figure S6. Extracted MS spectra of α -CS-E from HILIC-MS experiments presented in Figure 2 at pH a) 3.5 t_R 11.0-11.5 min, b) 4.0 t_R 10.0-10.4 min, c) 4.5 t_R 9.6-10.1 min and d) 5.0 t_R 5.0-5.4 min.

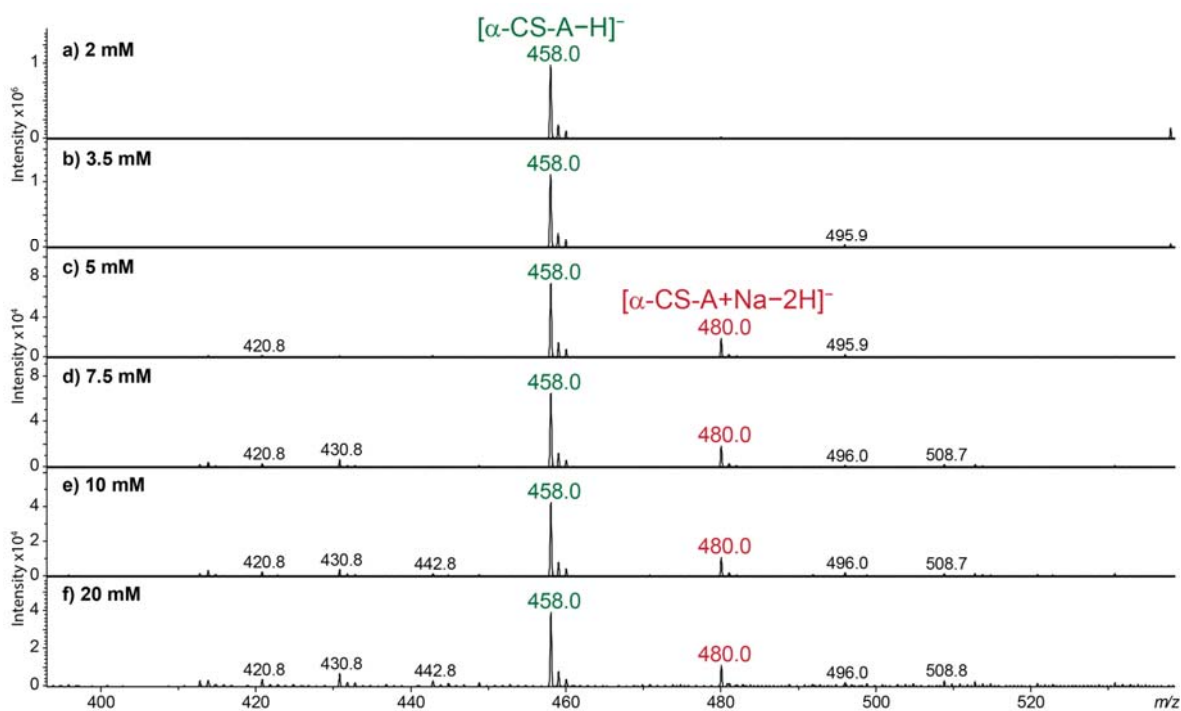


Figure S7. Extracted MS spectra of α -CS-A from HILIC-MS experiments presented in Figure 3 at ammonium formate concentrations of a) 2 mM t_R 2.2-2.5 min, b) 3.5 mM t_R 3.2-3.4 min, c) 5 mM t_R 5.3-5.6 min, d) 7.5 mM t_R 7.0-7.5 min, e) 10 mM t_R 8.8-9.2 min and f) 20 mM t_R 10.6-10.9 min. Note that in the case of a) the MS signal at m/z 458.0 is the sum of the contributions from α -CS-A, α -CS-C and β -CS-D and for b) the contributions from α -CS-A and α -CS-C.

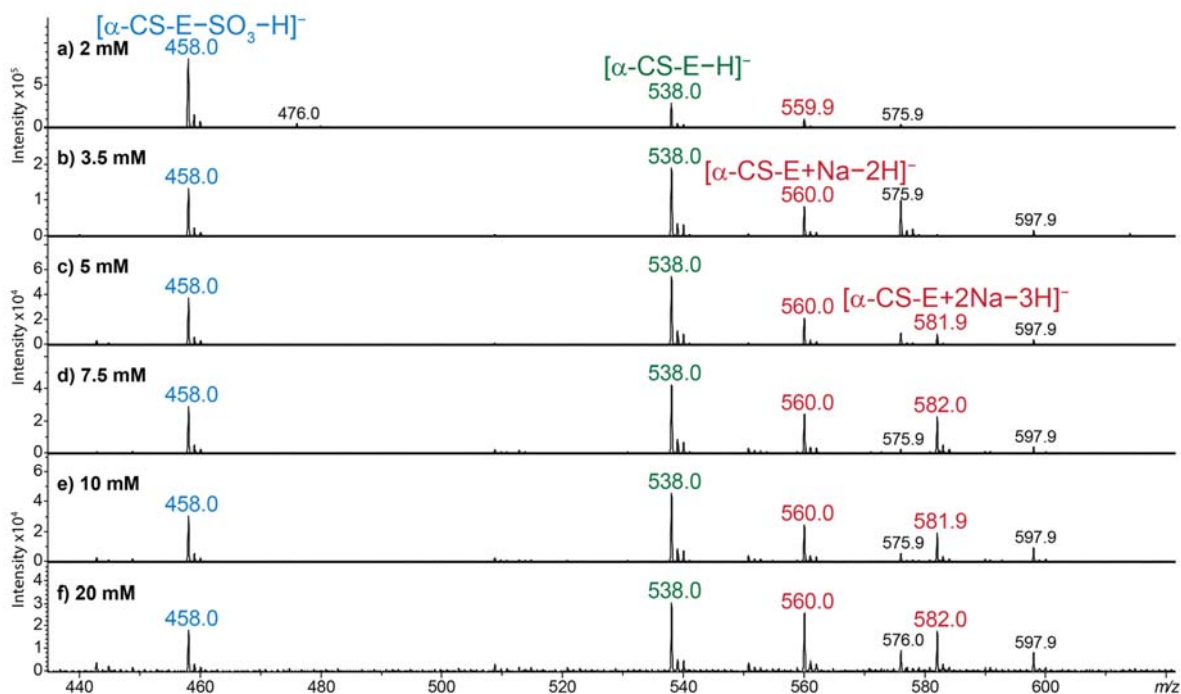


Figure S8. Extracted MS spectra of α -CS-E from HILIC-MS experiments presented in Figure 3 at ammonium formate concentrations of a) 2 mM t_R 2.7-2.9 min, b) 3.5 mM t_R 4.9-5.3 min, c) 5 mM t_R 10.0-10.4 min, d) 7.5 mM t_R 11.2-11.5 min, e) 10 mM t_R 12.3-12.6 min and f) 20 mM t_R 13.1-13.3 min. Note that in the case of a) the MS signal at m/z 458.0 is the sum of the contributions from α -CS-E, β -CS-A, and β -CS-C.

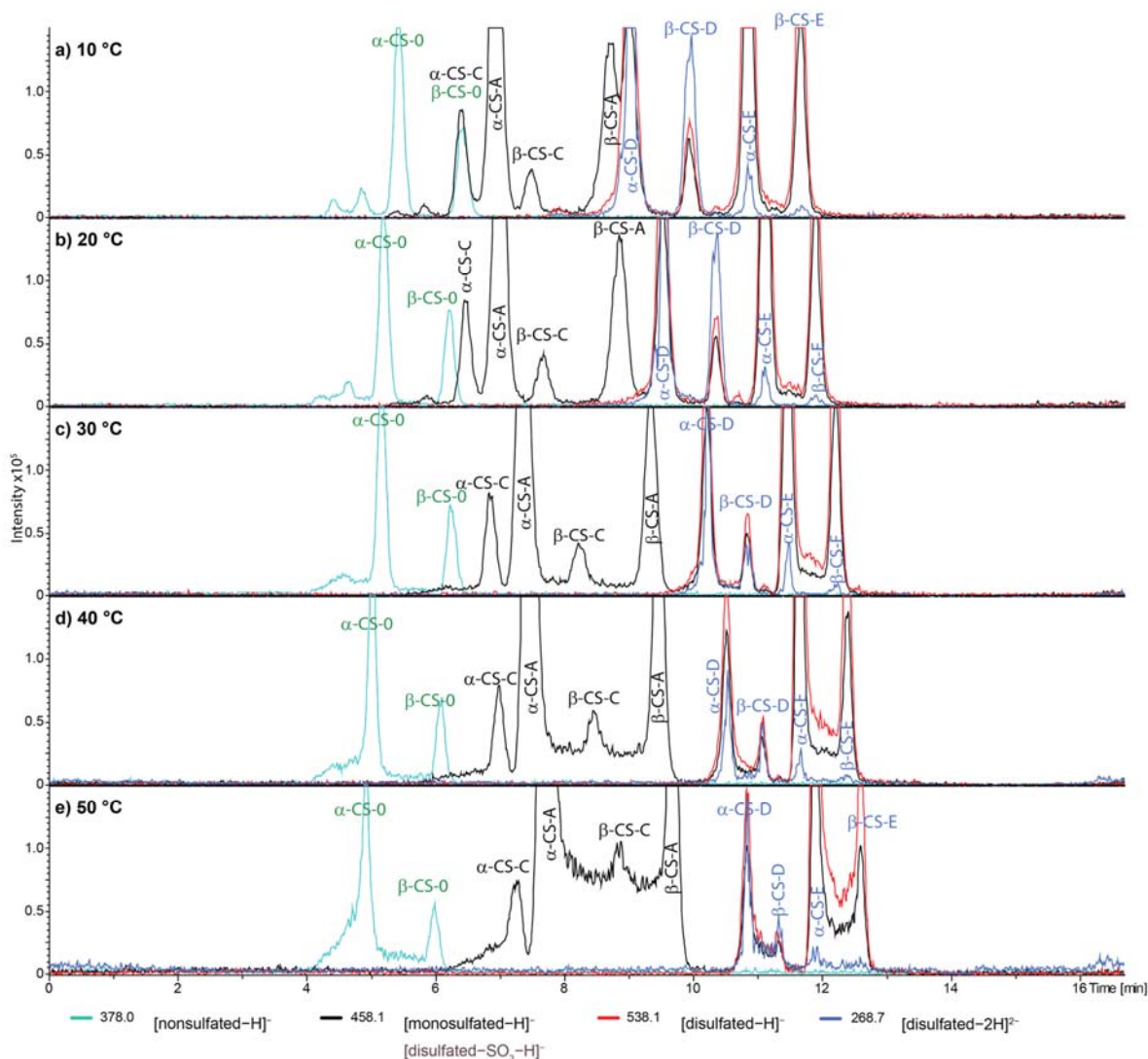


Figure S9. Intensity zooms from 0 to 150000 counts of EIC of deprotonated CS from HILIC-MS separation using the following chromatographic conditions: Solvent A: 7.5 mM of ammonium formate adjusted at pH 4.0, Solvent B: MeCN, injection volume: 2.5 μ L, flow rate: 300 μ L/min and column temperature: a) 10 °C, b) 20 °C, c) 30 °C, d) 40 °C and e) 50 °C. The gradient is: 0 min (24%A), 4 min (24%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 (24%A) followed by 8 min of reconditioning for a total analysis time of 25 minutes. Each chromatogram contains CS-0 at 10 μ M, CS-A at 4 μ M, CS-C at 2 μ M, CS-E and CS-D at 20 μ M in H₂O/MeCN 25:75. The color code of m/z values for each EIC is explain at the bottom of the Figure with intact ions annotated in black and in-source MS desulfated species annotated in dark red.

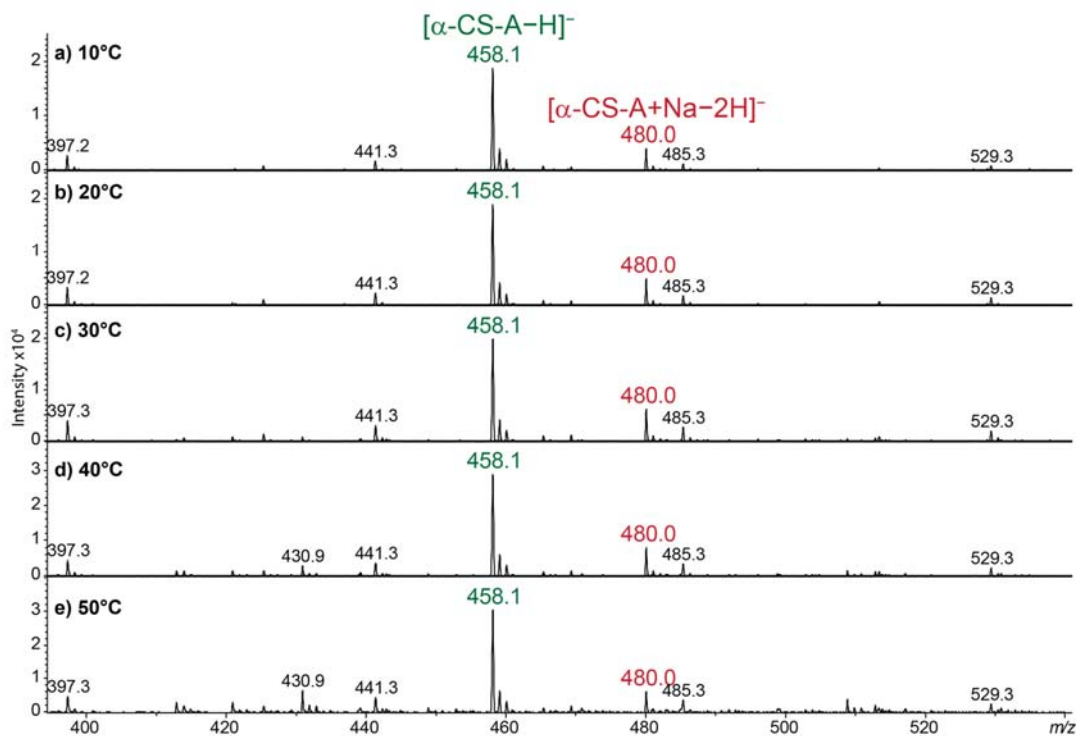


Figure S10. Extracted MS spectra of α -CS-A from HILIC-MS experiments presented in Figure 4 at a) 10 °C t_R 6.7-7.2 min, b) 20 °C t_R 6.8-7.2 min, c) 30 °C t_R 7.1-7.6 min d) 40 °C t_R 7.3-7.7 min and e) 50 °C t_R 7.5-8.0 min.

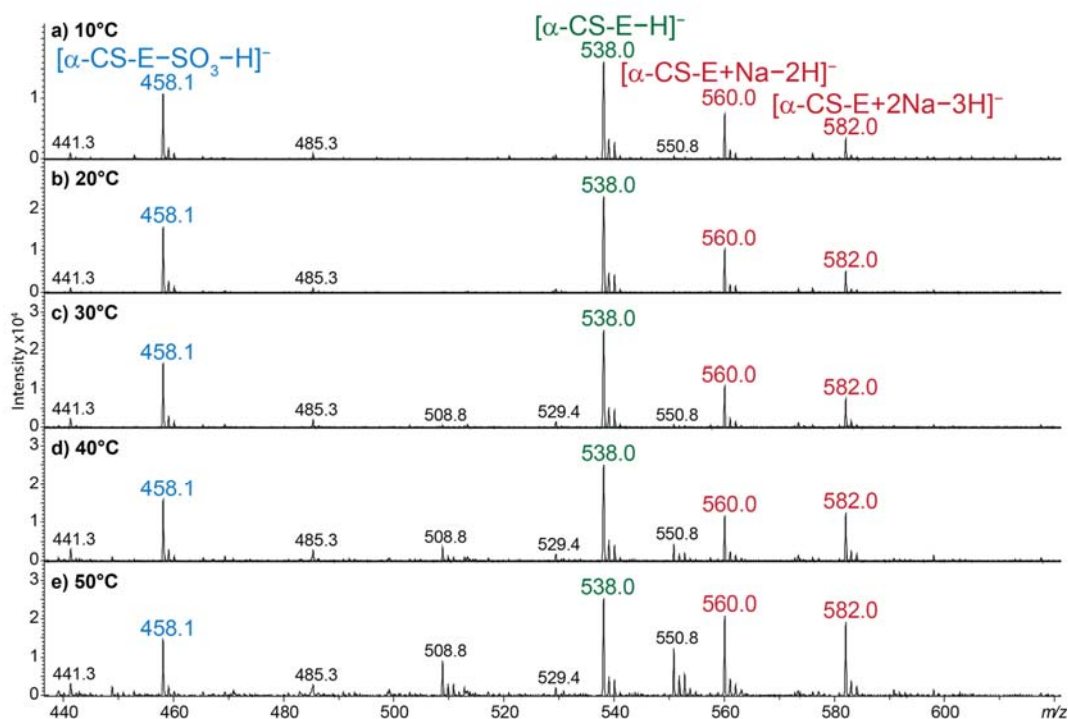


Figure S11. Extracted MS spectra of α -CS-E from HILIC-MS experiments presented in Figure 4 at a) 10 °C t_R 10.5-11.2 min, b) 20 °C t_R 10.9-11.3 min, c) 30 °C t_R 11.3-11.7 min d) 40 °C t_R 11.5-11.9 min and e) 50 °C t_R 11.8-12.1 min.

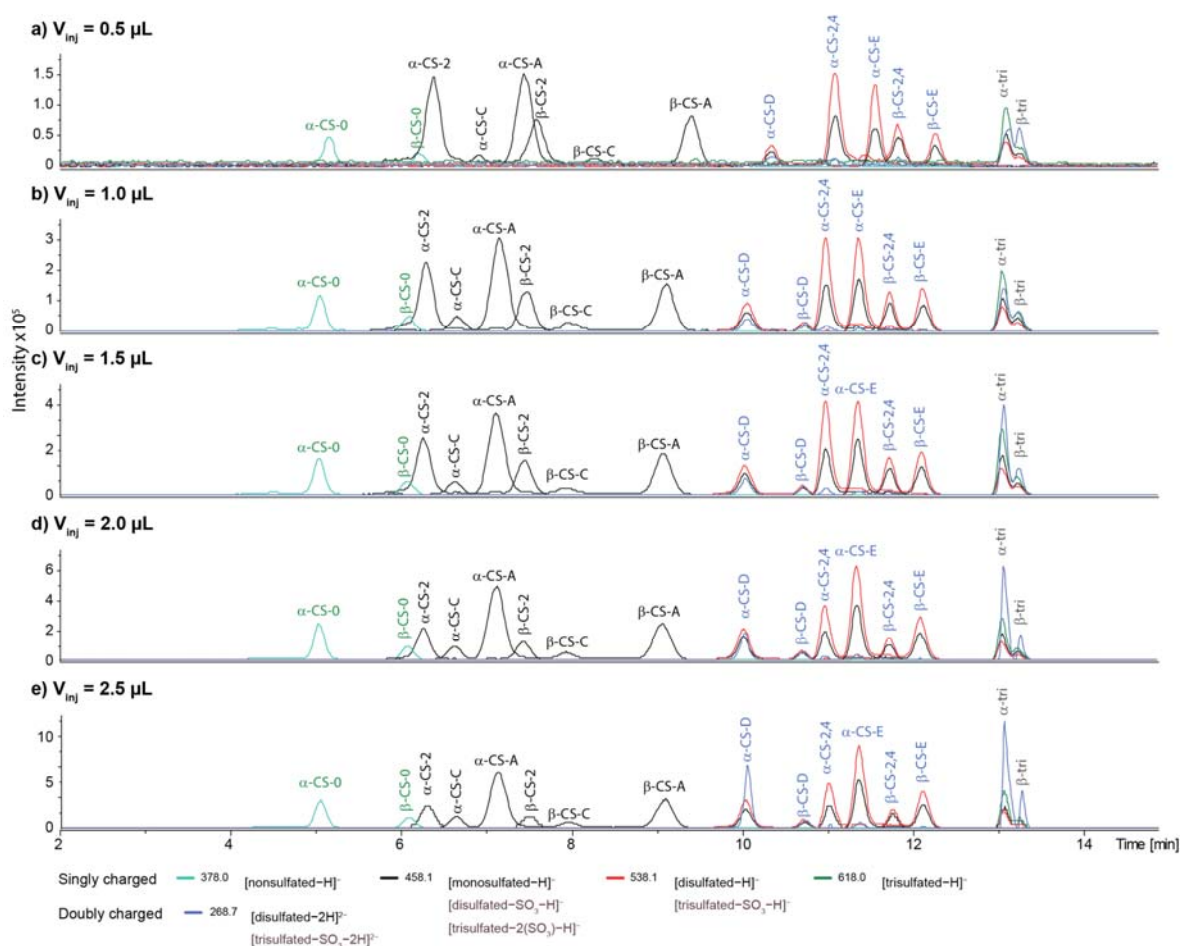


Figure S12. EIC of deprotonated CS from HILIC-MS separation using the following chromatographic conditions: Solvent A: 7.5 mM of ammonium formate adjusted at pH 4.0, Solvent B: MeCN, injection volume: a) 0.5 μL , b) 1.0 μL , c) 1.5 μL , d) 2.0 μL and d) 2.5 μL , flow rate: 300 $\mu\text{L}/\text{min}$ and column temperature: 20 $^{\circ}\text{C}$. The gradient is: 0 min (24%A), 4 min (24%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 (24%A) followed by 8 min of reconditioning for a total analysis time of 25 minutes. Each chromatogram is an overlay of two analysis containing different injected CS as followed: the first mixture contains CS-0 at 10 μM , CS-A at 4 μM , CS-C at 2 μM , CS-E and CS-D at 20 μM , and the second one CS-2 at 2 μM , CS-2,4 at 20 μM and the trisulfated CS at 50 μM in $\text{H}_2\text{O}/\text{MeCN}$ 25:75. The color code of m/z values for each EIC is explain at the bottom of the Figure with intact ions annotated in black and in-source MS desulfated species annotated in dark red.

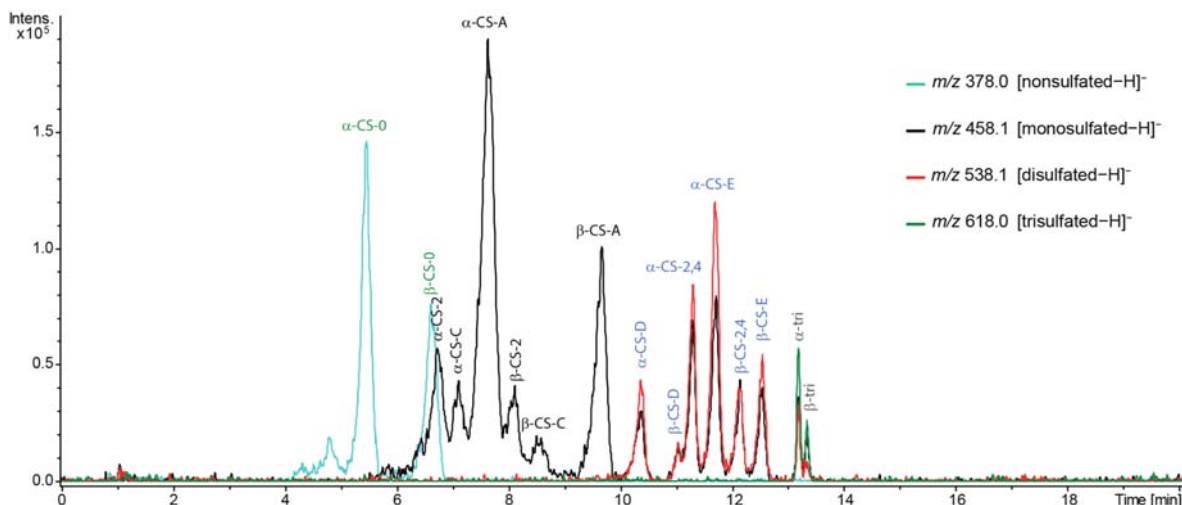


Figure S13. EIC of deprotonated CS from HILIC-MS separation using the following chromatographic conditions: Solvent A: 7.5 mM of ammonium formate adjusted at pH 4.0, Solvent B: MeCN, injection volume: 2.0 μ L, flow rate: 300 μ L/min and column temperature: 20 $^{\circ}$ C. The gradient is: 0 min (24%A), 4 min (24%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 (24%A) followed by 8 min of reconditioning for a total analysis time of 25 minutes. The CS sample contains CS-0 at 65 μ M, CS-A at 2.6 μ M, CS-C at 0.65 μ M, CS-E at 20 μ M, CS-D at 0.5 μ M, CS-2 at 0.35 μ M, CS-2,4 at 0.4 μ M and the trisulfated CS at 750 μ M in H₂O/MeCN 25:75. The color code of m/z values for each EIC is explain at the right of the Figure.

Table S1. Limit of detection observed for HILIC-MS analysis with the injection of 2 μ L of isomer mixtures in H₂O/MeCN 25:75

CS sample	LOD (pmol)	CS sample	LOD (pmol)
CS-0	14	CS-D	0.23
CS-2	0.53	CS-2,4	1.7
CS-A	0.55	CS-E	4.2
CS-C	0.27	CS-trisulfated	1180

Table S2. Comparison with other methods reported in the literature related to the separation of CS disaccharides. The list of publications is sorted by year.

CS disaccharide	Analytical method	Detection limit*	Run time	Treatment time	Separation efficiency	Reference
CS-0, CS-C, CS-A, CS-2,4 and CS-D	SAX-UV	1.7 µg/mL	45 min	-	baseline resolved	(1, 2)
CS-0, CS-C and CS-A	PGC-MS	0.01 ng	60 min	-	baseline resolved	(3)
CS-0, CS-C, CS-A, CS-2,4, CS-D and CS-E	SAX-UV	n/d	60 min	-	baseline resolved	(4)
CS-0, CS-C, CS-A, CS-2,4, CS-D, CS-E and trisulfated CS	HILIC-FLD	n/d	60 min	4 hours	baseline resolved	(5)
CS-A	PGC-MS	< 2 µg/mL	7.1 min	-	n/d	(6)
CS-0, CS-C, CS-A and CS-E	HILIC-MS	n/d	29 min	-	$\alpha < 1.5$	(7)
CS-C and CS-A	RPIP-MS	< 20 ng/mL	9 min	-	co-eluted	(8)
CS-C CS-2 and CS-A	HILIC-MS	3 ng/mL	8 min	-	$\alpha < 1.5$	(9)
CS-0, CS-C, CS-A, CS-2,4, CS-D, CS-E and trisulfated CS	HILIC-MS	0.3 ng	25 min	-	baseline resolved	This work

*Observed for mono-sulfated disaccharides

SAX: Strong anion exchange chromatography, PGC: Porous graphitic carbon, FLD: Fluorescence detector, RPIP: Reversed phase ion pairing, α : separation factor.

Table S3. Retention times, peak widths and peak area for singly- and doubly charged CS analogues obtained from HILIC-MS analysis presented on Figure 2. t_R and peak widths are in minutes and peak area are divided by 1.10^5 to simplify values comparison.

	α CS-0	β CS-0	α			β			α			β			α	β
	CS-2	CS-C	CS-A	CS-2	CS-C	CS-A	CS-D	CS-2,4	CS-E	CS-D	CS-2,4	CS-E	CS-tri	CS-tri		
pH 3.5																
t_R	5.2	6.3	6.4	6.8	7.8	8.2	9.0	9.8	10.6	11.3	10.8	11.9	12.6	13.2	13.3	
Peak width	0.4	0.4	0.5	0.8	0.5	0.4	0.5	0.5	0.5	0.5	0.5	0.4	0.4	0.2	0.2	
Area $[M-H]^-$	25	12	24	95	13	5.3	32	35	54	63	8.2	20	21	22	4.1	
Area $[M-2H]^{2-}$								9.5	ND	0.55	4.5	ND	ND	7.9	9.0	
pH 4.0																
t_R	4.4	5.3	4.8	5.1	5.5	5.7	6.1	7.0	7.4	9.3	10.2	8.9	10.6	11.0	12.1	12.7
Peak width	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.5	0.6	0.6	0.4	0.4	0.4	0.4	0.5	0.3
Area $[M-H]^-$	40	27	33	16	105	25	13	74	43	79	90	16	29	47	46	12
Area $[M-2H]^{2-}$									5.3	ND	1.1	11	ND	0.5	21	23
pH 4.5																
t_R	4.3	5.2	4.7	5.0	5.4	5.5	5.9	6.8	7.0	9.0	9.8	8.3	10.4	10.8	11.8	12.3
Peak width	0.4	0.4	0.4	0.3	0.4	0.4	0.3	0.4	0.6	0.5	0.6	0.6	0.3	0.3	0.4	0.4
Area $[M-H]^-$	44	16	59	17	109	31	6.2	59	44	110	97	10	29	27	59	16
Area $[M-2H]^{2-}$									39	7.6	5.6	17	4.8	1.8	116	74
pH 5.0																
t_R	3.4	4.0	3.0	3.2	3.4	3.4	3.6	4.1	3.6	4.7	5.2	4.1	5.7	6.4	8.7	10
Peak width	0.2	0.3	0.5	0.3	0.2	0.3	0.2	0.2	0.3	0.5	0.6	0.3	0.5	0.5	0.5	0.5
Area $[M-H]^-$	100	48	414	65	1040	265	ND	ND	154	224	388	33	39	99	167	17
Area $[M-2H]^{2-}$									459	216	281	309	165	29	748	126
pH 5.5																
t_R	3.4	4.0	3.0	3.2	3.4	3.4	3.7	4.2	3.7	4.6	5.3	4.1	5.5	6.4	8.4	9.8
Peak width	0.3	0.4	0.3	0.3	0.3	0.3	0.2	0.4	0.5	0.4	0.5	0.3	0.5	0.5	0.7	0.5
Area $[M-H]^-$	31	23	27	10	80	24	ND	ND	37	48	139	17	21	76	35	10
Area $[M-2H]^{2-}$									142	12	27	77	9.6	11	51	33
pH 6.0																
t_R	3.4	4.0	3.0	3.2	3.5	3.4	3.7	4.2	3.7	4.6	5.4	4.2	5.6	6.5	8.6	10.0
Peak width	0.3	0.4	0.4	0.2	0.3	0.3	0.2	0.4	0.3	0.5	0.5	0.3	0.5	0.5	0.7	0.4
Area $[M-H]^-$	42	23	116	19	201	73	ND	ND	43	66	148	8.3	18	46	76	9.8
Area $[M-2H]^{2-}$									565	213	59	209	25	3.5	205	39

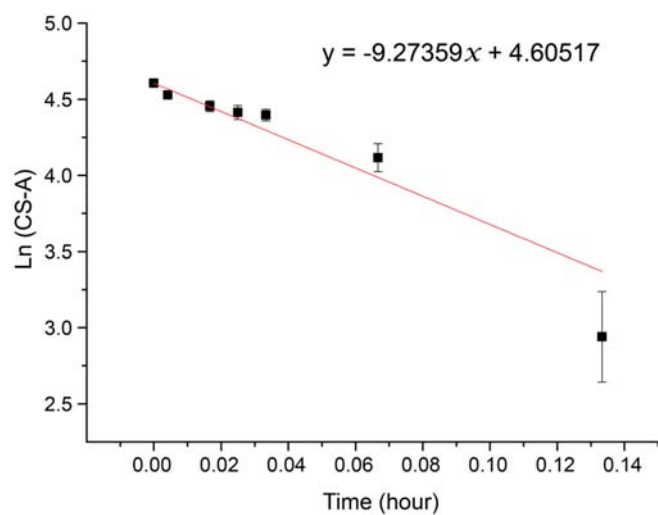


Figure S14. $\text{Ln}([\text{CS-A}]) = f(t)$ plots with the experimental errors to determine the rate coefficient of the desulfation reaction for CS-A.

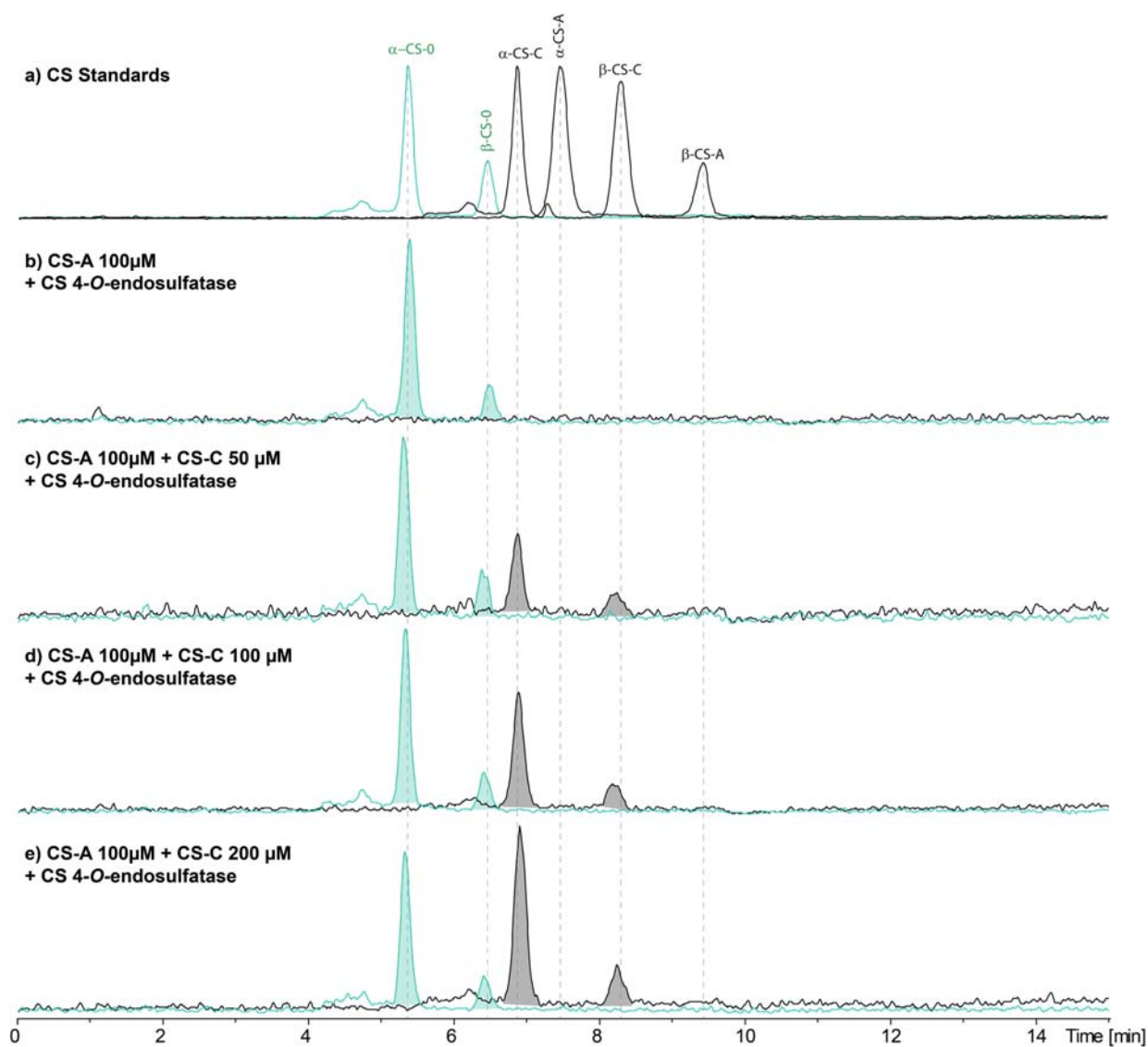


Figure S15. EIC of deprotonated CS from HILIC-MS separation of CS-0 in green (m/z 378.0) and monosulfated CS in black (m/z 458.0) from a) CS standards, and after the reaction of 100 μ M of CS-A with the CS 4-O-endosulfatase in presence of b) 0, c) 0.5, d) 1 and e) 2 equivalents of CS-C.

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