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1 **Ion mobility mass spectrometry enables the discrimination of**  
2 **positional isomers and the detection of conformers from cyclic**  
3 **oligosaccharides-metals supramolecular complexes**

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17 **ABSTRACT**

18           Cyclic oligosaccharides are well known to interact with various metals, able to form supra-  
19 molecular complexes with distinct sizes and shapes. However, the presence of various isomers in  
20 a sample, including positional isomers and conformers, can significantly impact molecular recog-  
21 nition, encapsulation ability and chemical reactivity. Therefore, it is crucial to have tools for deep  
22 samples probing and correlation establishments. The emerging ion mobility mass spectrometry  
23 (IM-MS) has the advantages to be rapid and sensitive, but is still in its infancy for the investigation  
24 of supramolecular assemblies. In the herein study, it was demonstrated that IM-MS is suitable to  
25 discriminate several isomers of cyclodextrins (CD)-metals complexes, used as cyclic oligosaccha-  
26 ride models. In this sense, we investigated branched 6-O- $\alpha$ -glucosyl- or 6-O- $\alpha$ -maltosyl- $\beta$ -cy-  
27 clodextrins ( $G_1$ - $\beta$ -CD and  $G_2$ - $\beta$ -CD) and their purely cyclic isomers: CD8 ( $\gamma$ -CD) and CD9 ( $\delta$ -CD). The  
28 corresponding collision cross section (CCS) values were deducted for the main positive singly and  
29 doubly charged species. Experimental CCS values were matched with models obtained from mo-  
30 lecular modelling. The high mobility resolving power and resolution enabled discrimination of  
31 positional isomers, identification of various conformers and accurate relative content estimation.  
32 These results represent a milestone in the identification of carbohydrate conformers that cannot  
33 be easily reached by other approaches.

34

35           **Keywords:** Cyclic oligosaccharides; Cyclodextrins; Conformers; Isomers; Supramolecular  
36 assembly; Ion-mobility.

37

## 38 1. INTRODUCTION

39 With cellulose, starch is among the most abundant polysaccharides present in the nature  
40 (Tester et al., 2004). Various bacterial enzymes such as  $\alpha$ -amylase and cyclodextrin glycosyltrans-  
41 ferase (CGTases), are able to degrade starch to produce lower size molecules i.e. oligosaccharides.  
42 Indeed, the aforementioned enzymes own unique capacity to catalyse the formation of  $\alpha(1\rightarrow4)$   
43 malto-oligosaccharides as linear (maltodextrins), but also circular ones (cyclodextrins; CDs). CDs  
44 are formed by the enzymatic conversion of polysaccharide by CGTases through intramolecular  
45 transglycosylation reaction, leading mainly to CDs with 6 ( $\alpha$ -CD), 7 ( $\beta$ -CD), or 8 ( $\gamma$ -CD)  $\alpha(1\rightarrow4)$   
46 linked D-glucopyranose units (Glc) as final products. In spite of these last ones, which are com-  
47 mercially available and have been extensively investigated,(Szejtli, 1998) it was reported that CDs  
48 with higher degree of polymerization (DP) (until >100) can be also obtained. Nonetheless, larger  
49 CDs (DP  $\geq 9$ ) are rapidly degraded by the enzyme in a reverse reaction to linear glucans which are  
50 subsequently converted to  $\alpha$ -,  $\beta$ - and  $\gamma$ -CD, leading to a negligible yield of the larger ones (Son-  
51 nendecker, Melzer, et al., 2019; Sonnendecker, Thürmann, et al., 2019; Terada et al., 1997; Te-  
52 rada Yoshinobu et al., 2001; M. Zheng et al., 2002). However, elegant approaches dealing with  
53 the templated enzymatic synthesis to promote some CD sizes have been very recently described  
54 (Erichsen et al., 2023; Samuelsen et al., 2022; Yang et al., 2021). Furthermore, incomplete depol-  
55 ymerisation/cyclization processes can also lead to branched  $\alpha$ -glycosylated-(1 $\rightarrow$ 6)CD (Ao et al.,  
56 2007; D. French et al., 1965; Taniguchi & Honnda, 2009; Terada et al., 1997). The main interest of  
57 CDs is based on their structure/properties relationship where they exhibit a doughnut shape with  
58 a hydrophobic cavity able to reversibly encapsulate several organic molecules (Kfoury et al., 2018;

59 Song et al., 2009), and disclose peripherally hydrophilic features that form an intra- and inter-  
60 hydrogen bonding network (Loftsson et al., 2005). Such properties drive the particularly attractive  
61 roles of CDs in several fields like food, pharmaceutical and cosmetic industries (Singh et al., 2002).

62 The efficiency of supramolecular host-guest complexes is tightly dependent on the struc-  
63 ture of the host molecule, though if presence of CDs with (bio)chemical modifications can be  
64 easily discriminated, the differentiation of isomeric forms of unmodified CDs can be a more chal-  
65 lenging task. In this sense, the first bottleneck is to achieve an acceptable separation to unambig-  
66 uously identify each species. Liquid chromatography (LC) was employed to achieve this, yielding  
67 contrasting outcomes. Hence, some partial separation was reached for positional isomers of di-  
68 glucosyl- $\beta$ -CD ( $(G_1)_2$ - $\beta$ -CD), maltotriosyl- $\beta$ -CD ( $G_3$ - $\beta$ -CD) (Koizumi et al., 1991; Okada et al., 1994),  
69 or also heterogeneous doubly branched  $\alpha$ -D-galactosyl-/ $\alpha$ -D-glucosyl- $\alpha$ -CD (Koizumi et al., 1986).  
70 On the other hand, complete separation was achieved in one run for positional isomers of di- $\alpha$ -  
71 D-glucosyl- $\gamma$ -CD ( $(G_1)_2$ - $\gamma$ -CD) (Koizumi et al., 1986; Tanimoto et al., 1995), as well as with a mixture  
72 of  $\beta$ -CD,  $\alpha$ -CD,  $G_1$ - $\beta$ -CD,  $G_2$ - $\beta$ -CD and  $(G_2)_2$ - $\beta$ -CD (Yamamoto et al., 1989), or with two distinct  
73 columns for  $\alpha$ -,  $\beta$ - or  $\gamma$ -CD and their maltosyl grafted derivatives (Shiraishi et al., 1989).  $^1\text{H}$  or  $^{13}\text{C}$   
74 NMR was also successfully applied to characterize  $\alpha$ -CDs and 6-O- $\alpha$ -glucopyranosyl- to 6-O- $\alpha$ -  
75 maltoheptaosyl- $\alpha$ -CD ( $G_1$ - $\alpha$ -CD to  $G_7$ - $\alpha$ -CD) (Ishizuka et al., 2004), as well as for doubly and triply  
76 branched glucosyl-CDs ( $(G_1)_2$ / $(G_1)_3$ - $\gamma$ -CD) (Koizumi et al., 1986, 1990, 1991; Tanimoto et al., 1995),  
77 maltosyl-CD (Abe et al., 1988; Okada et al., 1994), or longer branched glucosyl chains. (Hizukuri et  
78 al., 1989) While mass spectrometry (MS) is commonly used for the characterization of cyclodex-  
79 trins (CDs), there are relatively few studies that have focused on the analysis of their glycosyl

80 derivatives. Fast Atom Bombardment MS was applied for masses verification of glucosyl- to  
81 maltopentaosyl- $\alpha$ -CD (Hizukuri et al., 1989) or also various isomers of doubly branched  $\beta$ -CD (Abe  
82 et al., 1988; Koizumi et al., 1986, 1991). MS spectrum of  $G_2$ - $\beta$ -CD (Yamamoto et al., 1989), was  
83 also obtained by Secondary Ionization MS (SIMS), while electrospray coupled to quadrupole-  
84 time-of-flight (ESI-QTOF) instrument was successfully used for MS/MS of  $G_1$ - $\beta$ -CD (Xia et al.,  
85 2017). Yamagaki *et al.* reported the possibility to associate the relative intensities of fragment  
86 ions issued from the post-source decay (PSD) in MALDI-TOF MS with the chemical structures of  
87 three CD isomers, namely  $\gamma$ -CD,  $G_2$ - $\alpha$ -CD and  $(G_1)_2$ - $\alpha$ -CD (Yamagaki et al., 1996). The correlation  
88 seems to be caused by the difference in the number of cleavage sites at the glycosidic bond. It  
89 was found that the intensity of the PSD ion resulting from one cleavage is higher than that result-  
90 ing from two cleavages at this linkage. It can be hypothesized that energy is more widely dissi-  
91 pated in the latter case. The same authors have also given evidence that measurements at a re-  
92 duced acceleration voltage were advantageous to distinguish easily between the otherwise very  
93 similar PSD fragment spectra of  $G_1$ - $\beta$ -CD and  $(G_1)_2$ - $\alpha$ -CD (Yamagaki & Nakanishi, 1999). More re-  
94 cently, it has been demonstrated that two isomers of larger CD i.e. CD10 and  $G_1$ -CD9 can be dis-  
95 tinguished by a characteristic loss of 120.04 mass units. That corresponds to  $^{0,2}X_1$  cross ring cleav-  
96 age of a grafted non-reducing end (1 $\rightarrow$ 6) glucosyl unit, and obtained from higher energy collision  
97 dissociation (HCD) MS/MS step (Sonnendecker, Thürmann, et al., 2019). However, none of these  
98 aforementioned approaches allows both a direct discrimination of such CD isomers and a sub-  
99 milligram sample consumption.

100 During the last decade, a promising approach named ion mobility-MS (IM-MS) has been  
101 more widely introduced and could overcome previously described limitations. IM-MS is a 2D  
102 method, that is suitable to resolve glycan isomers (Bansal et al., 2020; Clowers et al., 2005; Ga-  
103 bryelski & Froese, 2003; Gaye et al., 2015; Harvey et al., 2018; Hofmann et al., 2015; Huang &  
104 Dodds, 2013; Li et al., 2013; Nagy et al., 2018; Przybylski & Bonnet, 2021; Pu et al., 2016; Rashid  
105 et al., 2014; Xie et al., 2021; X. Zheng et al., 2017). During IM-MS, the ions are separated not only  
106 according to their  $m/z$ , but also as function of their size and shape in the gas phase, which is  
107 portrayed by collision cross section (CCS) values in a given gas. IM-MS has been successfully ap-  
108 plied to the characterization of a large variety of linear and branched saccharidic isomers using  
109 various technological setups such as field asymmetric ion mobility spectrometry (FAIMS) (Gab-  
110 ryelski & Froese, 2003), traveling wave ion mobility (TWIM) (Harvey et al., 2018; Hofmann et al.,  
111 2015; Huang & Dodds, 2013; Rashid et al., 2014), drift tube ion mobility spectrometry (DTIMS)  
112 (Clowers et al., 2005; Gaye et al., 2015; Li et al., 2013; X. Zheng et al., 2017), improved serpentine  
113 ion pathway like structures for lossless ion manipulation (SLIM) (Bansal et al., 2020; Nagy et al.,  
114 2018), or also trapped ion mobility spectrometry (TIMS) (Przybylski & Bonnet, 2021; Pu et al.,  
115 2016; Xie et al., 2021). Since the first report in 1997 by Clemmer and Liu of the first drift time for  
116  $\alpha$ -,  $\beta$ - and  $\gamma$ -CD and their fragments, only a few studies focusing on IM-MS of CD have been re-  
117 ported (Liu & Clemmer, 1997).  $\alpha$ -,  $\beta$ - and  $\gamma$ -CD were analyzed by ESI-/MALDI-DTIMS (Fenn &  
118 McLean, 2011; Klein et al., 2018; S. Lee et al., 1997). More recently, ESI-TWIMS was successfully  
119 applied to study mono- to trimeric  $\beta$ -CD (Berland et al., 2014), as well as complexes involving  $\alpha$ -  
120 and  $\beta$ -CD with either *o*-, *m*- or *p*-coumaric acids (Kralj et al., 2009), amino- $\beta$ -CD/labile sesamins

121 (Sugahara et al., 2015),  $\alpha$ - or  $\beta$ -CD with piperine or curcumin (Nag et al., 2018), whereas such  
122 analysis with same CDs based complexes and amino acids were also reported (Chen et al., 2018;  
123 S.-S. Lee et al., 2018). Ding and colleagues have demonstrated the potential to use TIMS for en-  
124 antiomeric distinction of D/L amino acids by forming complexes with CDs and  $Mg^{2+}$  (Yang et al.,  
125 2022). Smith and colleagues have elegantly developed a coupling of TW-SLIM utilizing serpentine  
126 ultra-long path with extended routing (SUPER) for analysis of  $\alpha$ -CD with bile acids in negative and  
127 positive modes (Chouinard et al., 2018). Yamagaki and colleagues showed, using TWIM-MS, that  
128 the drift times of  $\gamma$ -CD,  $G_2$ - $\alpha$ -CD and  $(G_1)_2$ - $\alpha$ -CD, and their product ions with the same masses  
129 were almost the same in collision-induced dissociation (CID) MS/MS (Yamagaki & Sato, 2009). In  
130 contrast, the IM peak widths were suggested to serve as 'trend line' to discriminate between the  
131 three isomers. Indeed, the IM peak widths were sensitive to structural differences of the isomeric  
132 product ions. For example, the product ions  $[M-Glc_n+H]^+$  ( $n=0\sim6$ ) of  $\gamma$ -CD exhibited a linear cor-  
133 relation with their masses, whereas the product ions with larger and/or longer chains had broader  
134 peak widths (Yamagaki & Sato, 2009). Nevertheless, the aforementioned MS approaches only give  
135 partial and indirect information. Very recently, we successfully demonstrated the usefulness of  
136 ESI-TIMS-TOF to differentiate isomers of supramolecular complexes with tubular shape based on  
137 CDs with alkali metals (Przybylski & Bonnet, 2022). Moreover, with the same instrumentation  
138 Chakraborty *et al.* have fruitfully probed the isomerism of CD based oligomers with  $Cu^{2+}$   
139 (Chakraborty et al., 2023).



140 In the present work, we hypothesize that high-resolution IM-MS such as ESI-TIMS-TOF can  
141 be suitable to discriminate CD isomers as well as to be compatible with the timescale of intercon-  
142 version to unveil and quantify potential conformers. The ability of such high-resolution instru-  
143 ment to differentiate studied models of cyclic oligosaccharides according to their metal adduct,  
144 size, structure and shape feature was also investigated.

145

## 146 **2. MATERIALS AND METHODS**

### 147 **2.1. Chemicals and Materials**

148  $\gamma$ -Cyclodextrin was kindly supplied by Wacker Chimie S.A.S (Lyon, France). 6-*O*- $\alpha$ -Maltosyl- $\beta$ -cy-  
149 clodextrin (G<sub>2</sub>- $\beta$ -CD), LiCl, NaCl, KCl, RbCl and CsCl alkali salts were purchased from Sigma-Aldrich  
150 (Saint-Quentin Fallavier, France). 6-*O*- $\alpha$ -D-Glucosyl- $\beta$ -cyclodextrin (G<sub>1</sub>- $\beta$ -CD), was provided from  
151 ABCR Chemie. According to previously described procedures, large ring CD9 ( $\delta$ -CD) was produced  
152 by action on starch of a specifically genetically engineered CGTase followed by LC purification  
153 steps as described elsewhere (Sonnendecker et al., 2017; Sonnendecker, Melzer, et al., 2019;  
154 Sonnendecker, Thürmann, et al., 2019). Methanol of MS grade used for sample preparation was  
155 purchased from VWR (West Chester, PA, USA). Water was of ultrapure quality.

### 156 **2.2 Solvents**

157 Methanol used for sample preparation was of MS grade and was purchased from VWR (West  
158 Chester, PA, USA). Water was of ultrapure quality (18.2 M $\Omega$ ).

### 159 **2.3 Samples**

160 Stock solutions were made at 1 mM in water. Then, the solution was diluted to 1  $\mu$ M in metha-  
161 nol/water (1:1 v/v) with 0.3  $\mu$ M of one given salt or a mixture of them for further analysis.

## 162 2.4 TimsTOF™ experiments.

163 An ESI-TIMS-TOF™ instrument (Bruker Daltonics, Bremen, Germany) operating with oTOF control  
164 v5.0 software was used. The source temperature was held at 200°C, and the drying and nebuliz-  
165 ing gas (N<sub>2</sub>) operated at a flow rate of 3 L. min<sup>-1</sup> and at a pressure of 0.3 bar, respectively. The  
166 instrument was calibrated using Tuning Mix G24221 (Agilent Technologies, Les Ulis, France). Ap-  
167 plied voltages were +4 kV and -0.5 kV for capillary and endplate offset, respectively. The acqui-  
168 sition was achieved in the  $m/z$  300-4000 range with a centre at  $m/z$  500. TIMS separation depends  
169 on the gas flow velocity ( $v_g$ ), elution voltage ( $V_{elution}$ ), ramp time ( $t_{ramp}$ ), base voltage ( $V_{out}$ )  
170 and the electric field ( $\vec{E}$ ). The reduced mobility,  $K_0$ , can be calculated as follows (Eq. 1):

$$171 \quad K_0 = \frac{v_g}{\vec{E}} = \frac{A}{(V_{elution} - V_{out})} \quad (\text{Eq. 1})$$

172 The mobility calibration constant  $A$  was determined using known reduced mobilities of tuning  
173 mix components. The resolving power ( $R$ ) and resolution ( $r$ ) are defined as  $R = (1/K_0)/w$  and  
174  $r = 1.18 \times [(1/K_0)_2 - (1/K_0)_1]/(w_1 + w_2)$ , where  $w$  is the full peak width at half maximum.  
175 To improve separation efficiency, the scan rate ( $Sr = \Delta V_{ramp}/t_{ramp}$ ) was tuned by imex™ tech-  
176 nology. For this,  $t_{ramp}$  is automatically set as function of manually adjusted  $\Delta V_{ramp}$ . N<sub>2</sub> was used  
177 as buffer gas at funnel temperature ( $T = 305$  K) with  $v_g$  set by the pressure difference of 1.69  
178 mbar. A potential of 350 Vpp was applied to radially confine the trapped ion cloud. The measured

179 inverse reduced mobilities were converted into collision cross sections (CCS) using the Mason-  
180 Schamp equation (Eq. 2):

$$181 \quad \Omega = \frac{(18\pi)^{1/2}}{16} \times \frac{q}{(k_B \times T)^{1/2}} \times \left[ \frac{1}{m_i} + \frac{1}{m_g} \right]^{1/2} \times \frac{1}{N} \times \frac{1}{K_0} \quad (\text{Eq. 2})$$

182 where  $q$  is the ion charge,  $k_B$  is the Boltzmann constant,  $N$  is the gas number density,  $m_i$  is the  
183 ion mass, and  $m_g$  is the gas molecular mass. TIMS-MS spectra and mobilograms were analysed  
184 using Compass Data Analysis 5.1 (Bruker, Germany).

## 185 **2.6 ESI-TIMS-MS analysis of the cyclo-oligosaccharides**

186 Throughout this study, all cyclodextrins were analysed in the positive ion mode as singly or dou-  
187 bly charged ions with or without salt doping. All samples were continuously infused at 3  $\mu\text{L}\cdot\text{min}^{-1}$   
188 via a 250 mL syringe. All experiments were performed in quintuplicate and were reproducible  
189 across the replica measurements.

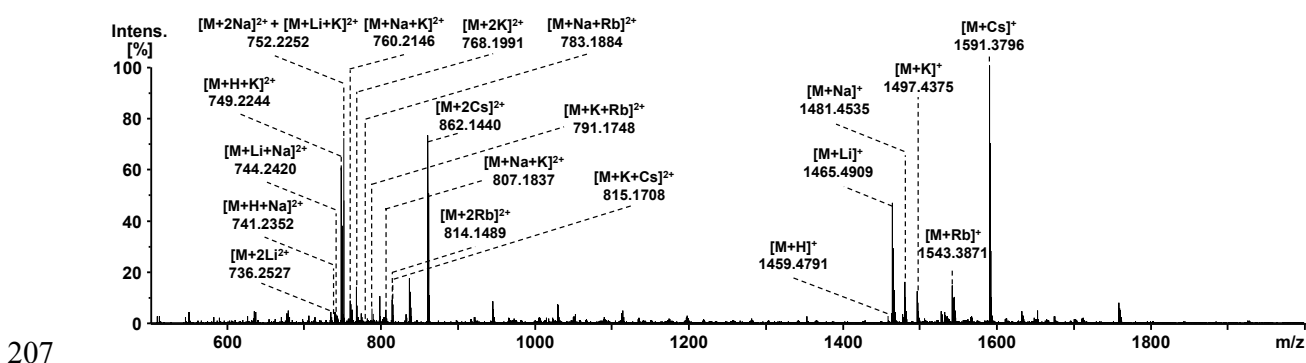
## 190 **2.7 Theoretical collision cross section calculations**

191 All initial geometry relaxations were performed using the Merck molecular force field (MMFF94)  
192 implemented in Avogadro (v1.95.1). Geometry optimization was finalized using density func-  
193 tional theory (DFT) calculations with NWChem (v7.0). Theoretical CCS calculations were carried  
194 out in IMoS (v.1.10) (Larriba & Hogan, 2013) using the average of ten processes by Elastic/Diffuse  
195 Hard Sphere Scattering (EHSS/DHSS) or Trajectory method Diffuse Hard Sphere Scattering (DTHSS  
196 with 4-inf potential) (Wei et al., 2019).

197

198 **3. RESULTS AND DISCUSSION**

199 Cyclodextrins like most of the neutral carbohydrates exhibit a strong affinity for metals, especially  
200 alkali and (post-)transition ones (Przybylski et al., 2015; Dossmann et al., 2021). Such attachment  
201 is advantageously used to detect the cyclodextrin as a wide variety of ionic complexes with vari-  
202 able stoichiometries which can be summarized as  $[n\text{CD}+m\text{X}]^{z+}$ , where n is the number of cyclodex-  
203 trins, m and X is the number and the studied metal, respectively, and z the number of charge.  
204 Due to its highly softness, ESI is the most suitable ionization method to transfer intact assemblies  
205 from solution to gas phase for MS analysis. ESI mass spectra showed numerous ions, as for exam-  
206 ple for  $\text{G}_2\text{-}\beta\text{-CD}$  doped with salts mixture (Fig. 1) or  $\gamma\text{-CD}$ ,  $\text{G}_1\text{-}\beta\text{-CD}$  and  $\gamma\text{-CD}$  (Fig. S1).



208 **Fig. 1.** ESI-MS spectra of  $\text{G}_2\text{-}\beta\text{-CD}$  ( $1\ \mu\text{M}$ ) with mixture of LiCl, NaCl, KCl, RbCl, and CsCl salts (each at  $0.3\ \mu\text{M}$ ).

209

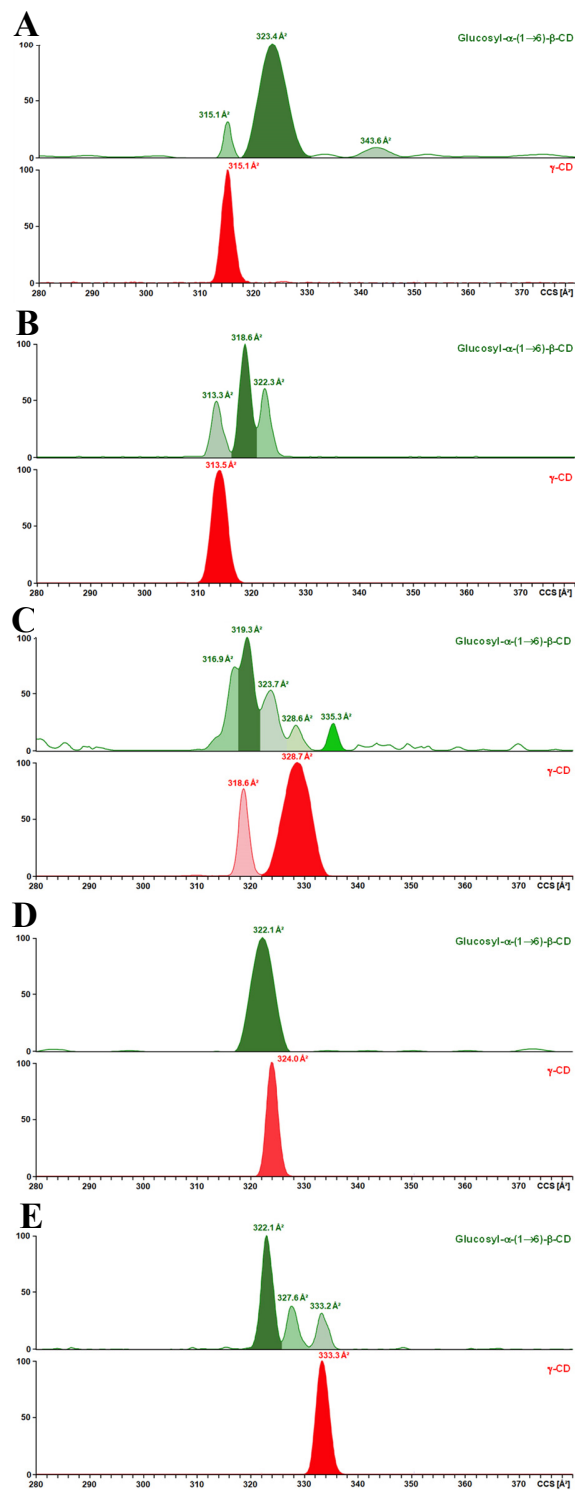
## 210 **3.1 Delineate the influence of usual metals and charge states as discrimination features**

### 211 *3.1.1 Singly charged species*

212 The charge states as well as the number and type of metals can strongly affect the result-  
213 ing assemblies in terms of size and conformation. TIMS analysis revealed that a clear differentia-  
214 tion between  $G_1$ - $\beta$ -CD/ $\gamma$ -CD and  $G_2$ - $\beta$ -CD/ $\delta$ -CD couples can be obtained by the addition of a single  
215 lithium, sodium, potassium, or cesium ion (Fig. 2-5). The high resolution of TIMS also provides  
216 the advantage to estimate the relative content of a given sample by the integration of each ion  
217 mobility peak.

218 *Case of  $G_1$ - $\beta$ -CD/ $\gamma$ -CD couple.*  $[G_1$ - $\beta$ -CD+Li] $^+$  revealed three well resolved peaks with a  
219 prominent one at 323.4  $\text{\AA}^2$  and two others exhibiting lower intensity at 315.1  $\text{\AA}^2$  and 343.6  $\text{\AA}^2$   
220 (relative content: 71/21/8, respectively) (Fig. 2A top and Table S1). Moreover, the high resolution  
221 ( $r$ ) and resolving power ( $R$ ) give clear evidences that the  $G_1$ - $\beta$ -CD sample can potentially contain  
222 a small part of  $\gamma$ -CD, showing a unique peak at 315.1  $\text{\AA}^2$  (Fig. 2A bottom and Table S1). Keeping in  
223 mind that only a single mobility peak was observed for singly lithiated  $\alpha$ -,  $\beta$ - and  $\gamma$ -CD,(Przybylski  
224 & Bonnet, 2022) the detection of two or more peaks can be rationally ascribed to distinct posi-  
225 tional and/or conformational isomers i.e. stereoisomers and/or conformers, respectively. The  
226 adduct with sodium led to three mobility peaks for  $G_1$ - $\beta$ -CD at 318.6, 322.3, and 313.3  $\text{\AA}^2$   
227 (47/30/23), respectively (Fig. 2B, top), while a single one at 313.5  $\text{\AA}^2$  was observed for  $\gamma$ -CD (Fig.  
228 2B, bottom). The higher peak resolution obtained with sodium confirmed the presence of a few  
229 amounts of  $\gamma$ -CD into the  $G_1$ - $\beta$ -CD sample, as previously observed for lithium addition. The intro-  
230 duction of potassium led to four unresolved peaks at 319.3, 316.9, 323.7, and 328.6  $\text{\AA}^2$

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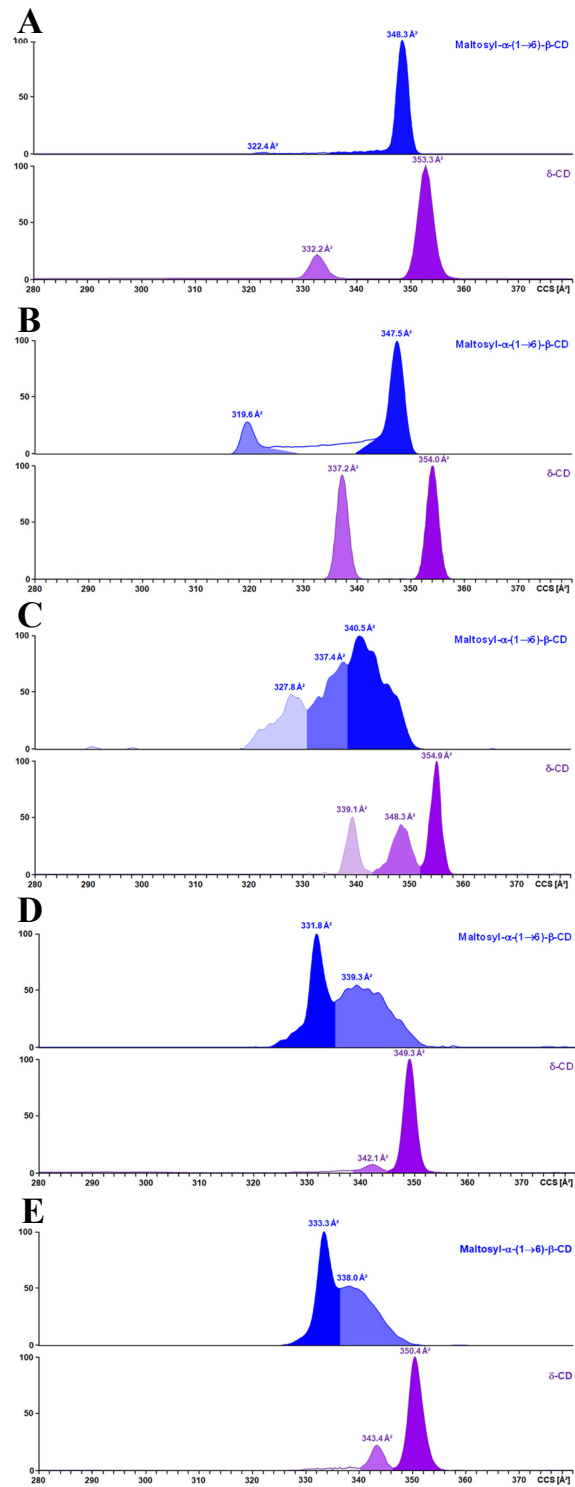
**Fig. 2.** TIMS based mobilograms and corresponding CCS for singly charged ions of  $G_1$ - $\beta$ -CD (top, green trace) and  $\gamma$ -CD (bottom, red trace) with A) lithium, B) sodium, C) potassium, D) rubidium and E) cesium.

258 (37/27/19/9) as well as a resolved one at 355.3 Å<sup>2</sup> (8) for G<sub>1</sub>-β-CD (Fig. 2C, top), and a couple of  
259 CCS at 328.7/318.6 Å<sup>2</sup> (64/36) for γ-CD were detected (Fig. 2C, bottom). Using rubidium showed  
260 a unique peak at 322.1 for G<sub>1</sub>-β-CD (Fig. 2D, top) and 324.0 Å<sup>2</sup> for γ-CD (Fig. 2D, bottom). The  
261 lower R value obtained for G<sub>1</sub>-β-CD in the case of rubidium can be attributed to a larger full width  
262 at half maximum, suggesting that the peak may contain both G<sub>1</sub>-β-CD and γ-CD species. None-  
263 theless, this impairs to serve as a suitable metal for straightforward discrimination. With cesium,  
264 which is the larger cation tested in this work, three quite well resolved peaks were detected for  
265 G<sub>1</sub>-β-CD at 322.1, 327.6 and, 333.2 Å<sup>2</sup> (59/22/19) (Fig. 2E, top), while a unique one at 333.3 Å<sup>2</sup>  
266 occurred for γ-CD (Fig. 2E, bottom). Conversely, protonated G<sub>1</sub>-β-CD led to a unique mobility peak  
267 corresponding to a CCS of 316.8 Å<sup>2</sup>, whereas at least two unresolved peaks, with the most intense  
268 at 324.1 Å<sup>2</sup> and a lower one at 317.2 Å<sup>2</sup> were detected for γ-CD (Fig. S1). Based on the standard  
269 deviation values (Table S1), the latter peak could be putatively ascribed to G<sub>1</sub>-β-CD, which ap-  
270 pears as a minor trace in the γ-CD sample.

271 *Case of G<sub>2</sub>-β-CD/δ-CD couple.* Concerning singly lithiated species of both G<sub>2</sub>-β-CD and δ-  
272 CD, two distinct couples of peaks were detected at 348.3 Å<sup>2</sup>/322.4 Å<sup>2</sup> (Fig. 3A, top) and 353.3  
273 Å<sup>2</sup>/332.2 Å<sup>2</sup> (Fig. 3A, bottom). For such couples, the relative content is 99/1 for the former and  
274 82/18 for the latter. Conversely, regarding G<sub>2</sub>-β-CD/δ-CD couple no trace of G<sub>2</sub>-β-CD was de-  
275 tected in the δ-CD sample. Similarly to lithium adduct, following the complexation with sodium,  
276 two couple of peaks were clearly observed both for G<sub>2</sub>-β-CD and δ-CD at 347.5/319.6 Å<sup>2</sup> (23/77)  
277 (Fig. 3B, top) and 354.0/337.2 Å<sup>2</sup> (48/52) (Fig. 3B, bottom), respectively.

278 However, in the case of G<sub>2</sub>-β-CD, no return to the baseline occurred between the two peaks,

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298 **Fig. 3.** TIMS based mobilograms and corresponding CCS for singly charged ions of G<sub>2</sub>-β-CD (top, blue trace) and δ-CD  
 299 (bottom, purple trace) with A) lithium, B) sodium, C) potassium, D) rubidium and E) cesium.



300 indicating a non-equilibrium state and the possible presence of intermediary species. Such phe-  
301 nomenon, also observed to in a less extent for  $[G_2\text{-}\beta\text{-CD+Li}]^+$  is similar to an exponential bridge  
302 smoothly merging two peaks already described in capillary electrophoresis.(Cherney et al., 2011)  
303 Regarding  $[G_2\text{-}\beta\text{-CD+K}]^+$ , three unresolved peaks could be deduced at  $\approx 327.8$ ,  $337.4$ , and  $340.5$   
304  $\text{\AA}^2$  (22/34/44) (Fig. 3C, top), while for  $[\delta\text{-CD+K}]^+$ , three well resolved ones at  $354.9$ ,  $348.3$  and  
305  $339.1 \text{\AA}^2$  (24/27/49) are distinguished (Fig. 3C, bottom). The mobilogram of  $[G_2\text{-}\beta\text{-CD+Rb}]^+$  re-  
306 vealed a clear peak at  $331.8 \text{\AA}^2$  and an only partially resolved distribution with a dumbbell shape  
307 centred around  $339.3 \text{\AA}^2$  (Fig. 3D, top), while  $[\delta\text{-CD+Rb}]^+$  exhibited two close but resolved peaks  
308 at  $349.3$  (93 %) and  $342.1 \text{\AA}^2$  (7 %) (Fig. 3D, bottom). Unfortunately, the mobility range of  $[G_2\text{-}\beta\text{-}$   
309  $\text{CD+Rb}]^+$  is too broad, encompassing the values obtained for  $\delta\text{-CD}$  and preventing a clear conclu-  
310 sion regarding the presence or absence of  $\delta\text{-CD}$  trace in the  $G_2\text{-}\beta\text{-CD}$  sample.  $[G_2\text{-}\beta\text{-CD+Cs}]^+$   
311 and  $[\delta\text{-CD+Cs}]^+$  species with CCS couple of  $333.3/338.0 \text{\AA}^2$  (67/33) (Fig. 3E, top) and  $350.4/343.4$   
312  $\text{\AA}^2$  (17/83) (Fig. 3E, bottom), respectively, showed similar behaviour to their corresponding ru-  
313 bidium adduct counterparts.

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315 Regarding both protonated  $G_2\text{-}\beta\text{-CD}$  and  $\delta\text{-CD}$ , a single peak was observed at  $345.2 \text{\AA}^2$  and  $350.0$   
316  $\text{\AA}^2$ , respectively (Fig. S1). As other neutral polyols, CDs present a weak affinity for protons, leading  
317 to quite low abundance and sometimes to more of one peak. This is due to the proton mobility  
318 and the absence of a unique and stable attachment site (Przybylski et al., 2015; Przybylski & Bon-  
319 net, 2022), demonstrating that singly protonated state is not a straightforward reference herein.  
320 Singly charged species with lithium and sodium allow us to unambiguously differentiate each

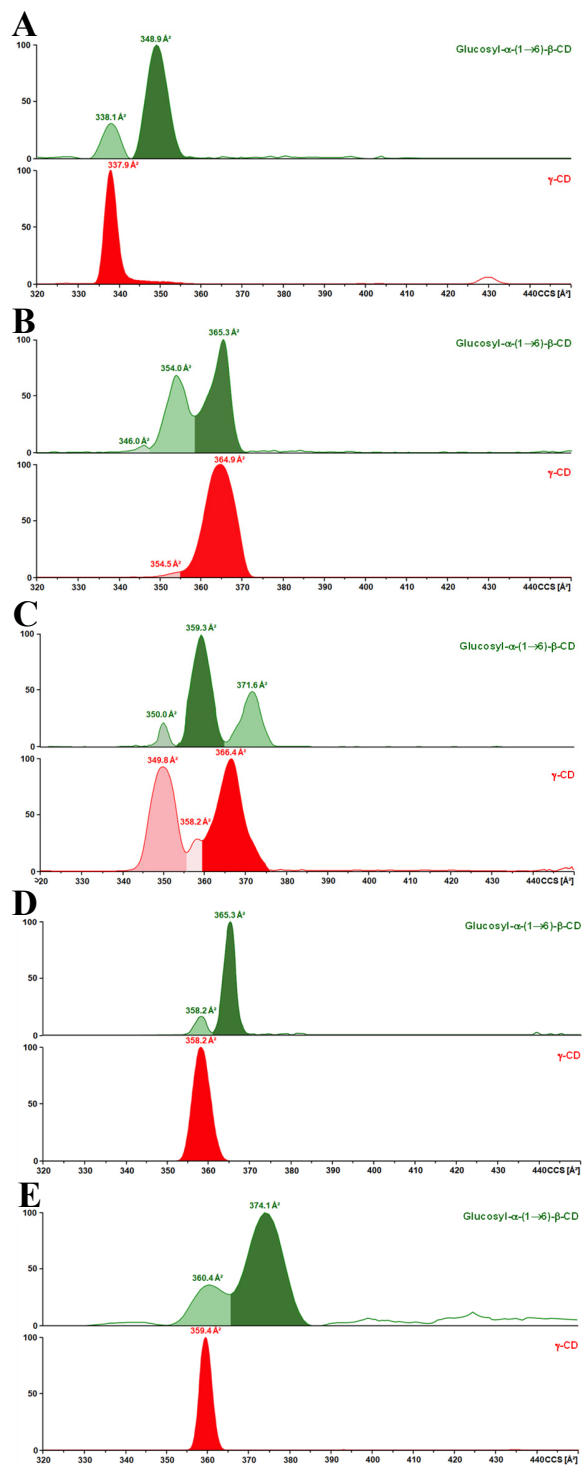
321 content of the two isomeric couples  $G_1$ - $\beta$ -CD/ $\gamma$ -CD and  $G_2$ - $\beta$ -CD/ $\delta$ -CD, as well as the identification  
322 of trace amounts of a specific structural isomer in other samples or the discovery of new con-  
323 formers. Nevertheless, with larger alkali metals like potassium, rubidium and cesium, more con-  
324 trasted results were obtained. Indeed, with such metals, analysis of  $G_1$ - $\beta$ -CD and  $G_2$ - $\beta$ -CD led to  
325 a large unresolved peak distribution. The occurrence of various conformations, which may or may  
326 not be consecutive to the unique attachment sites of metal-based cations, could explain this ob-  
327 servation. To verify such an assumption, doubly charged species with either the two same cations  
328 or different ones were investigated.

### 329 3.1.2 Homocationic doubly charged species

330 While the use of two cations generally led to higher CCS values, it was often accompanied by a  
331 decrease in  $R$  and  $r$ , which ranged from minor to substantial (Fig. 4-5, Table S2).

332 *Case of  $G_1$ - $\beta$ -CD/ $\gamma$ -CD couple.* Ternary complexes involving two lithium, led to a well re-  
333 solved couple peaks at CCS of 348.9/338.1  $\text{\AA}^2$  for  $G_1$ - $\beta$ -CD (Fig. 4A, top), while a single peak with  
334 a CCS equal to 379.9  $\text{\AA}^2$  occurred for  $\gamma$ -CD (Fig. 4A, bottom). Regarding doubly sodiated species,  
335 three partially resolved peaks occurred for  $G_1$ - $\beta$ -CD including two with high intensity at 365.3 (57  
336 %) and 354.0  $\text{\AA}^2$  (40 %) and a very low one at 346.0  $\text{\AA}^2$  (3 %) (Fig. 4B, top), while a prominent one  
337 at 364.9  $\text{\AA}^2$  (98 %) with a slight shouldering at 354.5  $\text{\AA}^2$  (2 %) takes place for  $\gamma$ -CD (Fig. 4B, bottom).  
338 As compared to singly potassiated  $G_1$ - $\beta$ -CD, the corresponding doubly charged species including  
339 two potassium, yielded clearer mobilogram traces, where three quite resolved peaks were ob-  
340 served at 359.3/371.6/350.0  $\text{\AA}^2$  (59/29/12) (Fig. 5C, top).  $[\gamma\text{-CD}+2\text{K}]^{2+}$  exhibited a couple of main  
341 peaks at 366.4/349.8  $\text{\AA}^2$  (45/41) and a less abundant one at 358.2  $\text{\AA}^2$  (15 %) (Fig. 5C,

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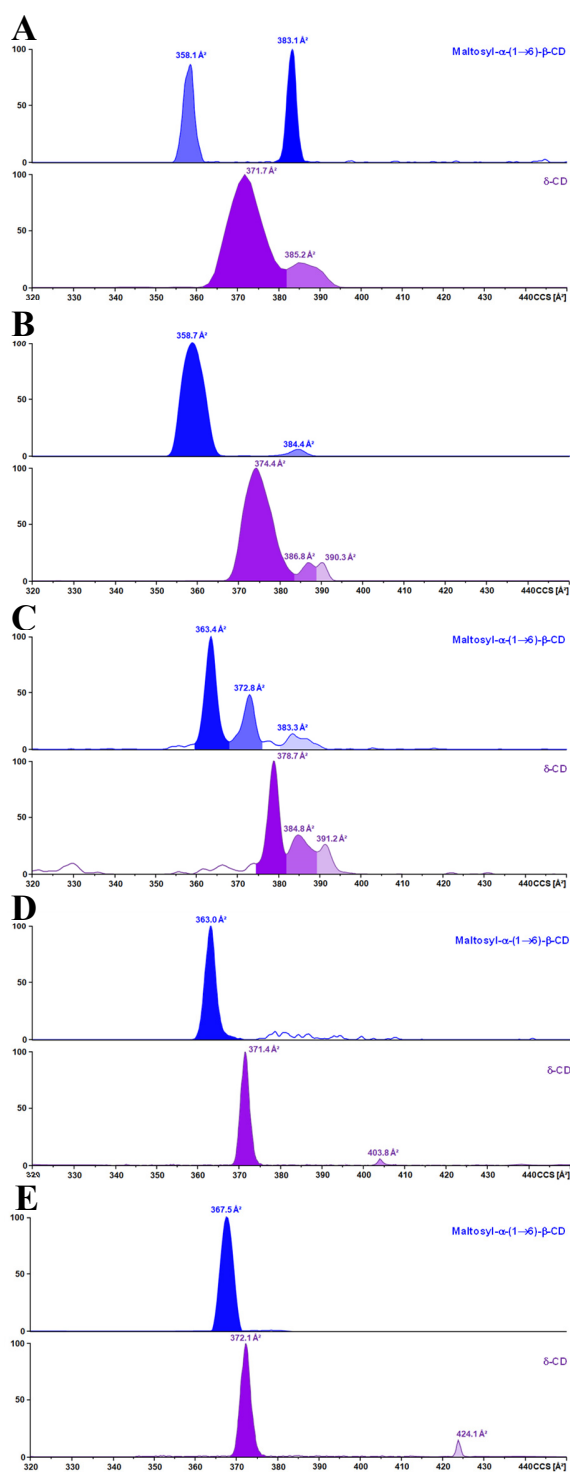


**Fig. 4.** TIMS based mobilograms and corresponding CCS for homocationic doubly charged ions of  $G_1$ - $\beta$ -CD (top, green trace) and  $\gamma$ -CD (bottom, red trace), with A) lithium, B) sodium, C) potassium, D) rubidium and E) cesium.

387 bottom). Similarly, at the difference of the corresponding singly charged species, the introduction  
388 of two rubidium with  $G_1\text{-}\beta\text{-CD}$  yielded two well resolved peaks with a CCS value of  $365.3 \text{ \AA}^2$  (86  
389 %) and  $358.2 \text{ \AA}^2$  (14 %) (Fig. 4D, top), while a unique one is present at  $358.2 \text{ \AA}^2$  for  $\gamma\text{-CD}$  (Fig. 4D,  
390 bottom). With two cesium, a quite close behaviour to two rubidium complexes was observed. In  
391 detail, two large and partially resolved peaks at  $374.1$  and  $360.4 \text{ \AA}^2$  (71/29) occurred for  $[G_1\text{-}\beta\text{-}$   
392  $\text{CD}+2\text{Cs}]^{2+}$  (Fig. 4E, top), while only a narrow peak at  $359.4 \text{ \AA}^2$  was present for  $[\gamma\text{-CD}+2\text{Cs}]^{2+}$  (Fig. 4E,  
393 bottom). Careful examination of CCS values both for singly and doubly charged ions with a given  
394 cation unambiguously confirmed the presence of  $\gamma\text{-CD}$  in the  $G_1\text{-}\beta\text{-CD}$  sample.

395 *Case of  $G_2\text{-}\beta\text{-CD}/\delta\text{-CD}$  couple.* As observed for their singly lithiated counterpart, complex-  
396 ation of  $G_2\text{-}\beta\text{-CD}$  with two lithium led to two well resolved peaks at  $383.1/358.1 \text{ \AA}^2$  (54/46) for  
397  $G_2\text{-}\beta\text{-CD}$  (Fig. 5A, top), while unresolved ones were obtained at  $371.3/385.2 \text{ \AA}^2$  (83/17) for  $\delta\text{-CD}$   
398 (Fig. 5A, bottom). The same trend was observed for  $[G_2\text{-}\beta\text{-CD}+2\text{Na}]^{2+}$  where a couple of distant  
399 peaks were clearly observed with major/minor abundance at  $358.7/384.4 \text{ \AA}^2$  (95/5) (Figure 5B,  
400 top). For  $\delta\text{-CD}$ , a main and large peak at  $374.4 \text{ \AA}^2$  (78 %) and two lower unresolved ones at  
401  $386.8/390.3 \text{ \AA}^2$  (11/11) were detected (Fig. 5B, bottom). Moreover, contrary to their correspond-  
402 ing singly charged species, a complete return to the baseline occurred between the two peaks  
403 from  $[G_2\text{-}\beta\text{-CD}+2\text{Li}]^{2+}$  and  $[G_2\text{-}\beta\text{-CD}+2\text{Na}]^{2+}$ . That suggests the existence of two well distinct and  
404 stabilized structures. As quoted out for  $G_1\text{-}\beta\text{-CD}/\gamma\text{-CD}$  couple, simultaneous complexation of two  
405 potassium tends to simplify the mobilogram traces. Indeed, three quite resolved peaks at  
406  $363.4/372.8/383.3 \text{ \AA}^2$  (Fig. 5C, top) were present for  $[G_2\text{-}\beta\text{-CD}+2\text{K}]^{2+}$ , while  $[\delta\text{-CD}+2\text{K}]^{2+}$  led to a  
407 major peak at  $378.7 \text{ \AA}^2$  (65 %) and two others ones with both lower abundance and weak

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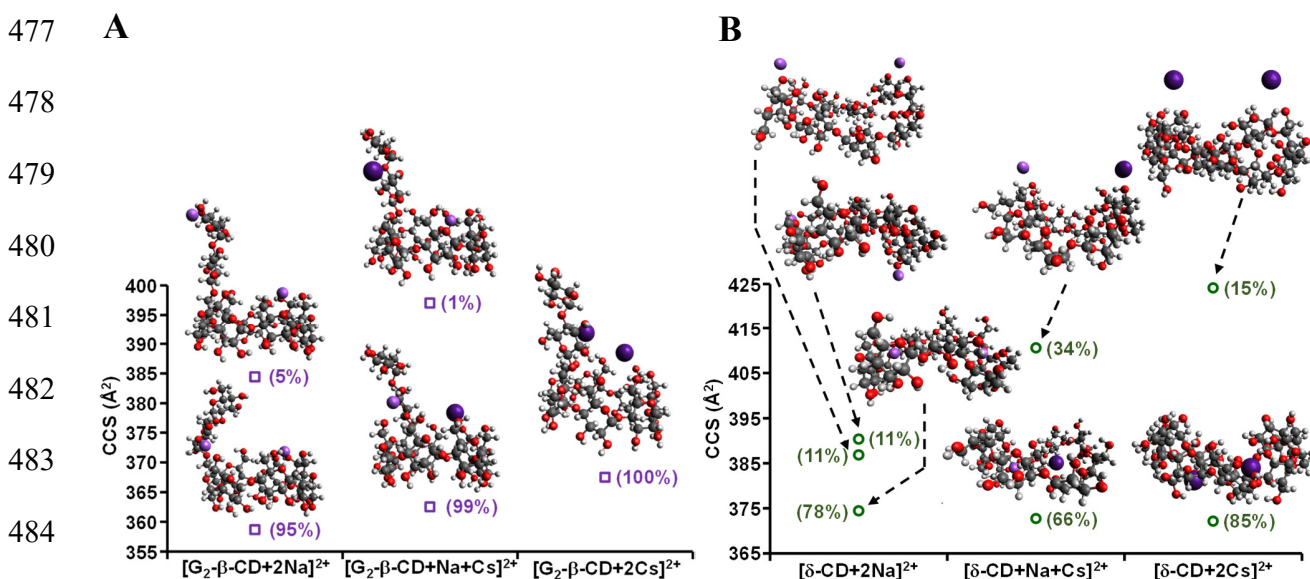
**Fig. 5.** TIMS based mobilograms and corresponding CCS for homocationic doubly charged ions of  $G_2$ - $\beta$ -CD (top, blue trace) and  $\delta$ -CD (bottom, purple trace) with A) lithium, B) sodium, C) potassium, D) rubidium and E) cesium.

452 resolution at 384.8 (19 %) and 391.2 Å<sup>2</sup> (17 %) (Fig. 5C, bottom). Regarding [G<sub>2</sub>-β-CD+2Rb]<sup>2+</sup>, a  
453 single peak was detected at 363.0 Å<sup>2</sup> (Fig. 5D, top), while for δ-CD a largely prominent one at  
454 371.4 Å<sup>2</sup> (96 %), and a very minor one at 403.8 Å<sup>2</sup> (4 %) (Fig. 5D, bottom) were found. The same  
455 conclusion can be drawn from the doubly cesiated species, where only one peak was detected at  
456 367.5 Å<sup>2</sup> for [G<sub>2</sub>-β-CD+2Cs]<sup>2+</sup> (Fig. 5E, top), and a couple of peaks at 372.1/424.1 Å<sup>2</sup> (85/15) for  
457 [δ-CD+2Cs]<sup>2+</sup> (Fig. 5E, bottom). The results on the doubly charged G<sub>2</sub>-β-CD/δ-CD couple, con-  
458 firmed that there is no clear evidence of the presence of δ-CD in the G<sub>2</sub>-β-CD sample.

### 459 3.1.3 Heterocationic doubly charged species

460 Considering heterocationic doubly charged species, i.e. complexes involving one cy-  
461 clodextrin with two different cations (Table S2), some observations can be done: i) None ion as-  
462 cribed to [CD+Li+X]<sup>2+</sup> or [CD+H+Y]<sup>2+</sup> (where X = H, Rb or Cs and Y = Li, Rb or Cs), was detected for  
463 the four analysed samples, ii) all obtained CCS values are not necessarily included between those  
464 of their corresponding homocationic doubly charged analogues ([CD+X+Y]<sup>2+</sup> compared to  
465 [CD+2X]<sup>2+</sup> and [CD+2Y]<sup>2+</sup>, where X and Y are two different cations), iii) the number of detected  
466 CCS can be different of their corresponding homocationic doubly charged counterparts (Table S2  
467 and Figs. S3-S4). The observed results can be explained by the high mobility and weak proton  
468 affinity of CDs, as well as by the conformational changes induced by the attachment of lithium,  
469 rubidium, and cesium ions to the CDs. Additionally, the nonlinear variations in CCS values be-  
470 tween homo- and heterocationic doubly charged series with similar cations could be due to in-  
471 creased rigidity or flexibility of both the main rim and any branched α(1→6) glucose/maltose.  
472 This would be possibly driven by a first attachment of a given cation, before a second occurred.

473 Anyway, several distinct cases can be observed. For example, doubly sodiated  $G_2\text{-}\beta\text{-CD}$  resulted  
 474 in two different conformations each featuring a sodium stabilized by the primary rim. The second  
 475 sodium was located either adjacent to the primary rim and the branched maltose or at the non-  
 476 reducing end of this last one at  $358.7$  (95 %) and  $384.4 \text{ \AA}^2$  (5 %), respectively (Fig. 6A).



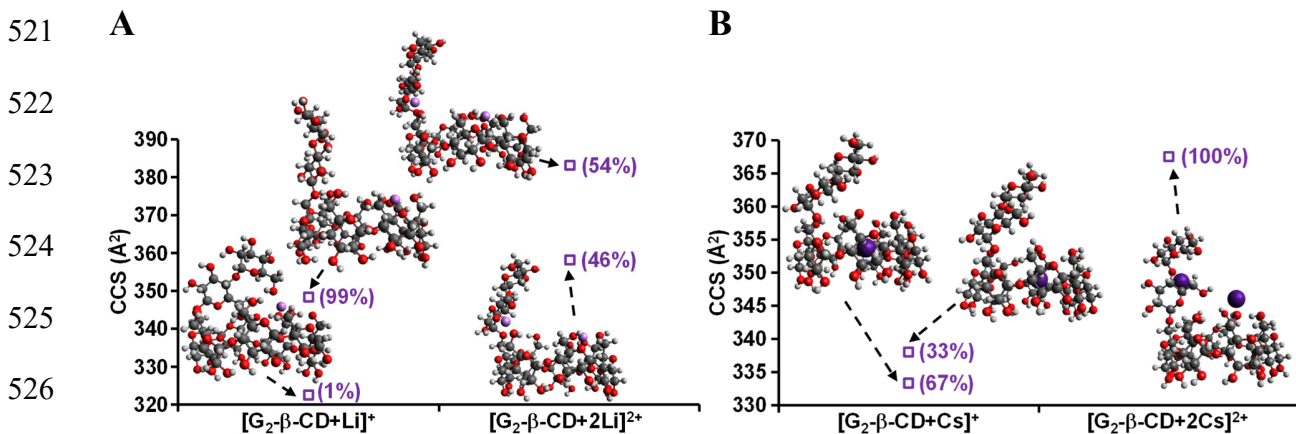
485 **Fig. 6.** Evolution of CCS and ascribed theoretical conformations of doubly charged species  $[CD+2Na]^{2+}$ ,  
 486  $[CD+Na+Cs]^{2+}$  and  $[CD+2Cs]^{2+}$  for A)  $G_2\text{-}\beta\text{-CD}$  (violet square) and B)  $\delta\text{-CD}$  (green circle). The number in brackets  
 487 indicates the relative content of each supposed conformation.  
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490 The formation of a supramolecular complex comprising a sodium and a cesium resulted in the  
 491 emergence of two distinct conformations. It involves a first cation stabilized by the primary rim  
 492 and a second one by the grafted maltose backbone leading to the corresponding couples Cs/Na  
 493 at  $362.5 \text{ \AA}^2$  (99 %) and Na/Cs at  $397 \text{ \AA}^2$  (1 %), respectively. Regarding  $[G_2\text{-}\beta\text{-CD}+2Cs]^{2+}$ , a unique  
 494 peak was observed at  $367.5 \text{ \AA}^2$ . Such CCS value was ascribed to a single but a lesser stable con-  
 495 formation than previous ones, emphasizing some limits in accommodation of both CD rim and  
 496 branched maltose, portraying the difficulty to successfully form and detect complex involving

497 two cesium ions. Conversely, the presence of two both flipped linkages and twisted rings in the  
498  $\delta$ -CD confers a high degree of conformational flexibility, enabling the observation of three distinct  
499 peaks corresponding to doubly sodiated forms at 374.4 Å<sup>2</sup> (78 %), 376.8 Å<sup>2</sup> (11 %) and, 390.3 Å<sup>2</sup>  
500 (11%), respectively (Fig. 6B). Following the previous order, the three potential conformers can be  
501 basically described as functions of the position of the two sodium with respect to  $\delta$ -CD such as:  
502 centre-centre, top-top, and top-bottom, respectively. The substitution of one sodium by a larger  
503 ion such as cesium ( $[\delta\text{-CD}+\text{Na}+\text{Cs}]^{2+}$ ), still led to a structural rearrangement entailing only two  
504 peaks at 372.7 Å<sup>2</sup> (66 %; centre-centre) and 410.6 Å<sup>2</sup> (34 %; top-top). It can be quoted out that  
505 the most abundant peak (centre-centre) matches very well with a more compact structuration  
506 than for the three observed  $[\delta\text{-CD}+2\text{Na}]^{2+}$  ions. This output suggests that the CCS of the supra-  
507 molecular complex is not solely determined by the size of the cation, but also by the CD's capacity  
508 to accommodate optimal template. This assumption is supported by the results from the doubly  
509 cesiated ions, where a major peak (85 %) exhibits a slightly smaller CCS at 372.1 Å<sup>2</sup> (centre-centre)  
510 than the heterocationic sodiated/cesiated analogues. On the other hand, a lower intense peak  
511 at 424.1 Å<sup>2</sup> (15 %; top-top) was also detected. Hence, the aforementioned hypothesis which can  
512 explain the difference in the number of detected CCS based peaks between hetero- and homo-  
513 cationic doubly charged species, can also be valid for the comparison between some homoca-  
514 tionic doubly charged species, and their singly charged analogues. For example, with the smaller  
515 alkali metal studied herein, the singly lithiated G<sub>2</sub>- $\beta$ -CD showed a main peak at 348.3 Å<sup>2</sup> (99 %)  
516 and a minor peak with a more compact structure at 322.4 Å<sup>2</sup> (1 %) (Fig. 7A). Structurally, lithium  
517 is stabilized in both cases by three contact points with oxygen from the primary rim. The only



518 slight difference between the two peaks is that in the minor peak lithium is more centred at the  
 519 top of the deformed cavity and additionally stabilized by the primary oxygen from the bending  
 520 of the maltose chain.



527  
 528 **Fig. 7.** Evolution of CCS and ascribed theoretical conformations of  $G_2\text{-}\beta\text{-CD}$  (violet square) as singly and doubly  
 529 species with A) lithium and B) cesium. Number in brackets indicates the relative content of each supposed conforma-  
 530 tion.  
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532 A more contrasted picture from the bending of the maltose chain was obtained with  $[G_2\text{-}\beta\text{-}$   
 533  $CD+2Li]^{2+}$ , with two peaks highlighting a balanced content at  $358.1 \text{ \AA}^2$  (46 %) and  $383.1 \text{ \AA}^2$  (54 %).  
 534 In the first case, one lithium is centred at the top of the deformed cavity and the second at the  
 535 linkage between the CD core and orthogonal orientated maltose. In the second case, a similar  
 536 structuration occurred, but with lesser inwardly curved maltose. In any case, lithium is stabilized  
 537 by three proximal oxygens of the rim, as well as an additional oxygen located opposite to the  
 538 linkage between CD core and maltose. Considering the largest metal, i.e. cesium,  $[G_2\text{-}\beta\text{-CD+Cs}]^+$   
 539 ion yielded two distinct CCS at  $333.3 \text{ \AA}^2$  (67 %) and  $338.0 \text{ \AA}^2$  (33 %), while  $[G_2\text{-}\beta\text{-CD+2Cs}]^{2+}$  exhib-  
 540 ited a unique peak at  $367.5 \text{ \AA}^2$  (Fig. 7B). For singly charged ions, cesium was centred in the more  
 541 or less deformed cavity linked to a bent maltose chain, while for doubly charged ions, one cesium

542 is located at the top of the deformed cavity and the second cesium is stabilized by a maltose  
543 chain that is both twisted and bent.

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### 545 **3.2 Differentiation of isomers according to metalation**

#### 546 **3.2.1 Discrimination of structural isomers**

547 Considering complexes with the following stoichiometry CD:number of metals, previous  
548 studies have shown that, as either the larger the size of the CDs (from  $\alpha$ -,  $\beta$ - to  $\gamma$ -CD (Przybylski  
549 & Bonnet, 2022), or the higher the methylation degree on  $\beta$ -CD (Przybylski et al., 2015), the  
550 higher the proportion of 1:2 complexes relative to 1:1 complexes increased. This is consistent  
551 with the results observed in the current study, which indicate that the proportion of 1:2 com-  
552 plexes relative to 1:1 complexes increases continuously from  $\gamma$ -CD to  $\delta$ -CD and from  $G_1$ - $\beta$ -CD to  
553  $G_2$ - $\beta$ -CD, as evidenced by the sum of the relative intensities observed by MS for all alkali metal  
554 cation-based CDs. Nonetheless, contrasted results were obtained regarding the trends for the  
555 isomeric couples i.e.  $G_1$ - $\beta$ -CD/ $\gamma$ -CD and  $G_2$ - $\beta$ -CD/ $\delta$ -CD (Table S1-S2). For the former couples, lith-  
556 ium, sodium and cesium are suitable to unambiguously discriminate any singly charged structural  
557 isomers. Of particular interest, high-resolution  $r$  (1.4-1.7, Table S1) analysis revealed the presence  
558 of a small proportion of  $\gamma$ -CD in the  $G_1$ - $\beta$ -CD sample. Conversely, potassium and rubidium did not  
559 give a satisfactory resolution to differentiate the two structural isomers and, consequently are  
560 not appropriate for such purpose. Due to overlapping peaks, it was not possible to unambigu-  
561 ously identify the first CCS of  $G_1$ - $\beta$ -CD from that of  $\gamma$ -CD ( $318.6 \text{ \AA}^2$ ) when using potassium as the  
562 alkali metal cation (Fig. 2C). With rubidium, the unique peak from  $G_1$ - $\beta$ -CD sample resulted in a

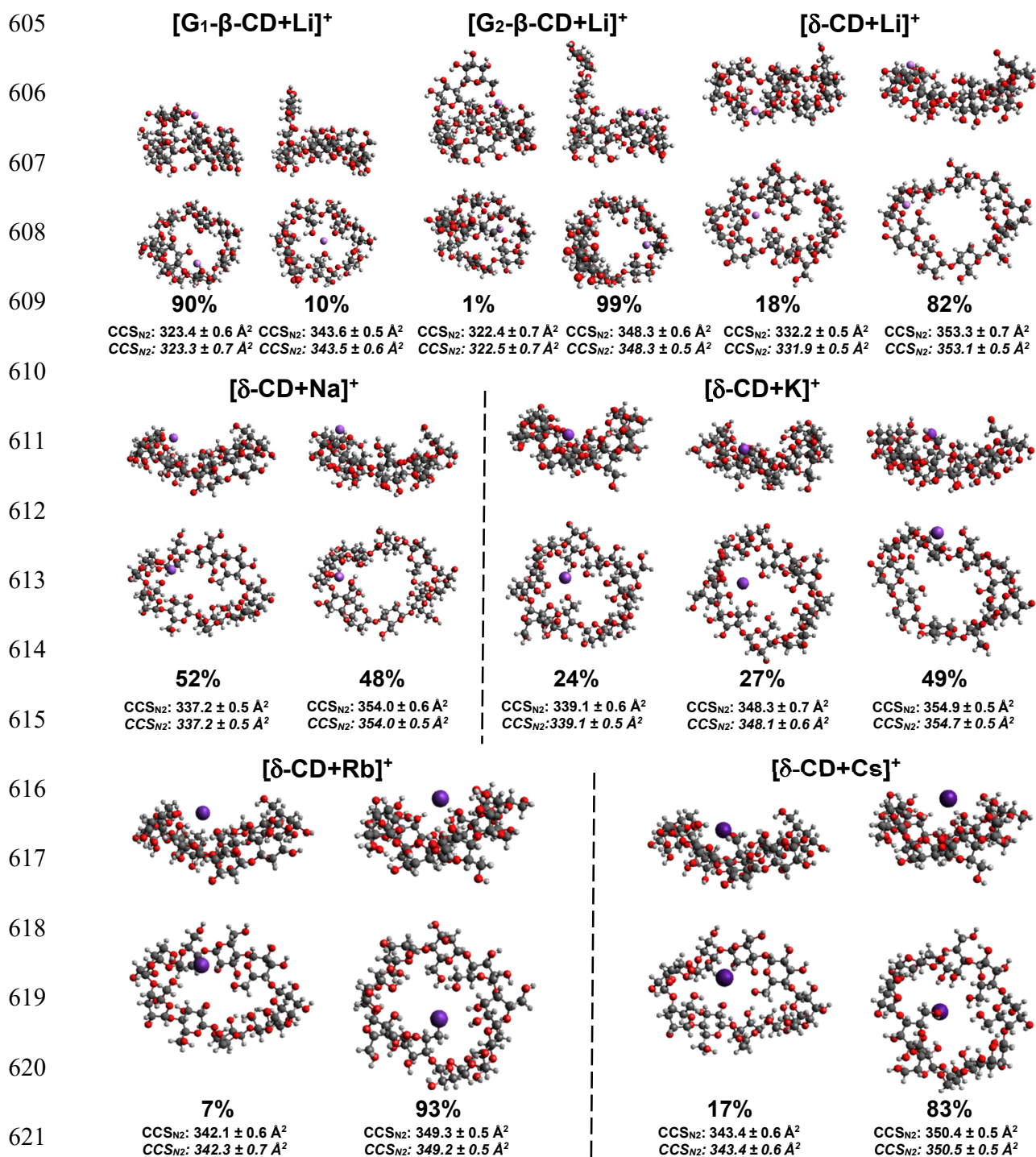
563 peak width that was too large to allow a clear identification of the dedicated CCS value of  $\gamma$ -CD,  
564 as previously mentioned (Fig. 2D). Regarding the latter couple,  $G_2$ - $\beta$ -CD/ $\delta$ -CD, only lithium and  
565 sodium produced satisfactory results in differentiating the two peaks from  $G_2$ - $\beta$ -CD or  $\delta$ -CD, with  
566  $r$  values of 1.3-1.8 and 1.4-3.8, respectively (Fig. 3). Due to a large number of unresolved peaks,  
567 potassium, rubidium and cesium can only be used to partially highlight differences between  $G_2$ -  
568  $\beta$ -CD and  $\delta$ -CD. Indeed, this can be achieved merely by taking into account the lower and higher  
569 CCS, respectively. Therefore, only the following alkali metal cation pairs provide sufficient differ-  
570 entiation between  $G_2$ - $\beta$ -CD and  $\delta$ -CD: 327.8/354.9  $\text{\AA}^2$  ( $r$ : 4), 331.8/349.8  $\text{\AA}^2$  ( $r$ : 3.7) and  
571 333.3/350.4  $\text{\AA}^2$  ( $r$ : 3.3) for potassium, rubidium and cesium, respectively (Fig. 3).

572         At a glance, the mobilograms issued from the simultaneous presence of two identical cat-  
573 ions within the various CD complexes tend to reduce the diversity of possible structures and thus,  
574 clarify the distribution of the CCS based peaks (Fig. 6). This could be attributed to a higher likeli-  
575 hood of certain stabilized structures occurring. Most of herein investigated homocationic doubly  
576 charged ions support an easier differentiation of the two CD couples compared to their singly  
577 charged analogues, as attested by the CCS values. Nonetheless, regarding the deducted mobility  
578 resolution, a more contrasted conclusion can be drawn. Hence, according to a given alkali metal  
579  $r$  values were between 0.7-1.5 and 0.5-3.1 for  $G_1$ - $\beta$ -CD/ $\gamma$ -CD (Fig. 4) and  $G_2$ - $\beta$ -CD/ $\delta$ -CD (Fig. 5),  
580 respectively. Altogether, the aforementioned data have clearly demonstrated that the ability to  
581 efficiently discriminate structural isomers of cyclodextrins must be grasped according to both (i)  
582 the given metal, (ii) the number of adducted metals, and of course, (iii) the considered isomers  
583 couple. Interestingly, we noted that lithium and sodium seem to be most suitable for both  $G_1$ - $\beta$ -

584 CD/ $\gamma$ -CD and G<sub>2</sub>- $\beta$ -CD/ $\delta$ -CD couples as well as both singly and homocationic doubly charged spe-  
585 cies. Regarding resolution, G<sub>1</sub>- $\beta$ -CD/ $\gamma$ -CD with lithium/sodium,  $r$  is 1.7/1.4 and 0.9/1.3 for singly  
586 and homocationic doubly charged species, respectively, while for G<sub>2</sub>- $\beta$ -CD/ $\delta$ -CD,  $r$  is 1.7/1.4 and  
587 0.9/1.3 for singly and homocationic doubly charged species, respectively. In addition, the good  
588 to high resolution  $r$  (0.3-6.4) through a given mobilogram, offers, most of the time, the possibility  
589 to estimate the relative content of each species in a given sample. Hence, once the peaks that  
590 may correspond to structural isomers have been identified, it is necessary to determine the iden-  
591 tity of those remaining. In this sense, the hypothesis of the presence of conformational isomers  
592 must be investigated.

### 593 **3.2.2 Revealing the presence of conformers**

594 If the observation of the profound effect from the charge site on an ion's gas-phase be-  
595 haviour i.e. distinct protonation site isomers, the well-known protomers, is very well described  
596 for proteins and peptides by ion mobility, the influence of cationization sites on cyclic oligosac-  
597 charide structure remains quite under-investigated. The glucopyranose units within CDs are very  
598 flexible as a consequence of the rotation and rapid proton exchange of the primary and second-  
599 ary hydroxyl groups, and the C5-C6 bond shows a high degree of rotation, which can be also  
600 supported to some extent by the glycosidic bond rotation. These intrinsic characteristics involve  
601 that many conformers can co-exist, especially in equilibrium in an aqueous solution, as investi-  
602 gated by theoretical studies (Gamboa-Carballo et al., 2017; Lipkowitz, 1998). Except for potas-  
603 sium,  $\gamma$ -CD exhibits only one peak with all other studied metals suggesting that only one con-  
604 former can be detected. Conversely, considering G<sub>1</sub>- $\beta$ -CD analysis, and excluding peaks that may



622 **Fig. 8.** Side and top views of potential CD/alkali metal structures-based conformers obtained by theoretical ap-  
 623 proaches and matching with experimental CCS. The estimated content was given below each structure (%) as well  
 624 as the experimental (top, plain) and theoretical (bottom, italic) CCS<sub>N2</sub> values. Color coding: very light violet; lithium  
 625 (first row) light to dark violet; sodium, potassium, rubidium and cesium (second and third row), pink; carbon, gray;  
 626 oxygen, light red; hydrogen, white.

627 be provided by  $\gamma$ -CD traces, singly charged ion yields to two or more peaks (Fig. 1). Molecular  
628 modelling computation allowed us to ascribe the experimental CCS values to potential structures.  
629 For example,  $[\text{G}_1\text{-}\beta\text{-CD+Li}]^+$  yielded to two peaks at  $323.4 \pm 0.6$  and  $343.6 \pm 0.5 \text{ \AA}^2$  ( $r$ : 2.1). The  
630 former is quite in a closed form (Fig. 5,  $[\text{G}_1\text{-}\beta\text{-CD+Li}]^+$ , 90 %), with grafted glucose unit acting as a  
631 bent lid, trapping lithium between it and side oxygens from the primary rim. The latter appears  
632 to have an open structure (Fig. 5,  $[\text{G}_1\text{-}\beta\text{-CD+Li}]^+$ , 10 %) where lithium is stabilized at the top centre  
633 of the primary rim. In this last arrangement, the side glucose block is perpendicularly oriented to  
634 the primary rim, and the secondary rim exhibits a concave distortion. Regarding  $[\text{G}_2\text{-}\beta\text{-CD+Li}]^+$ ,  
635 the main form, by far, shows a quite perpendicular orientation of the grafted maltose on one side  
636 of the primary rim and a stabilization of the cation at the opposite side.

637 In the case of  $\delta$ -CD, the very high flexibility of the glycosidic backbone allows it to adopt  
638 several conformations. Indeed,  $\delta$ -CD is the first member of the relatively under-investigated  
639 large-ring cyclodextrins (LRCD) where both glucose flips and linkage kinks occurred (French &  
640 Johnson, 2007; Raffaini & Ganazzoli, 2007; Saenger et al., 1998). Such enhanced degree of free-  
641 dom offers the opportunity for a given cation to be stabilized at the primary rim, at a so-called  
642 pseudo-secondary rim (designed as such due to the contribution of a flipped glucose unit) or also  
643 in the centre of a more compacted structure. For  $[\delta\text{-CD+Li}]^+$ , the identified structure highlighted  
644 that lithium can be located at the primary rim or at a pseudo- secondary rim, with content equal  
645 to 82 and 18 %, respectively (Fig. 8,  $[\delta\text{-CD+Li}]^+$ ). Switching from lithium to sodium still leads to  
646 two distinct conformations both with sodium located at the primary rim. In the first conformation  
647 (Fig. 8,  $[\delta\text{-CD+Na}]^+$ , 52 %), the cation attachment site at the  $n$  and  $n+1$  units resulted from two

648 flips towards the exterior of the cavity of glucose at the  $n-1$  and  $n+2$  positions. This induced a kink  
649 of the glycosidic bonds between  $n-1/n$  and  $n+1/n+2$  units, allowing a tight coordination of  $\text{Na}^+$   
650 with two primary oxygens ( $\text{O}\cdots\text{Na}$ : 2.187 and 2.207 Å). In the second conformation (Fig. 8, [ $\delta$ -  
651  $\text{CD}+\text{Na}$ ] $^+$ , 48 %), the cation was also coordinated at  $n$  and  $n+1$  unit, but involved a three-point  
652 interaction. To elaborate further, the coordination of sodium in the second conformation in-  
653 volved the two primary oxygens of  $n$  and  $n+1$  (2.175 and 2.313 Å), and oxygen from glycosidic  
654 bonds between  $n$  and  $n+1$  (2.477 Å). This coordination was made possible due to a more pro-  
655 nounced deformation of the cyclodextrin, with glucose at  $n+2$  and  $n-3$  flipping towards the inte-  
656 rior of the cavity. The observed CD enlargement resulting from the more pronounced defor-  
657 mation of the cyclodextrin strongly supports the higher CCS value of the second conformation  
658 compared to the first. With potassium, which presents a higher ionic radius than sodium and  
659 lithium (137 versus 99 and 59 pm, respectively), a greater distance between the cation and ox-  
660 ygens is needed to achieve the most stable non-covalent complex. Three distinct conformations  
661 were delineated. The less abundant involves three  $\text{O}\cdots\text{K}$  bonds: one secondary oxygen O(1) of  
662 the  $n$  units (2.617 Å), and two primary ones O(6) from  $n-2$  (2.801 Å) and  $n+3$  (2.756 Å) which both  
663 own two glucose units tilted by  $\sim 90^\circ$  towards the interior of the cavity (Fig. 8, [ $\delta$ - $\text{CD}+\text{K}$ ] $^+$ , 24 %).  
664 The second one is only slightly more intense than the first one, and also stabilized by three-points  
665 interaction as O(1) of the  $n$  units (2.572 Å), and two O(6) from  $n-1$  (2.815 Å) and  $n+3$  (2.688 Å)  
666 both also exhibiting bent glucose towards the cavity's centre (Fig. 8, [ $\delta$ - $\text{CD}+\text{K}$ ] $^+$ , 27 %). The prom-  
667 inent conformation, exhibiting the highest CCS, was ascribed to an extended boat shape. In such  
668 an arrangement, potassium is situated within a pocket rich in oxygen at the outer edge of the

669 primary rim, where two closer O(6)···K bonds occur than in the previous structures, originating,  
670 from  $n$  (2.492 Å) and  $n+1$  (2.616 Å) units (Fig. 8,  $[\delta\text{-CD+K}]^+$ , 49 %). Regarding larger cations like  
671 rubidium ( $r_i = 152$  pm), two different conformations can be deduced from CCS values. In the  
672 minor one, two oxygens from the primary rim from  $n$  (3.346 pm) and  $n+1$  (3.710 pm) units inter-  
673 act with the cation, while at the exact opposite side of the rim ( $n+3$ ) glucose is again tilted as for  
674  $[\delta\text{-CD+K}]^+$  (Fig. 8,  $[\delta\text{-CD+Rb}]^+$ , 7 %). The prominent conformation presents an important distortion  
675 of the cavity leading to a quite concave shape. Rubidium was located quite in the bottom centre  
676 of the cavity, by bonding at O(1) of  $n$  unit (3.818 Å), O(2) of  $n+4$  (4.039 Å), and two O(6) from  $n+3$   
677 (3.960 Å) and  $n-1$  (4.078 Å). The two O(6) involved result again from perpendicularly twisted glu-  
678 copyranose (Fig. 8,  $[\delta\text{-CD+Rb}]^+$ , 93 %). It must be noted that the examination of CCS values from  
679  $[\delta\text{-CD+Rb}]^+$ , shows that a more compact structure occurred for the minor conformation of  $[\delta\text{-}$   
680  $\text{CD+Rb}]^+$ , than the two less abundant ones from  $[\delta\text{-CD+K}]^+$ . On the other hand for the major one,  
681 the structure seems to be slightly larger than the aforementioned less abundant  $[\delta\text{-CD+K}]^+$  ion,  
682 but more tightened than the main  $[\delta\text{-CD+K}]^+$  complex. Such results strongly suggest a non-negli-  
683 gible effect of rubidium as a compacting agent. As for rubidium, the larger alkali metal studied  
684 here, cesium ( $r_i = 167$  pm), led to two discernible conformational states. The minor conformation  
685 showed a boat shape similar to the prominent  $[\delta\text{-CD+K}]^+$  conformation, but with a smaller CCS  
686 value (343.4 versus 354.9 Å<sup>2</sup>). Cesium is coordinated to two O(6) from the  $n$  (3.831 Å) and  $n+1$   
687 units (4.024 Å) as well as to the hemi-acetal oxygen from  $n-1$  (4.168 Å), while the  $n+3$  glucose is  
688 tilted towards the interior of the cavity (Fig. 8,  $[\delta\text{-CD+Cs}]^+$ , 17 %). Regarding the main confor-  
689 mation, the glucose rim adopts a twisted ribbon shape, which is circular in nature, creating a



690 clamp-like structure with the cation centred in the middle. The unique landscape of the main  
691 conformation arises from the rich electron density of the cyclodextrin, which enables multiple  
692 interactions. Specifically, the interaction between O(1) and O(2) of the  $n$  unit (at 3.997 and 4.317  
693 Å, respectively), along with the O(6) of both  $n-4$  and  $n+4$  units (at 4.001 and 4.335 Å, respectively),  
694 facilitates the centering of the cation in the middle of the deformed cavity. This interaction is  
695 further favoured by the twisted glucose units at positions  $n-1$  and  $n+4$ , which cause the O(6)  
696 groups to orient towards the centre of the cavity.

#### 697 **4. CONCLUSIONS**

698 In this study, we have unambiguously demonstrated that ESI-TIMS-TOF is an effective  
699 method for distinguishing positional isomers of macrocyclic carbohydrates, such as cyclodextrins.  
700 This capability also provides the opportunity to identify and quantify any residual traces of other  
701 isomers, allowing for an estimation of the purity of the investigated sample. However, many pre-  
702 cautions must be taken with the choice of the dedicated metal as well as the selected charge  
703 state, according to target both resolving power and resolution. As for the CCS values, it is apparent  
704 that the CD-metal complexes are not exclusively governed by the size of the cation. Indeed, the  
705 presence of not negligible flexibility of the main rim and grafted chain for  $G_1$ - $\beta$ -CD and  $G_2$ - $\beta$ -CD  
706 and more enhanced distortion for  $\gamma$ -CD and  $\delta$ -CD strongly influence the size/shape of the resulting  
707 complexes.

708 In contrast to the smaller CD with quite syn-relative conformations of the glucopyranoside  
709 units,  $\gamma$ -CD and  $\delta$ -CD and larger CD show a high propensity to form band flips and kinks leading to  
710 twisted glucose units. These conformers are expected to be in equilibrium in aqueous solution

711 (Lipkowitz, 1998). NMR, which is the reference technique to study conformational changes, is a  
712 quite slow spectroscopic technic when compared to the timescale of conformational changes of  
713 CDs (Schneider et al., 1998). Indeed, the magnetic pulses are applied to an enormous number of  
714 molecules, which means that NMR measurements are averaged over time and space. These lim-  
715 itations imply that signals from atoms located at the same position on different glucopyranose  
716 units are not distinguished in an NMR spectrum. For this reason, NMR spectra will always lead to  
717 symmetrical CDs, which makes this technic not suitable for conformers study. In the present in-  
718 vestigation, the use of electrospray coupled with trapped ion mobility mass spectrometry allows  
719 to by-pass the short timescale of interconversion, which can preclude the separation of confor-  
720 mational isomers in most cases. Such a combination allows us to capture a snapshot portraying  
721 the solution. It relies on the sequential “freezing” ability of the molecular state from solution and  
722 high resolution separation according to shape/size, as previously demonstrated for carbohydrates  
723 and CDs (Przybylski & Bonnet, 2021, 2022; Sastre Toraño et al., 2021). It also seems to be reason-  
724 able to assume that the several dozen nanosecond timescales required for potential CD confor-  
725 mational switching is compatible with this observation (Gotsev & Ivanov, 2007). For a given ion,  
726 the matching of CCS values with molecular modelling approaches, permitted to highlight the pres-  
727 ence of several stable conformers. For a more accurate sample characterization, those last results  
728 suggest considering not only the CCS value for a given sample, but also the distribution of the  
729 resulting peaks pattern. Nonetheless, some pending questions remain, what is the behaviour with  
730 higher-valence metals such as transition or noble ones? Is it the type and the location of the cat-  
731 ion that induce the various conformation or the prior most adapted conformations which allow

732 the best match attachment of the cation? Also, how many conformers can be distinguished and  
733 identified with other grafting locations, such as CD10 and larger?

#### 734 **CONFLICTS OF INTEREST**

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

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