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1 **Off-line coupling of capillary isotachopheresis separation to IRMPD spectroscopy for**
2 **glycosaminoglycans analysis: application to the chondroitin sulfate disaccharides model solutes**

3

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16 microscale separation

17

18

19 **Abstract**

20 Glycans analysis is challenging due to their immense structural diversity. Isotachopheresis was
21 investigated as separation method for the purification of isobaric sulfated disaccharides prior to their
22 characterization by Mass Spectrometry (MS) and tunable IR multiple photon dissociation (IRMPD).
23 This proof of feasibility study was applied to the separation and characterization of chondroitin
24 sulfate (CS) disaccharides. ITP separation conditions were optimized. Separation starts using a 2.5
25 mM chloride ions and 10 mM glycine at pH 3.2 solution as leading electrolyte and a terminating
26 electrolyte composed of formic acid 2.5 mM and glycine 10 mM at pH 3.5. The CS disaccharides
27 sample were prepared in the terminating electrolyte. The length of injection was also investigated in
28 order to create longer plateau-like bands of pure solutes. This strategy was helpful for collecting
29 fraction at such microseparation scale. Indeed, capillary ITP affords the injection of few tens of
30 nanoliter of sample. Fractionation of the CS disaccharides mixture in isolated ITP bands and
31 collection of solutes were successfully done using a HPC coated fused silica capillary of 1m-length
32 and 75 μm of internal diameter. Collected fractions in a final of volume 10 μL were analyzed by CZE,
33 tandem MS and IRMPD spectroscopy. The purity of each fraction is higher than 90% and is well-
34 adapted to IRMPD characterization.

35

36 **1. Introduction**

37 A general trend in analytical chemistry concerns the development of original approaches opening
38 new perspectives in the context of complex molecules analysis and/or characterization. Such
39 analyses are driven, for example, by the pressing demand of biologists investigating biomolecules
40 and bio-molecular interactions as well as pharmacists qualifying new biologic drugs that enter the
41 pharma market, to mention just a few. Dealing with complex samples, oligosaccharides analysis (also
42 referred to as carbohydrates or glycans) are very exciting due to their inherent structural complexity
43 and importance in biological and physiological processes through the regulation of their various
44 protein partners. [1,2] Glycosaminoglycans (GAGs) are of particular importance in this context. Their
45 presence on the cell membranes and in the extracellular matrix plays a critical role in the intercellular
46 communication in organisms [3,4]. The GAG family, composed of 5 members: hyaluronan (HA),
47 heparan sulfate (HS; including the highly sulfated form heparin), chondroitin sulfate (CS), dermatan
48 sulfate (DS) and keratin sulfate (KS), are linear, highly charged, polysaccharides, composed of
49 repeating disaccharides units. Understanding the structure of GAGs *via* building blocks composition,
50 blocks connectivity, stereochemical configuration and sulfation patterns, represents a major
51 analytical challenge.[5] Standard approaches for GAGs characterization, rely on depolymerization

52 reactions (chemical or enzymatic reactions) followed by fractionation for their further structural
53 identification by sensitive mass spectrometry and nuclear magnetic resonance experiments [6,7].
54 Such bottom-up approaches benefit from optimal fractionation methods. Chromatographic methods
55 such as size-exclusion chromatography (SEC) and anion exchange chromatography (AEX) as well as
56 capillary electrophoresis are widely used and even coupled to one another for the separation of
57 heterogeneous mixtures before their characterization. Each have their pros and cons and there is still
58 great way of improvement in the sample preparation [6]. Underivatized oligosaccharides are
59 hydrophilic species that can be separated in ion exchange (IEX), ion-pair chromatography (IPC) and
60 HILIC modes. With respect to mass spectrometric detection, HILIC is usually preferred as this
61 chromatographic mode, compared to IEX and IPC, does not suffer from ionization suppression effects
62 due to mobile phase components. But this separation mode has not always been effective in
63 resolving isomeric oligosaccharides [6,8]. With regard to sample volume, standard chromatographic
64 autosamplers require often few tens to hundreds microliters of sample volume even when low
65 volume of sample (i.e. 1 μ L) is injected. Capillary electrophoresis is an alternative and powerful
66 separation technique for and ionized compounds. Its separation mechanism depends on the ratio of
67 the ionic charge to the hydrodynamic radius that determines the effective mobility of ionic species in
68 a given background electrolyte. The high peak efficiencies in CE lead to very narrow peaks (a few
69 seconds) requiring high sampling rate detection systems. Isotachopheresis (ITP) is a powerful but
70 often overlooked electrokinetic separation technique because its unique plateau-like signal signature
71 may be disturbing for chromatographers accustomed to work with peak signals. This technique is
72 amenable to work with small volume of crude samples [9] (no additional sample preparation needed
73 before analysis) and produces pure solute bands (without impurities like salts or organic modifiers
74 that impede subsequent MS characterization). Capillary ITP was successfully applied to the
75 preconcentration and microseparation of heparin and heparan sulfate samples on-line coupled to
76 NMR detection via a 1-mm microcoil probes wrapped around the fused silica capillary [10,11]. The
77 plateau-like signals of ITP was well-adapted to such specific microcoil NMR probes and the relatively
78 long acquisition time required for NMR (1.4 s). IRMPD spectroscopy benefits from the sensitivity of
79 Mass Spectrometry and the structural resolution of IR spectroscopy: it is a powerful tool to deduce
80 detailed structural information on the monosaccharide content and stereochemistry of the glycosidic
81 linkages [10–12]. However, the acquisition time required to obtain an IR spectrum using this
82 technique is several minutes, which is very long compared to standard in-line detectors of separation
83 methods. Therefore, the online coupling of IRMPD with peak signal analytical separation methods is
84 a tremendous challenge. [16]

85 In this context, we propose capillary ITP as microseparation technique prior to IRMPD
86 characterization. Capillary ITP was investigated due to its plateau-like signals that allow more time
87 for relatively long acquisition time for the detection system or for the collection of pure bands of
88 solutes for off-line coupling with sophisticated characterization methods at the microscale. The proof
89 of feasibility was demonstrated with the separation of two chondroitin sulfate disaccharide isomers,
90 namely CSA ($\Delta U(1-3)\text{GalNAc},4\text{S}$) and CSC ($\Delta U(1-3)\text{GalNAc},6\text{S}$). Chondroitin Sulfates like other GAGs
91 display substantial diversity within tissues and molecular diversity across different tissues and over
92 developmental time [3] and are involved in controlling neurogenesis, neuronal plasticity and
93 regeneration after injury to the central nervous system for example. In addition, it was demonstrated
94 that the specificity of interaction of CS with proteins depends on the level and distribution of
95 sulfation [4]. The ITP conditions were optimized for the separation of these two CS disaccharides
96 isomers. Collected fractions were then characterized by CZE to determine the purity yield. Finally, the
97 collected fractions were analyzed by IRMPD by nanospray infusion, demonstrating the effectiveness
98 of such coupling at the microscale.

99

100 **2. Materials and methods**

101

102 *2.1. Reagents and chemicals*

103 Methanol (HPLC grade), triethylamine (TEA), dipotassium hydrogen phosphate (K_2HPO_4), O-
104 phosphoric acid, sodium hydroxide (NaOH), acetic acid, hydroxypropylcellulose (HPC, Mw ~100,000),
105 hydrochloric acid, glycine, formic acid were purchased from Sigma Aldrich (France). Chondroitin
106 sulfate disaccharide standards $\Delta U(1-3)\text{GalNAc},4\text{S}$ (CSA) and $\Delta U(1-3)\text{GalNAc},6\text{S}$ (CSC) were purchased
107 from Iduron (Alderley Edge, UK). Stock solutions of each chondroitin sulfate disaccharides (CSA and
108 CSC) were prepared in ultra-pure water at 100 mM. Acetic acid (AcOH) was purchased from Riedel-de
109 Haënn (Riedel-de Haënn, Seelze, Germany). All aqueous solutions were prepared using >18 M Ω -cm
110 water (Millipore, Molsheim, France). Polyimide (TSP)- coated fused-silica capillaries were purchased
111 from Polymicro Technologies (Polymicro Technologies, Arizona, USA).

112 *2.2. HPC Coating protocols*

113 Capillary liquid manipulations were controlled by pressure using the microfluidic flow controller
114 MFCS-100 system (Fluigent, France). This system is useful to fill, wash and dry capillaries.

115 The HPC coating procedure follows the protocol proposed by Koval et al [17]. Briefly, a 5 % (w/v) HPC
116 aqueous solution was flushed through 1 m of polyimide coated fused silica capillary (75 μm ID and
117 375 OD μm) at 5 bars pressure. The capillary was placed in a gas chromatography oven (HP 5890
118 series II GC system) heated at 60°C, and flushed with nitrogen at 1 bar pressure, for 10 minutes. The
119 temperature was increased up to 140°C with a 5°C/min ramp and kept at 140°C for 20 min.

120 2.3. ITP experiments

121 ITP experiments were carried out on an Agilent HP3DCE instrument (Agilent Technologies,
122 Waldbronn, Germany) equipped with a diode array detector (detection at 230 nm).

123 Separations were done in a 35-cm long HPC coated fused silica capillary (75- μm id / 365 μm od).

124 Otherwise stated in the text, ITP experiments were performed using a pH 3.2 leading electrolyte
125 composed of HCl 2.5 mM, glycine 10mM and a pH 3.5 terminating electrolyte composed of formic
126 acid 2.5 mM and glycine 10 mM. Mixtures of CSA and CSC at 1 mM each were prepared in the
127 terminal electrolyte. Injections were done under hydrodynamic pressure mode.

128 2.4. CZE experiments

129 Capillary zone electrophoresis experiments were carried out on an Agilent 7100 CE instrument
130 (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector. Separations
131 were done in a 35-cm length HPC coated fused silica capillary (75- μm id / 365 μm od). Prior to each
132 injection, the capillary was equilibrated by flushing with the background electrolyte (formic acid 50
133 mM and glycine 100 mM pH 3.24) for 30 sec. Samples were injected under hydrodynamic mode by
134 applying a 5 mbar pressure for 1s. Separation was done at - 20 kV.

135 2.5 Mass Spectrometry and IRMPD spectroscopy

136 Off-line coupling experiments were realized by injecting 22 nl of the 1 mM chondroitin sulfate
137 disaccharides mixture (corresponding to 44 pmol of each chondroitin sulfate disaccharides). Bands
138 collection of CSA and CSC after ITP separation were done in 10 μL H₂O and diluted in 10 μL MeOH. MS
139 analysis was carried out in a commercial mass spectrometer (ThermoFinnigan LTQ XL) equipped with
140 a nanospray ionization source set in negative mode (production of deprotonated ions) and a linear
141 ion trap analyzer. Tandem MS experiments are performed via collision induced dissociation (CID) in a
142 helium collision gas. A similar setup used for IRMPD analysis was previously described by Schindler *et*
143 *al.*[18]. In brief, the mass spectrometer is modified to allow irradiation of the trapped ions by a laser
144 beam produced by a YAG-pumped tunable IR OPO/OPA. This enables in situ gas-phase IRMPD
145 (InfraRed Multiple Photon Dissociation) spectroscopy of the trapped ions. Excitation of a vibrational

146 mode by the laser induces photofragmentation, which is detected by mass spectrometry. The photo-
147 fragmentation yield is calculated using the formula: $IRMPD\ yield = -\log[I_{parent} \div (I_{parent} + I_{fragment})]$,
148 where I_{parent} is the intensity of the parent ion and $I_{fragment}$ is the total intensity of the fragments
149 detected after laser excitation. The fragmentation yield is then monitored as a function of the
150 wavenumber to retrieve the absorption spectrum of the analyte. The spectral range 2700-3700 cm^{-1}
151 was scanned at 1 cm^{-1}/s , which correspond to an acquisition time of 17 minutes. In these conditions
152 mass-selected ions were isolated and irradiated for 500 ms per photofragmentation MS spectrum
153 and MS spectra were not averaged. The reference IRMPD spectra of pure CSC and CSA standards
154 were measured on a different setup, as described in [13].

155

156 **3. Results**

157 *3.1. Determination of the optimal ITP separation conditions*

158 *- Choice of the electrolytes (pH and nature)*

159 As for other differential migration methods, the condition for ITP separation is that the ionized
160 solutes in the sample will separate whenever their migration rates in the mixed state are different.

161 Figure 1

162 The two chondroitin sulfate disaccharides will better separate if the pH in the mixing zone increases
163 the difference in their respective effective mobility. The electrophoretic mobility of the two
164 chondroitin sulfate disaccharides was thus first investigated in Capillary Zone Electrophoresis in acidic
165 conditions due to the weak acidic properties of the CS disaccharides (carboxylic acid and sulfate
166 groups)[19]. Figure 1 presents the relative electrophoretic mobility difference between the two CS
167 disaccharides at a pH ranging from 2.18 to 4.06. The largest relative electrophoretic mobility
168 difference was observed between pH 3.21 to 3.45. This pH range was thus considered optimal for the
169 ITP separation and was fixed for the preparation of the leading electrolyte and terminal electrolyte.

170 A high effective mobility constituent such as chloride (strong ionic species commonly used in ITP)
171 must be used as leader. The pH of the leading electrolyte was adjusted using glycine to pH 3.2. To
172 cope with the objective to form long “plateau-like” bands of pure solute, low chloride leading
173 electrolyte concentrations were investigated (ranging from 1 to 10 mM). The lowest concentration
174 that can be used is however limited by the minimal current that can be carried by the electrolyte and
175 controlled accurately (down to 1 μA) by the CE instrument. The best compromise for chloride
176 concentration versus applied current was 2.5 mM.

177 Considering the mobility of the chondroitin sulfate disaccharides in this pH range, formate anions
178 were used as constituent of the terminating electrolyte. Formic acid (pKa 3.75, formate
179 electrophoretic mobility $5.66 \cdot 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$)[20] has an effective mobility below pH 3.5 lower than
180 $2.18 \cdot 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$, which is a lower mobility value than the mobility of CS disaccharides.

181 The terminating electrolyte was thus prepared by adjusting the pH of the formic acid solution with
182 glycine as counter buffering and counter constituent.

183

184 - *Sample zone optimization*

185 *ITP separation of CS disaccharides injected individually.* In first approach, each chondroitin was
186 injected separately (Figure 2A, blue and orange plot) after conditioning the capillary with the leading
187 electrolyte (HCl 2.5 mM, Glycine (10 mM) pH 3.5). ITP separation was initiated by applying a constant
188 current between the inlet side of the capillary immersed in the terminating electrolyte and its outlet
189 sideimmersed in the leading electrolyte. While contactless conductivity was also tested, the
190 difference in conductivity of the two CS disaccharide zones is quite low. Hence it is preferred to
191 monitor the separation using UV detection. A plateau was detected at the optimal wavelength of 230
192 nm for the two CS disaccharides. The two CS disaccharides present a relatively different absorbance
193 that is useful to attribute the signal obtained to the chondroitin passing through the detector. The
194 two CS disaccharides were then injected successively (CSC was injected in first position). Two
195 plateaus were obtained and the UV signal for each chondroitin (Fig2A, purple plot) was comparable
196 to the signal observed for the individual ITP separated CS disaccharides. UV detection is useful for
197 monitoring electrokinetic separation using commercial instrument as the UV detection is done in-
198 capillary, the two CS disaccharides are detected at 230 nm and can be differentiated.

199 Figure 2

200 *ITP separation of CS disaccharides in mixture.* A mix of the two CS disaccharides solubilized in water
201 was injected according to the previous experimental conditions. Only one plateau was observed
202 (Figure 2B, grey plot). The two CS disaccharides were not separated in these experimental conditions.
203 The pH of the sample zone is also an important experimental parameter for the separation of ionic
204 species in ITP [21]. In order to trigger the separation at the early stage of the ITP separation, the pH
205 of the sample zone was adjusted to 3.2-3.5. The CS were diluted in the terminating electrolyte to
206 adjust the pH. The buffering of the sample zone separation allowed to successfully separate the CS
207 disaccharides (Fig 2B, dark plot). The ITP separation leads to the formation of two plateaus of
208 increasing UV absorbance signals. The more mobile CSC solute is detected first and CSA second.

209 3.2. Fractionation/collection of the ITP separated CS disaccharides

210 - *Optimization of the injection volume*

211 For in-line or off-line coupling, preparation of long band lengths of pure solute would be obviously
212 highly valuable. This is ever true at the miniaturized scale where collecting fraction is not easy. In
213 order to maximize the band width, a 1-m long HPC coated capillary (75 μm ID) was used. Indeed, the
214 length of capillary needed to separate efficiently the two CS disaccharides increases with the
215 quantity injected. A 1-m column is the longest capillary compatible with our instrumental set-up. This
216 is due to the low leading electrolyte concentration used. Indeed, it is difficult to maintain constant
217 current during the separation using longer capillary.

218 Figure 3

219 The sample volume was increased linearly by adjusting the injection pressure during the injection
220 step (Figure 3). Up to 30 mbar, the separation led to the formation of two plateaus. At 40 mbar, two
221 plateaus were still detected but the boundary between the two plateaus seemed less pronounced
222 and at higher injection pressure conditions (50 mbar) 3 plateaus were observed. This intermediate
223 plateau corresponds to a mixing zone of CS A and CS S between the two pure bands in formation. For
224 the following experiments, the injection conditions were set to 30 mbar for 10 sec. The linear velocity
225 of mobile phase at 30 mbar was estimated to be 0.05 cm/sec using leading electrolyte. This
226 corresponds to an injection plug length of 5 mm. After separation, the band lengths (plateau) were
227 about 12 mm (this was estimated by measuring the duration of plateaus under pressure after their
228 formation by ITP).

229 In the context of miniaturized separation methods, the optimization of the ITP separation conditions
230 led to increase the injection volume up to about 22 nL and the formation of individual bands of
231 solutes of about 12 mm highlighting the potential interest of such methods for sample preparation at
232 the miniaturized scale.

233

234 - *Fractionation of the two chondroitins and analysis of each fraction by CZE*

235 Figure 4

236 Under the optimized separation conditions, the two CS disaccharides were well-separated and
237 formed two plateaus of more than 1 cm length. Fractionation of the sample after separation was
238 conducted by collection of a fraction of each plateau in 10 μL of water. From the experimental point
239 of view, the ITP separation was stopped before complete elution of the CS disaccharides from the

240 capillary. The outlet vial was then replaced by a vial containing a low volume conical insert filled with
241 10 μ L of water. The fraction of pure solute selected was eluted under hydrodynamic flow mode (50
242 mbar). The two fractions were recovered using two different ITP separations. The hydrodynamic flow
243 is convenient to accurately fractionate a part of the band, however it was supposed that during this
244 step, all interfaces (CSC/CSA, CSA or CSC/electrolyte) undergo band diffusion. This is expected to mix
245 the two separated chondroitins and thus affect their purity. Purity of each collected fraction was
246 assessed by capillary zone electrophoresis (Figure 4 B). Regarding the electrophoregram obtained
247 using a chondroitins standard solution, two pairs of chondroitin peaks were detected. Each pair was
248 attributed to chondroitin sulfate anomer forms. The first peak pattern corresponds to the CSC
249 chondroitin sulfate and the second one to the CSA. Each collected fraction presents a major
250 chondroitin sulfate isomer. Fraction 1 correspond to the CSC isoform with a purity of 89.6% and
251 fraction 2 corresponds to the CSA isoform with a purity of 94.6 %. The isolation of oligosaccharide
252 components for structural investigations must have a purity greater that 80% to 90 %. Moreover,
253 organic impurities or salts that can impede the characterization must also be minimized [6]. These
254 are generally observed in HPLC separations for example. ITP seems well-adapted for the separation
255 and purification of CS samples.

256

257 3.3. IRMPD analysis

258 Each collected fraction was characterized by IRMPD spectroscopy. The two disaccharides (CSA and
259 CSC) derived from chondroitin sulfate are challenging to characterize as they possess two
260 neighboring sites of sulfation on the *N*-acetyl galactosamine that is O6 and O4. Infrared multiple
261 photon dissociation spectroscopy (IRMPD) was recently applied to the characterization of isomeric
262 glycosaminoglycan disaccharides from heparins and chondroitin sources and has shown great
263 potential as a novel structural tool for carbohydrates [13]. However, this powerful technique requires
264 relatively long time of acquisition, which is not convenient for coupling with most separation
265 methods [16,22]

266 Figure 5

267 The MS/MS spectra of CSA and CSC are shown in the top layer of Figure 5. The singly deprotonated
268 precursor ions are observed at m/z 458. CSA features a main fragment at m/z 300, which
269 corresponds to the sulfated GalNAc moiety and is generally considered as its diagnostic fragment ion;
270 and CSC features a main fragment at m/z 282, which is generally considered as its diagnostic
271 fragment ion. [22]. [23,24]. Several authors reported that the fragmentation patterns are highly
272 dependent on the experimental conditions and MS/MS analysis must be carried out with extreme

273 caution. Yet, LC coupled to MS and/or tandem MS analysis is the most widespread approach for the
274 analysis of glycosaminoglycan composition [25].

275 The use of the IR vibrational spectrum of molecular ions(sensitive towards structural details) enables
276 the investigation of functional groups, intra- or intermolecular interactions and molecular
277 conformations bringing additional information compared to the MS/MS analysis. The IR spectrum
278 presents intrinsic features, which do not depend on the MS conditions, that benefits to the
279 saccharides analysis by recording IR fingerprints of the CS disaccharides for unambiguous
280 characterization. The IRMPD spectra of the two collected fractions show an apex at 3410 cm^{-1} and
281 3375 cm^{-1} attributed to the CSA and CSC disaccharides respectively, which is in good agreement with
282 reference spectra of CS disaccharides standards [13]. In the specific case of CSA and CSC, the MS/MS
283 fingerprint is sufficient to distinguish between the isomers, and the differences in the IRMPD
284 signatures are relatively subtle. However, IRMPD has proven useful to distinguish between a variety
285 of isomers when tandem MS is ambiguous. [14] Therefore, the presented example supports that the
286 fractionation of tens of nL of isobaric saccharides mixtures using ITP can be applied to the off-line
287 collection of high purity compounds for further IR spectroscopic characterization. This demonstrates
288 the pertinence of such coupling strategy at the microscale separation.

289

290 **4. Conclusion**

291 Electrokinetic separation of isomeric chondroitin sulfate disaccharides (CSA and CSC) at the
292 microscale was achieved by isotachopheresis and fractions of high purity were collected for further
293 structural analysis. ITP generates plateau-like bands of purified compounds which is particularly
294 advantageous for collecting sample at the microscale or for coupling with long acquisition time
295 detection methods. In contrast with ion exchange chromatography, ITP does not require the use of
296 high salt concentration that can interfere severely with Mass Spectrometry. Such proof of feasibility
297 aims at bringing an original coupling strategy with sophisticated spectroscopic characterization
298 method of oligosaccharides. The molecular characterization of such complex molecules requires
299 combined approaches of separation and characterization. Optimization of the ITP conditions allows
300 the separation of two isobaric CS disaccharides from few tens of nL sample solution and their
301 collection in $10\text{ }\mu\text{L}$. The purity of the collected fraction, more than 90 %, is well-adapted to off-line
302 coupling with gas phase IR action spectroscopy. This case study validates this proof of feasibility
303 study using two similar CS disaccharides GAGs precursor. In order to fully respond to biological
304 demand, the in-line coupling ITP-IRMPD spectroscopy would be further investigated allowing
305 miniaturized and automated analysis of complex samples.

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391

392 **Figure Captions:**

393 Figure 1. Plot of the relative electrophoretic mobility difference between CS A and CS C as a function
394 of the pH of the glycine/HCl electrolyte. Electrophoretic mobilities were determined by capillary zone
395 electrophoresis using the procedure described by Vigh et al. [18].

396 Figure 2. Isotachophoregrams of each individual chondroitin (CSA and CSC at 1 mM) injected
397 separately and injected in stacked configuration (CSC + CSA) (A) and ITP separation of CS
398 disaccharides in mixture in water or in the terminating electrolyte (B). Conditions for (A): HPC-coated
399 capillary length: 50 cm, Injection 50 mbar for 3 sec. Applied Current = -4 μ A and in mixture (CSA and
400 CSC at 1 mM each in water (grey) and solubilized in the terminating electrolyte (black)). Conditions
401 for (B) : ITP of CS disaccharides solubilized in water was realized in a 45.9 cm-length HPC-coated
402 capillary, injection 5 mbar for 8 sec. Applied Current = - 5 μ A. ITP of CS disaccharides solubilized in
403 terminating electrolyte was realized in a 58.7 cm length HPC-coated capillary, injection 20 mbar for
404 4sec). Applied Current = - 5 μ A.

405 Figure 3. Influence of the injection pressure of CS disaccharides mixture at 1 mM solubilized in the
406 terminating electrolyte, separation at -3 μ A, HPC-coated fused silica capillary (75 μ m id, total length
407 1m), UV detection at 230 nm, injection time (10 sec), corresponding injection volumes 7.8 to 39 nL.

408 Figure 4. ITP separation of chondroitin sulfate disaccharides (CSA and CSC) in a 1-m long HPC-coated
409 capillary and fractionation of the separated solutes. Injection of chondroitin sulfate disaccharides at 1
410 mM each in terminating electrolyte, separation under -3 μ A, detection at 230 nm, injection 30 mbar/
411 10 sec (A). CZE separation of the two collected fractions and of the mixture of the two chondroitin
412 sulfate disaccharides. HPC Fused silica capillary (75 μ m id, 30-cm total length, 21.5 cm-length to
413 detection), UV detection at 230 nm, BGE: Formate 50 mM, glycine 10 mM, pH 3.5, injection CS
414 standard mixture (20 μ M in terminating electrolyte) (-10kV, 5 sec) injection of the CS fraction (-10kV,
415 10 sec), response time 0.063 sec (40 Hz).

416 Figure 5. IRMPD analysis of fraction 2 (A) and fraction 1 (B). Top panels: CID spectra of singly
417 deprotonated disaccharides (m/z 458). Center panels: IRMPD signatures of the m/z 458 ions. Lower
418 panels: reference IRMPD spectra of pure CSA (A) and CSC (B) disaccharide standards. Strips
419 corresponding to the maximum of absorption of CSA (light red) and CSC (light blue) standards are
420 overlaid for facile structural identification.









