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## ABSTRACT

The aim of this study was to determine the impact of the origin and the manufacturing processes of the chiral stationary phases (CSPs) on their chromatographic behaviors. Hence, four chiral stationary phases based on immobilized tris (3,5-dichlorophenylcarbamate) derivative of cellulose supplied by four different manufacturers were evaluated. A set of twenty-nine compounds, including commercially available and in-house synthesized compounds, with a broad range of lipophilicity and polarity was chosen. Three main parameters were evaluated on all stationary phases: retention factor, selectivity and loading capacity. This work highlighted that the retention factor strongly varied according to the manufacturer. Regardless of the characteristic of the tested compounds *i.e* neutral, acidic or basic, there was a trend in retention ability of the four chiral stationary phases: retention was increasing from CHIRAL ART Cellulose-SC, REFLECT I-Cellulose C, Chiralpak IC to Lux i-Cellulose-5. On the contrary, selectivity did not follow the same trend as retention. The difference in selectivity between each column towards one compound was quite low while the difference in resolution depended on the nature of the compounds investigated and was significant in certain cases. Finally, the four different columns presented similar and high loading capacity.

## 52 1. Introduction

53 In pharmaceutical industry, the separation of enantiomers is an ever-growing field as  
54 enantiomers of the active substance can present different activities and toxicities, having a  
55 great impact on the processes of drug discovery and development. Consequently, in the last  
56 decades a strong endorsement could be observed, in the development of enantioselective  
57 syntheses and the enantioselective separation methods of new drugs.

58 The most important enantioseparation techniques are High-Performance Liquid  
59 Chromatography (HPLC) and Supercritical Fluid Chromatography (SFC), which has taken  
60 over the field. Chiral SFC has been providing for almost 30 years an efficient pathway to  
61 check the chiral purity of compounds, but also to provide enantiomerically pure materials for  
62 synthesis, active pharmaceutical ingredients and drugs [1-5].

63 The selection of an appropriate chiral stationary phase (CSP) represents an important step in  
64 the development of most chromatographic methods. There is a large range of CSPs, utilized  
65 for chiral resolution including polysaccharides. Polysaccharides (cellulose and amylose)  
66 derived chiral stationary phases have proved their usefulness as chiral selector in HPLC as  
67 well as in SFC, and a wide range of enantiomeric compounds have been successfully  
68 separated on these CSPs. These coated polysaccharide-based CSPs are the most popular  
69 choice firstly due to their large loadability (with saturation capacity ranged between 7.5 to  
70 more than 100 mg/g CSP) which is extremely important for preparative scale chromatography  
71 and secondly because this coated polysaccharides technology that run out of patent, has  
72 caused a revival of activity from many different manufacturers. Non-chlorinated  
73 polysaccharide-based CSPs have successfully resolved a vast majority of chiral compounds in  
74 a broad variety of structural classes [6-8], providing small and large quantities of  
75 enantiomerically pure active pharmaceutical ingredients for *in-vivo* and toxicological studies  
76 [9-10]. However, these phases have one major disadvantage: they are more or less soluble in

77 many organic solvents (tetrahydrofuran or chlorinated solvents, amongst others) thus reducing  
78 the choice of organic modifier. However, drawbacks, such as the limited resistance of these  
79 coated phases, have been overcome by immobilizing the polysaccharide derivatives, thus  
80 allowing a greater choice of co-solvent. The immobilized versions of the existing coated  
81 phases are of the utmost importance for the preparative scale for two reasons: i) on the one  
82 hand, new co-solvents can improve resolution through improved efficiency, and ii) on the  
83 other hand, can in particular cases considerably improve the solubility of the sample, which in  
84 turn allows to increase the productivity [11-13].

85 More recently introduced chlorinated polysaccharide CSPs (either coated or immobilized) can  
86 provide additional selectivity to the non-chlorinated CSPs. Chankvetadze's studies [14-16]  
87 and Zhang's work [17] on the effect of various groups on the phenyl moiety of different  
88 carbamate CSPs, have shown a link between the structural properties and the separation  
89 performance of the CSPs. The inductive effect of the chlorine atoms, strong electron  
90 withdrawing groups (EWG), can enhance the tendency of the hydrogen atoms to form  
91 hydrogen bonds, which can modify the chemical properties of the CSP, and improve the  
92 chiral recognition. Since separation performance of a chromatographic column strongly  
93 depends on the properties of the stationary phase and therefore results from the manufacturing  
94 process, it is important to have reliable information for an adequate selection of the brand.

95 The aim of our study was a practical comparison of cellulose tris (3,5-  
96 dichlorophenylcarbamate) columns purchased from four different manufacturers to evaluate  
97 their chromatographic performances. For this comparison, a set of different chromatographic  
98 parameters for different racemates were evaluated. In this study both commercially available  
99 and in-house synthesized compounds, containing one chiral center were used. Preparative  
100 chromatography has emerged as an asset in the synthesis of preclinical drug candidates and  
101 SFC has become a widely accepted technique for the small-scale preparation (hundreds of

102 milligrams) of pure enantiomers of novel drug candidates needed for preclinical and early  
103 clinical studies [18]. Therefore, the loadability of each column was also studied, keeping in  
104 mind that this work is not intended to explain the differences observed because the  
105 manufacturing process is confidential and might be the same anyway, but rather to give  
106 practical information to users in order to save time in method development.

## 107 2. Material and methods

### 108 2.1 Chemicals

109 Methanol (MeOH), ethanol (EtOH), propan-2-ol (iPrOH) and acetonitrile (ACN) were of  
110 HPLC grade and were purchased from Carlo-Erba Reagents (Val-de-Reuil, France). Carbon  
111 dioxide (CO<sub>2</sub>) of 99.995% purity was purchased from Linde (Saint Priest, France).  
112 Pharmaceutical compounds and triethylamine (TEA) were purchased from Sigma-Aldrich  
113 (Saint-Quentin Fallavier, France) and compounds 21-29 (proprietary structure, Figure 1) were  
114 synthesized internally.

### 115 2.2 Sample solutions

116 For analytical screening, solutions of samples were prepared in methanol at 1 mg/mL. For the  
117 loading study, solutions of samples were prepared in methanol at 10 mg/mL (excepted 1  
118 mg/mL for lenalidomide). The solutions were always degassed by an ultrasonic bath and  
119 filtered through a 0.45 µm PTFE syringe-filter (15 mm diameter) before use.

### 120 2.3 Supercritical Fluid Chromatography Instrument

#### 121 2.3.1 Stationary phases

122 Four chiral analytical columns of cellulose *tris* (3,5-dichlorophenylcarbamate) from different  
123 suppliers were used for this study.

124 Chiralpak IC was purchased from Chiral Technologies Europe (Illkirch, France), Lux i-  
125 Cellulose-5 from Phenomenex® (Le Pecq, France), CHIRAL ART Cellulose-SC from YMC  
126 Europe (Cluzeau Info Labo, Sainte-Foy-La-Grande, France) and the REFLECT I-Cellulose C

127 from Regis Technologies (Thermo Fisher Scientific, Villebon-Courtaboeuf, France). All  
128 columns had dimensions of 250x4.6 mm with 5  $\mu$ m particle size. The polysaccharide was  
129 immobilized on a silica-gel support.

### 130 2.3.2. Chromatographic system and conditions

131 The chromatographic system used was an SFC-PICLAB hybrid 10-20 apparatus (PIC  
132 Solution, Avignon, France) equipped with an autosampler comprising a 48-vials plate (model  
133 Alias, Emmen, Netherlands), three model 40 P pumps: two for CO<sub>2</sub> and a third for the  
134 modifier (Knauer, Berlin, Germany), a column oven with a Valco ten-position column  
135 selection valve, and a Valco six-position solvent switching valve. The system was also  
136 composed of a Smartline 2600 diode array detector (DAD) (Knauer, Berlin, Germany).  
137 Detection wavelength was set at 254 nm. The system was controlled, and the data was  
138 acquired with the SFC PicLab Analytic Online v.3.1.2 software (PIC Solution, Avignon,  
139 France). The volume injected was 20  $\mu$ L. For the loading experiment, the injected volumes  
140 were 50, 100, 250, 485  $\mu$ L, respectively. During the separation optimization, the used flow  
141 rate was 4 mL/min (excepted for acebutolol (cpd 19) for which the flow rate was 6 mL/min)  
142 and the mobile phase was always CO<sub>2</sub>-modifier mixture with 15% of methanol as modifier.  
143 The exceptions being lenalidomide, compound 25, compound 26 and compound 27 for which  
144 the modifier percentage was 30% instead of 15%. For compounds 5, 11, 12, 15, 16, 17 and  
145 18, 5% of methanol was also tested to avoid the co-elution with the solvent peak in order to be  
146 able to evaluate the selectivity. For acebutolol (cpd 19) and 1-(1-naphthyl)ethylamine (cpd  
147 20), 0.3% of triethylamine (TEA) was added to the co-solvent. All analyses were run in  
148 isocratic mode. The column oven temperature was 40 °C and the outlet pressure was  
149 maintained at 150 bar for all the experiments.

150 Retention times were average values of two replicate determinations. The data was processed  
151 by SFC New Data Manager V.1.8.0 software (Pic Solution, Avignon, France).

### 152 3. Results and discussion

153 In this work, four chiral stationary phases based on immobilized tris (3,5-  
154 dichlorophenylcarbamate) derivative of cellulose, coming from four different manufacturers,  
155 were evaluated and cited along the manuscript as four columns for the sake of clarity. For all  
156 of them, the polysaccharide was immobilized to the surface of a silica matrix. This work  
157 notably investigated the practical impact of the different brands on retention, selectivity,  
158 efficiency, resolution and loading capacity. Figure 1 shows the selected compounds with a  
159 broad range of lipophilicity and polar surface area. All the chromatographic parameters are  
160 summarized in Table 1. The direct comparison of the chromatographic parameters was  
161 enabled by i) the supercritical conditions used (high percentage of co-solvent and high back  
162 pressure) permitting to limit the compressibility of the mobile phase, thus limiting density  
163 variation along the column, and therefore the polarity and ii) the similar dimensions of the  
164 four columns. The inlet pressure was noticed for each column leading to a pressure drop of 37  
165 bar on Lux i-Cellulose-5, of 25 bar on Chiralpak IC, of 26 bar on REFLECT I-Cellulose C  
166 and of 24 bar on CHIRAL ART Cellulose-SC column (these values will be discussed later in  
167 the manuscript).

#### 168 3.1 Comparison of retention factors

169 First of all, the graph displayed in Figure 2 showed that the retention factors of the second  
170 enantiomer eluted ( $k_2$ ) varied a lot according to the column supplier, e.g. the retention factor  
171 of omeprazole (compound 1) ranged from 15.9 on CHIRAL ART Cellulose-SC provided by  
172 YMC to 28.3 on Lux i-Cellulose-5 provided by Phenomenex with intermediate values for  
173 both Chiralpak IC and REFLECT I-Cellulose C from Daicel and Regis, respectively.

174 Remarkably, the most retentive column was Lux i-Cellulose-5 from Phenomenex for all the  
175 selected compounds (except for compounds 18 and 28) regardless of the nature of the analyte,  
176 neutral, basic or acid. In addition, the retentiveness of the four columns in decreasing order

177 was always the same going from Lux i-Cellulose-5 (pink plots), Chiralpak IC (orange plots),  
178 REFLECT I-Cellulose C (blue plots) to CHIRAL ART Cellulose-SC (green plots). Meaning  
179 that the retention factors and retention times as well, are higher on the former column and  
180 lower on the latter one. This behavior is exemplified for acebutolol (compound 19) in Figure  
181 S1 (in Supplementary Information). In enantioselective chromatography, retention can result  
182 from either achiral interactions, or chiral interactions, or both. The main adsorbing site of the  
183 cellulose tris (3,5-dichlorophenylcarbamate) derivative CSP is the polar carbamate group  
184 which can interact with analytes through hydrogen bonds and dipole-dipole interactions [19].  
185 The hydrogen bonds are involved in both non-enantioselective and enantioselective  
186 interactions. For this series of compounds, the difference of retention of the second  
187 enantiomer eluted on the four different columns compared may be the consequence of a  
188 difference in achiral or chiral interactions. However, first, a similar trend was observed for the  
189 retention of the first enantiomer eluted of each racemate ( $k_1$ ) as for the second eluted  
190 enantiomer ( $k_2$ ). This means that a difference of retention for the same compound was  
191 observed between the four stationary phases whatever the enantiomers analyzed. Secondly,  
192 the graph on Figure 3, depicting the  $k_2$  versus the selectivity ( $\alpha$ ) for all the compounds,  
193 showed that there was no link between retention and selectivity. This observation allowed to  
194 conclude that the difference in retention capacity of the CSPs was more linked to achiral  
195 interactions than to chiral interactions and confirmed that retention was generally not  
196 correlated with enantio-resolution because strong interactions may occur between analytes  
197 and CSPs while these interactions do not necessarily contribute to the enantioselectivity. The  
198 difference of retentions between the four columns may be the result of the difference in  
199 manufacturing process used by the supplier. Indeed, Khater *et al.* [20] have shown in an  
200 article dedicated to chlorinated polysaccharide stationary phases that the retention of the latter  
201 depended obviously on the nature of the polysaccharide and the type of derivatizing group



202 attached to the glucose moieties of the polysaccharide, but also depended on the  
203 manufacturer. Indeed, two stationary phases with the same chiral selector can exhibit different  
204 retentions towards solutes. According to Khater *et al.*, the difference linked to the  
205 manufacturers was probably due to deviations in the manufacturing procedure between the  
206 different suppliers. In our study, significant differences of retentions were observed between  
207 the four columns, while the polysaccharide and the side chains are the same for all the CSPs  
208 leading to the same conclusion about the manufacturers. One of the explanations could be  
209 linked to the process used to immobilize the polysaccharide on the support. Indeed, several  
210 methods were used to immobilize the polysaccharide derivatives on silica gel: (i) Okamoto *et*  
211 *al.* [21-22] developed a method using diphenyl diisocyanate as a cross linking agent and (ii)  
212 the covalent attachment of cellulose derivative via an epoxide moiety [23]. Another  
213 possibility (iii) was the process developed by Francotte consisting in exposing the coated  
214 phases to photochemical [24] or thermal treatment. A last way (iv) which was a  
215 polymerization of a mixed 10-undecenoyl-3,5-dimethylphenylaminocarbonyl derivative of  
216 cellulose on the matrix was depicted by Minguillon *et al.* [25]. The process of immobilization  
217 of the four columns evaluated in this work is confidential or patented. However, it is obvious  
218 that the immobilization procedure used by the different suppliers could vary with an impact  
219 on the quantity of chiral selector immobilized (carbon load percentage). In the same manner,  
220 the three-dimensional structure of the polysaccharide after immobilization can vary, resulting  
221 in some differences on the retention. In addition, as explained by Khater *et al.* [26], the  
222 immobilization process led to the presence of residual hydroxyl groups on the polysaccharide  
223 and the percentage of hydroxyl groups may be different according to the CSPs with an impact  
224 on the retention. Finally, the nature of the silica gel may vary with a diversity of pore sizes,  
225 surface area, with the use of bare silica or aminopropyl silica or even different pre-treatment  
226 of the support leading to a diversity of physical properties of the stationary phases. All these

227 specificities of the four columns **resulted** in different retention capacities. In conclusion,  
228 although the chiral selector **was** the same in all the four columns, a large difference in the  
229 ability to retain the compounds **was** observed.

### 230 3.2 Comparison of selectivity

231 Just like the retention factors were different on the columns for each of the tested compounds,  
232 the trend **in selectivity was** also **diverse**.

233 Firstly, unlike the retention factor, which varied a lot for this set of compounds, the difference  
234 of selectivity between each column **towards a compound was** quite low as exemplified in  
235 **Figure S2** (in Supplementary Information) for compound **3** (lenalidomide) for which the  
236 selectivity values **were** equal to 2.44, 2.12, 2.39 and 2.31 (Table 1), respectively for Chiralpak  
237 IC, Lux i-Cellulose-5, CHIRAL ART Cellulose SC and REFLECT I-Cellulose C. **Those four**  
238 **values of selectivity represented a relative standard deviation of 6.07% which was the highest**  
239 **calculated among the 29 compounds**.

240 Secondly, the column providing the highest selectivity **was** not the same towards all the  
241 compounds. For the compound **20**, the best separation factor was obtained **within Chiralpak**  
242 **IC**. The CHIRAL ART Cellulose-SC column **provided** the best selectivity for the compound  
243 **14**, while the best separations of the compounds **4** and **25** were obtained on REFLECT I-  
244 Cellulose C and Lux i-Cellulose-5 respectively. Nevertheless, as illustrated in **Figure S3a** (in  
245 Supplementary Information), **Chiralpak IC gave** the best selectivity for **29.2%** of the **tested**  
246 compounds and the highest number of compounds separated ( $\alpha > 1$ ) **was** obtained **on the**  
247 **CHIRAL ART Cellulose-SC and REFLECT I-Cellulose C columns** with **58.7%** of separation  
248 (**figure S3b**, in Supplementary Information). **Remarkably**, for eight racemates no separation  
249 was achieved **on any of the four studied** columns. The chiral recognition ability of the  
250 columns **was** different towards compounds **28** and **29**. Indeed, the enantiomers of the  
251 compound **28 were** separated both on CHIRAL ART Cellulose-SC and REFLECT I-Cellulose

252 C, while no separation was observed on Chiralpak IC and Lux i-Cellulose 5 in the  
253 implemented conditions (Figure S4a, in Supplementary Information). Identically, for the  
254 compound **29**, the enantiomers were not separated on Chiralpak IC while they were partially  
255 separated on the three other columns in the implemented conditions (Figure S4b, in  
256 Supplementary Information).

257 Regarding the fifteen compounds separated on the four columns, an average separation factor  
258 was calculated for each column to give a higher recognition ability for Chiralpak IC for those  
259 compounds. Hence, despite the same chiral selector, some differences were observed between  
260 these four different columns. Indeed, the conditions used to immobilize the chiral selector are  
261 probably different for the four columns with an impact on the chiral recognition. Like the  
262 coating conditions on silica gel significantly influence their chiral recognition ability, the  
263 process used to immobilize the polysaccharide may impact the chiral recognition too [27].  
264 The utilization of different processes of immobilization may change the polymer  
265 configuration and/or the supramolecular structure of the polysaccharide. The high order  
266 structure of the polysaccharide is probably more or less maintained according to the  
267 manufacturer with an impact on the recognition ability. In conclusion, most of the time  
268 selectivity followed the same trend from one column to another, except for compounds **28** and  
269 **29**.

### 270 3.3 Comparison of plate number and resolution

271 The efficiency and resolution values were reported in Table 1 (Supplementary Information).  
272 For a fair comparison, only the 14 fully baseline resolved compounds were chosen. Mainly  
273 because efficiency can be wrongly evaluated for non-separated peak (whose peak width can  
274 be falsely increased by a beginning of separation of both enantiomers).  
275 The plate number was slightly different between the four columns for every compound.  
276 However, these discrepancies were not deeply studied and no conclusion was drawn from this

277 observation because the inlet pressure measured for every column was not the same (as  
278 depicted before) resulting in a difference of diffusion coefficient in the mobile phase with a  
279 potential impact on the efficiency.

280 Like the selectivity, the difference of resolution between each column towards a compound  
281 depended on the compound itself as explained by Figure 4. Some discrepancies were  
282 observed as the relative standard deviation were calculated for the four resolution values  
283 obtained on the four columns for each compound. For instance, the difference between the  
284 four columns was equal to 6.24% for compound **7** (indoprofen) which was quite low, meaning  
285 that the four results obtained on different manufacturer columns were similar. But for some  
286 compound this difference reached 17.56% for compound **1** (omeprazole) with resolution  
287 values equal to 10.27; 6.64; 9.01 and 9.39 and even 19.78% for compound **2** (thalidomide)  
288 with values equal to 4.39; 2.82; 4.52; 3.89 which was significant. For each compound, the  
289 four columns were classified either first or second position (out of four) depending on the  
290 resolution obtained. Among the fourteen compounds, the Chiralpak IC appeared seven times  
291 in first position and four times in second position, allowing the best resolution for eleven  
292 compounds out of fourteen. This column was followed by the CHIRAL ART Cellulose SC  
293 column with four first positions and three second positions. But with watching the Figure 4  
294 and Table 1, there were no drastic differences between columns, at two exceptions. For  
295 compound **28** a full resolution occurred for two columns (CHIRAL ART Cellulose SC and  
296 REFLECT I-Cellulose C) while no enantiomeric separation was observed nor on Lux i-  
297 Cellulose-5 neither on Chiralpak IC in the implemented conditions (Figure S4, in  
298 Supplementary Information). And for compound **29** for which the enantiomers were not  
299 separated on Chiralpak IC while they were partially separated on the three other columns in  
300 the implemented conditions (Figure S4b, in Supplementary Information).

301 Among the 29 compounds studied in this work, high resolutions were achieved on the four  
302 columns for compounds **1** (omeprazole); **2** (thalidomide); **4** (warfarin); **14** (tropicamide); **20**  
303 (1-(1-Naphthyl)ethylamine)) and in-house compounds **21** and **22**, under 15% MeOH and for  
304 compounds **3** (lenalidomide) and in-house compound **26** under 30% MeOH, with various  
305 retention times.

### 306 3.4 Comparison of the loading capacity

307 The results displayed in the three previous sections showed a significant difference of  
308 retention, differences of resolution and slight difference of selectivity between the four  
309 columns for the tested compounds. In addition to retention, selectivity and resolution, loading  
310 capacity can be evaluated because it is a significant parameter in preparative chromatography.  
311 Indeed, the loading capacity of the stationary phase will have an impact on the productivity of  
312 a separation [13]. Thus, the impact of increasing the amount injected on the four columns was  
313 assessed. Two methods can be used to overload a sample on a column: volume overload or  
314 mass overload. For volume overload, a feed solution was prepared by dissolving the  
315 compound with a defined concentration and then increasing volumes of the solution were  
316 injected until the peaks touched at the base (touching band) before resolution will be lost.  
317 Sometimes poor peak shape was obtained under high volume of injection. For mass overload,  
318 the sample volume was kept constant while the mass of compounds dissolved in this volume  
319 was increased progressively. Usually volume overload is used in preparative chiral separation  
320 in discovery because the method development is faster, explaining why this process was  
321 chosen in this work. Five compounds were selected, based on i) their high-resolution result  
322 under 15% MeOH as co-solvent for all of them (excepted 30% for lenalidomide) and ii) on  
323 shorter retention times (when similar resolution values were observed). Therefore  
324 omeprazole, warfarin, lenalidomide, tropicamide and the in-house compound **22** were  
325 selected.

326 To illustrate this loading capacity linked to the baseline return (or touching band), **the**  
327 **following** representations were chosen: i) overloaded chromatograms and ii) the plot of the  
328 separation criterion (S) in function of the amount injected.

329 Firstly, considering the overloaded chromatograms, they **were** represented for the in-house  
330 compound **22** (**Figure 5**) and for omeprazole (**Figure 6**). As shown in **Figures 5** and **6**,  
331 increasing volume of feed solution (10 mg/mL) **impacted** the peak shape. This modification of  
332 the peak shape may lead to a reduction of the resolution with, for some compounds, the  
333 observation of one broad peak instead of two for the larger injected volume. For the in-house  
334 compound **22**, a loss of baseline separation **was** observed for 2.50 mg injected (250 $\mu$ L of feed  
335 solution) on the four columns. Remarkably, for omeprazole, the retention times at the peak  
336 ends **were** constant whatever the injection volume, and the retention times at the beginning of  
337 the peak **decreased** in proportion to the injection volume. For the analysis of omeprazole  
338 (**Figure 6**), a baseline resolution **was** observed for 4.85 mg injected (485 $\mu$ L) on Chiralpak IC  
339 while a slight overlap of the peaks **was** observed for the three additional columns. However,  
340 the phenomenon **was** more the consequence of a lower selectivity on the three columns  
341 compared to Chiralpak IC **and** a lower retention on **the CHIRAL ART Cellulose-SC column**  
342 than a question of loadability since any significant difference in the peak shapes **was**  
343 observed.

344 As the resolution is difficult to determine for broad and asymmetric peak shape, the  
345 measurement of the separation criterion (S) was also used to monitor the quality of the  
346 separation of two successive peaks according to the injected amount [28]. S is defined as the  
347 difference between the retention time at the beginning of the second peak ( $t_{B2}$ ) and the  
348 retention time at the end **of** the first peak ( $t_{E1}$ ); peaks are baseline resolved if S is positive:  $S =$   
349  $t_{B2} - t_{E1}$ . The graphs for omeprazole and in-house compound **22** on **Figure 7** (and for  
350 lenalidomide, tropicamide and warfarin on **Figure S5**, in Supplementary Information) **showed**

351 the same trend for all the compounds **regardless column brand**. The increase in injected  
352 volume **caused** a reduction of the separation criterion with no significant difference observed  
353 between the four columns.

354 The loading capacity of a stationary phase towards a compound **strongly depends on the**  
355 selectivity ( $\alpha$ ) but is also impacted by the efficiency of the column and the retention factor. In  
356 other words, it also depends on the resolution. In addition, the loadability of a stationary phase  
357 is a function of the number of accessible interaction sites per mass unit of phase, so is  
358 dependent of the density of chiral selectors or carbon load [29]. The **behavior** of the four  
359 columns towards increasing injected amount **didn't** differ substantially, leading to the  
360 conclusion that the four columns have quite similar density of chiral selectors.

#### 361 **4. Conclusion**

362 The comparison of the different immobilized cellulose *tris* (3,5-dichlorophenylcarbamate)  
363 columns *i.e.* Chiralpak IC, Lux i-Cellulose 5, CHIRAL ART Cellulose-SC and REFLECT I-  
364 Cellulose C, by use of 29 representative racemic mixtures **showed** some differences. Indeed,  
365 the retention factors **were** significantly different with a higher retention on the Lux i-Cellulose  
366 5 and with the lowest retention on Chiral ART Cellulose-SC **for all compounds (excepted**  
367 **compounds 18 and 28, see Figures 2 and S4a)**. The best selectivity of each of the four  
368 columns **depended on the nature of the compounds but differences between selectivity values**  
369 **were minor**. Besides, the Chiralpak IC column showed the highest rate of separation.  
370 Concerning **the** resolution parameter, **once again**, the best resolution of each of the four  
371 columns depended on the compounds **but differences between resolution were quite**  
372 **significant for compounds 1 (omeprazole), 2 (thalidomide), 3 (lenalidomide) and in-house 27**  
373 **with standard relative deviation comprised between 11% and 20%**. Finally, for the loading  
374 capacity, no strong difference **was registered** between the different columns, thus for small-  
375 scale separation each of them can be chosen. This study **showed** that despite the same chiral

376 selector, the four stationary phases from different manufacturers were not identical probably  
377 due to differences in the manufacturing process such as the immobilisation of the  
378 polysaccharide that we do not intend to further explain here, as these processes are  
379 confidential. From a practical point of view, if one needs high retention capacity from its  
380 column, then choose a Lux i-Cellulose-5, on the contrary if your compounds elute too late,  
381 one should opt for CHIRAL ART SC-cellulose. If one has an important amount of racemates  
382 to separate then the Chiralpak IC seems the most appropriate column and will certainly lead to  
383 a high success rate in terms of selectivity.

384 The comparison of the chromatographic parameters obtained on the four immobilized  
385 columns with those obtained on the coated version of this chiral selector would have been  
386 interesting to determine the impact of the immobilization. But to be realistic, the coated and  
387 the immobilized columns should be provided by the same manufacturer. In addition, as  
388 explained by S. Carradori, this coated CSP (named as Sepapak-5 from Sepaserve GmbH  
389 affiliated to Phenomenex) is no longer commercially available, due to high solubility of this  
390 polysaccharide in mobile phase consisted of *n*-hexane/*i*PrOH mixture [30].

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484 **Figure Captions**

485

486 Figure 1: Molecular structures, names and numbers of the analyzed compounds within the  
487 SFC study.

488 Figure 2: Retention factor for the 29 compounds with the four CSPs. ● acidic compounds,  
489 ■ neutral compounds, ▲ basic compounds.

490 Figure 3: Selectivity vs retention factor for the 29 compounds on the four stationary phases.

491 Figure 4: Resolution for the 29 compounds with the four CSPs. ● acidic compounds, ■  
492 neutral compounds, ▲ basic compounds.

493 Figure 5: Chromatograms of the analysis of the in-house compound **22** on the four columns  
494 (Chiralpak IC, Lux i-Cellulose 5, CHIRAL ART Cellulose-SC and REFLECT I-Cellulose C).  
495 Conditions: mobile phase: CO<sub>2</sub>/MeOH 85/15; flow rate: 4 mL/min; temperature: 40 °C; outlet  
496 pressure: 150 bar; sample concentration: 10 mg/mL; sample volume: from 20 μL to 250 μL;  
497 λ= 254 nm.

498 Figure 6: Chromatograms of the analysis of omeprazole on the four columns (Chiralpak IC,  
499 Lux i-Cellulose 5, CHIRAL ART Cellulose-SC and REFLECT I-Cellulose C).  
500 Conditions: mobile phase: CO<sub>2</sub>/MeOH 85/15; flow rate: 4 mL/min; temperature: 40 °C; outlet  
501 pressure: 150 bars; sample concentration: 10 mg/mL; volume injected: from 20 μL to 485 μL;  
502 λ= 254 nm.

503 Figure 7: Separation criterion (S) vs injected amount on the four CSPs (Chiralpak IC, Lux i-  
504 Cellulose 5, CHIRAL ART Cellulose SC and REFLECT I-Cellulose C) for omeprazole (10  
505 mg/mL from 20 μL to 485 μL) and in-house compound **22** (10 mg/mL from 20 μL to 250  
506 μL).

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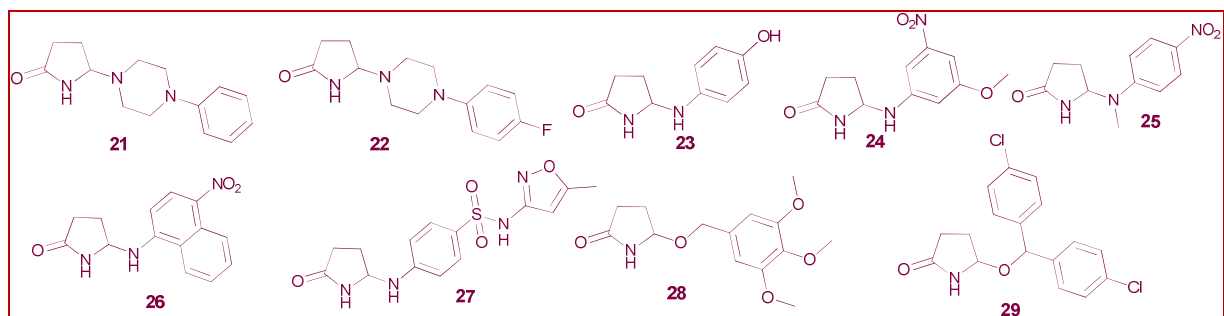
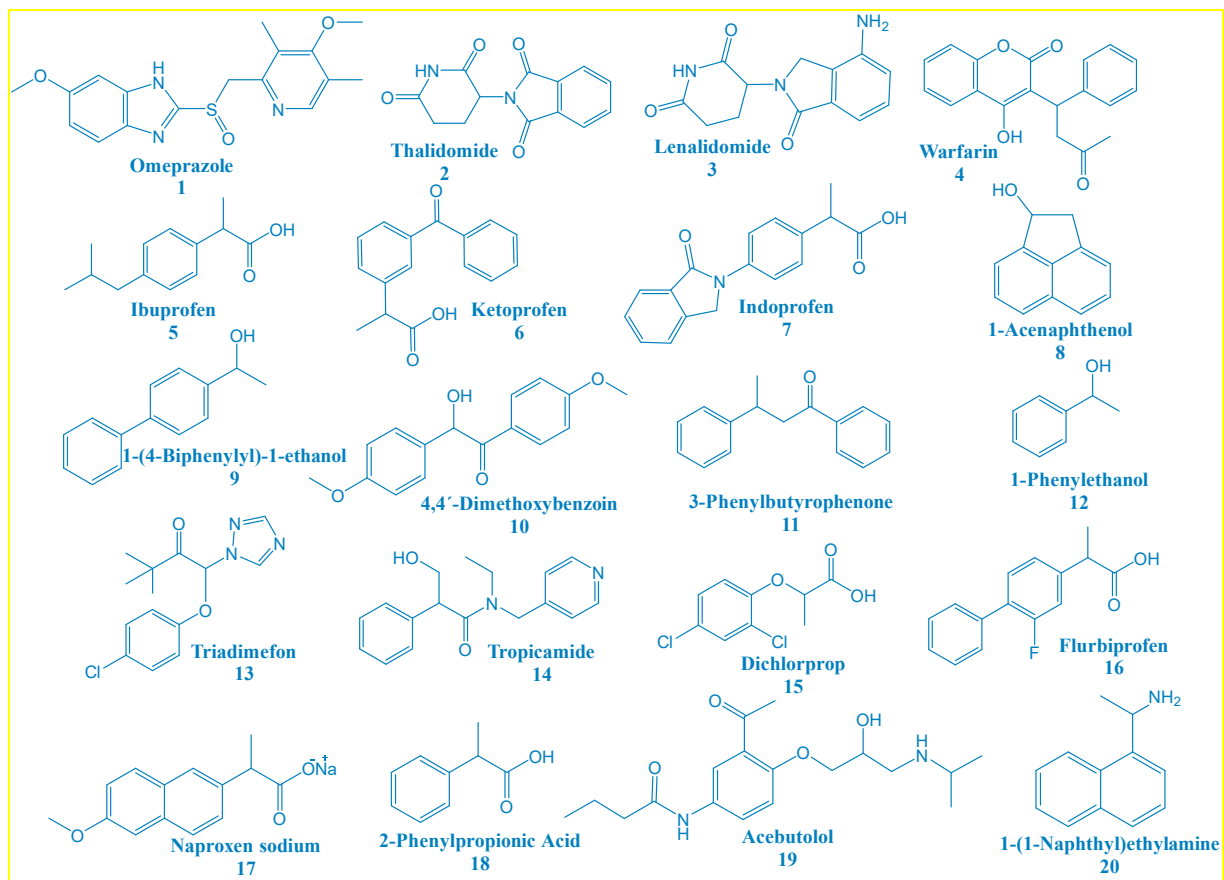


Figure 1

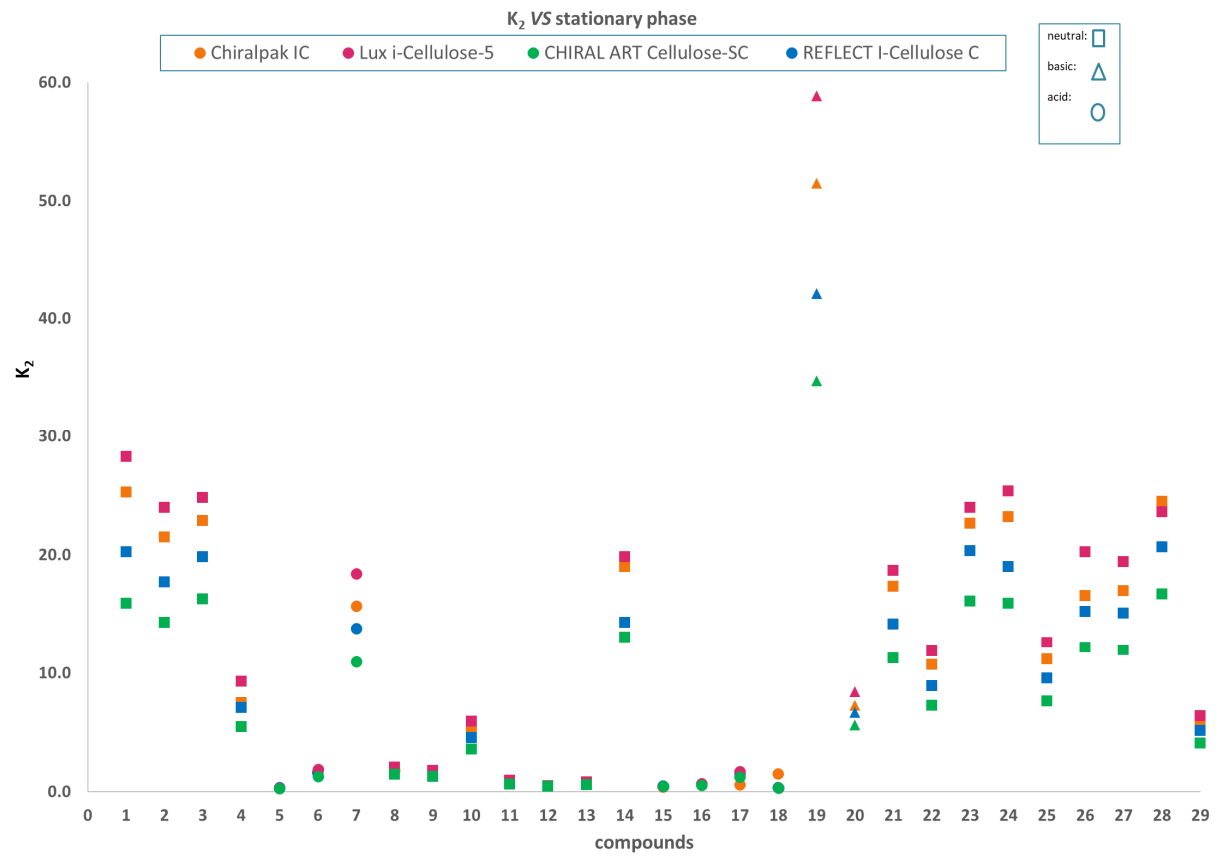


Figure 2

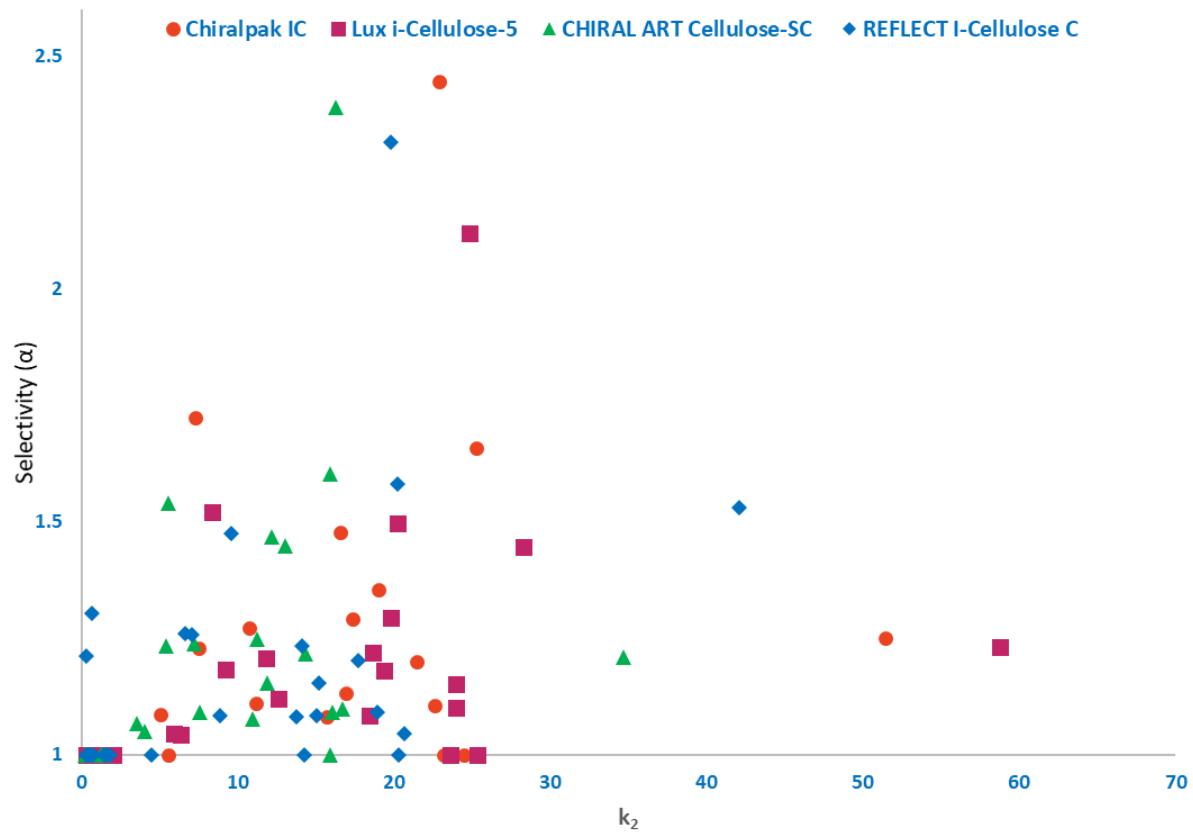


Figure 3



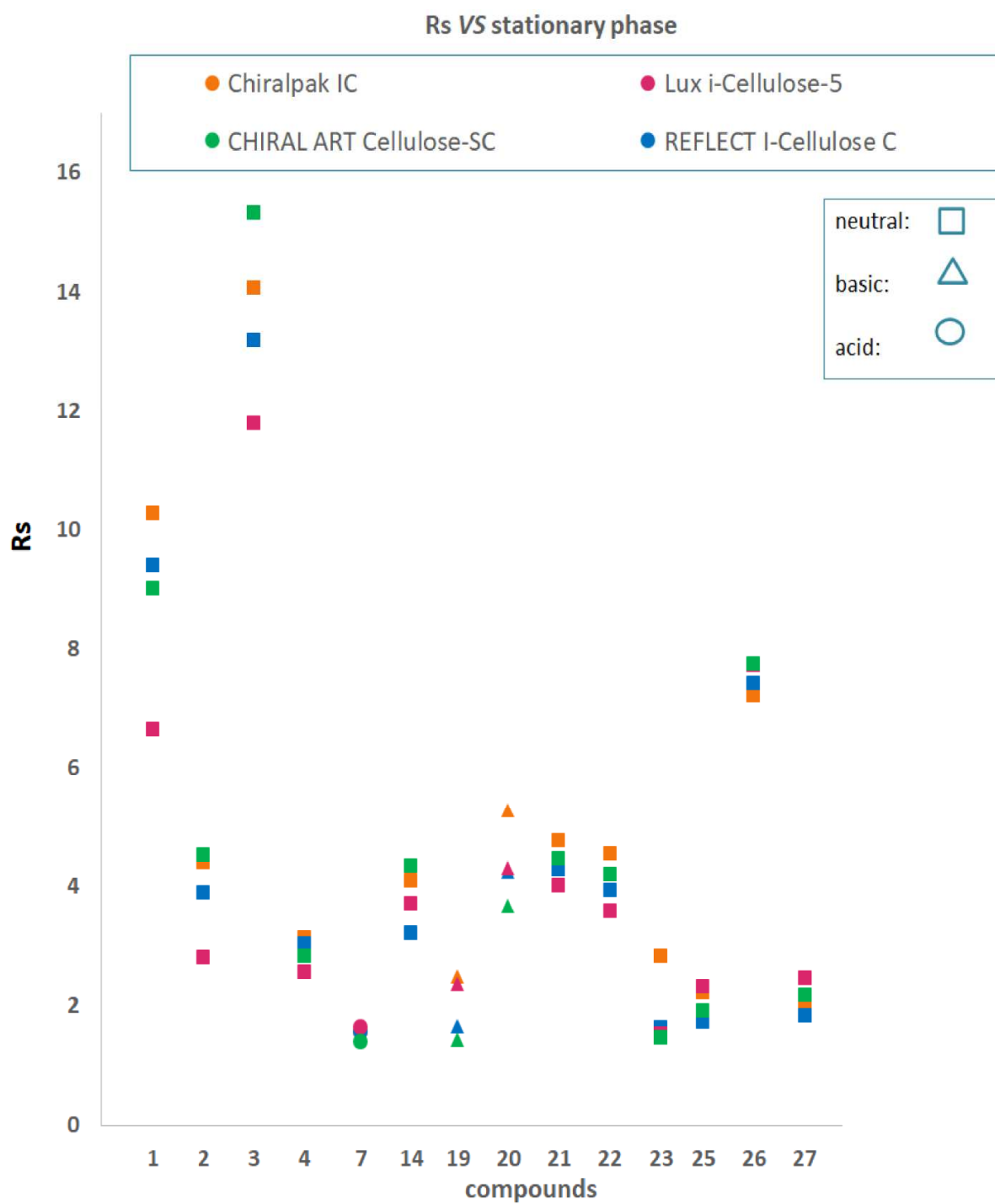
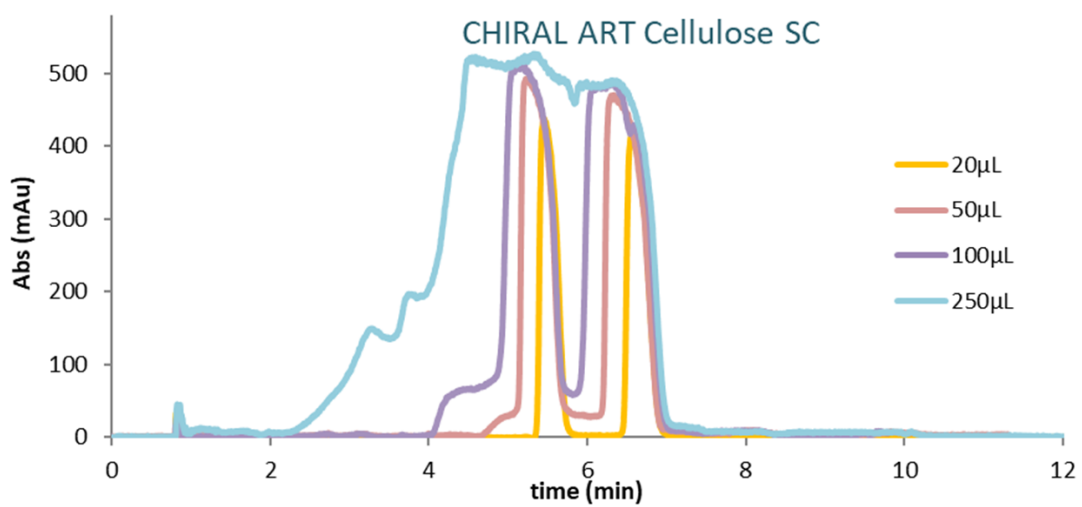
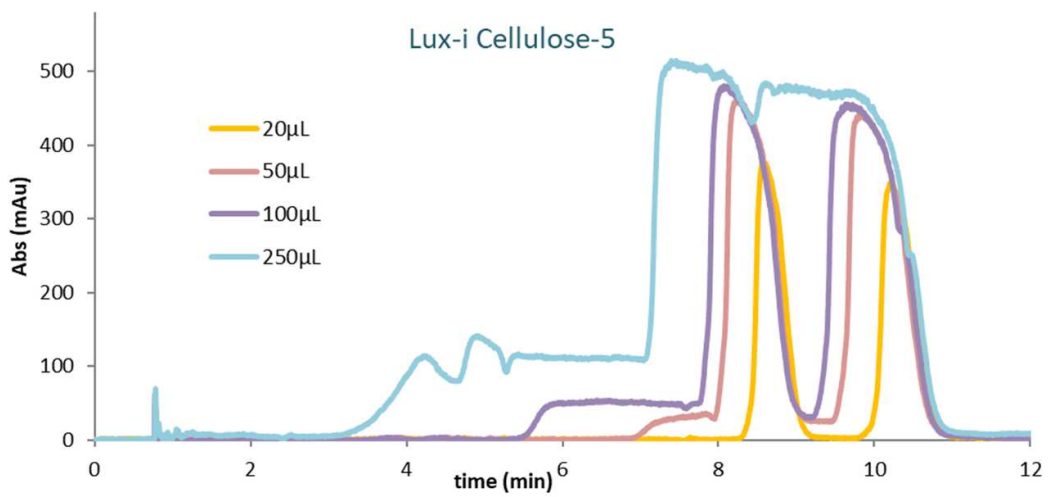
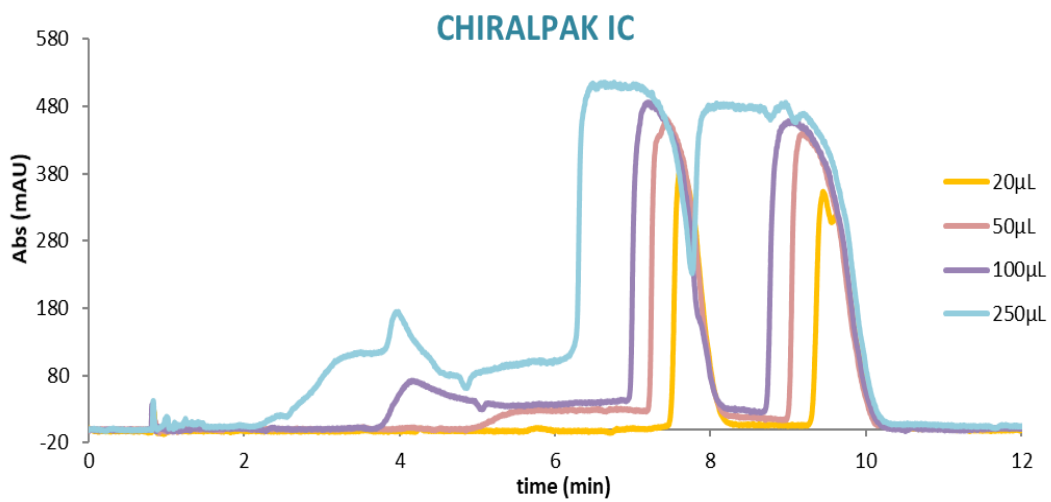


Figure 4



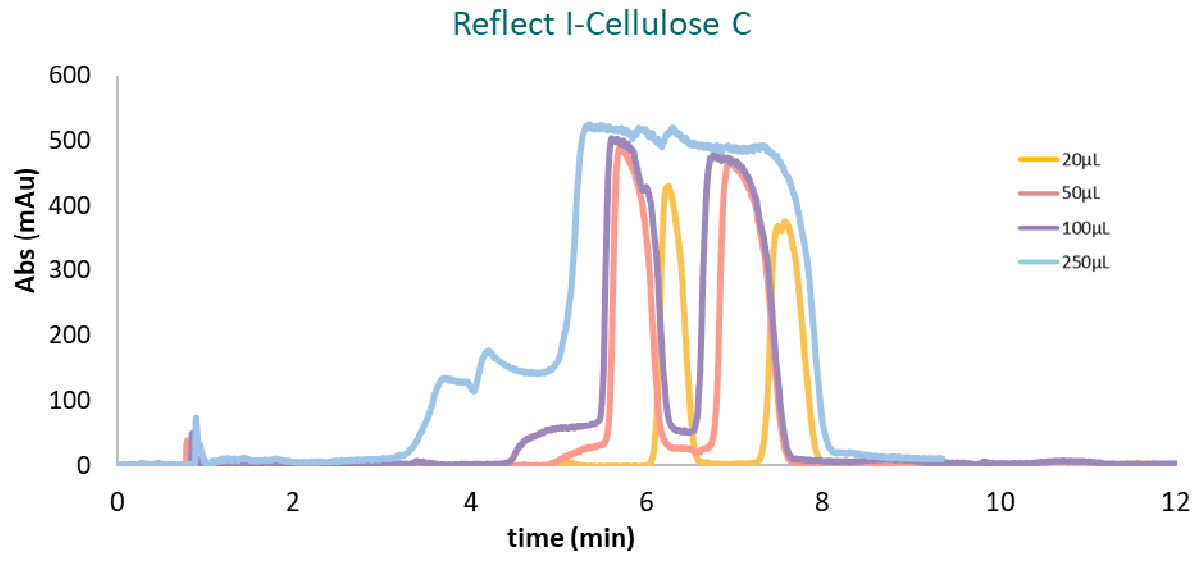
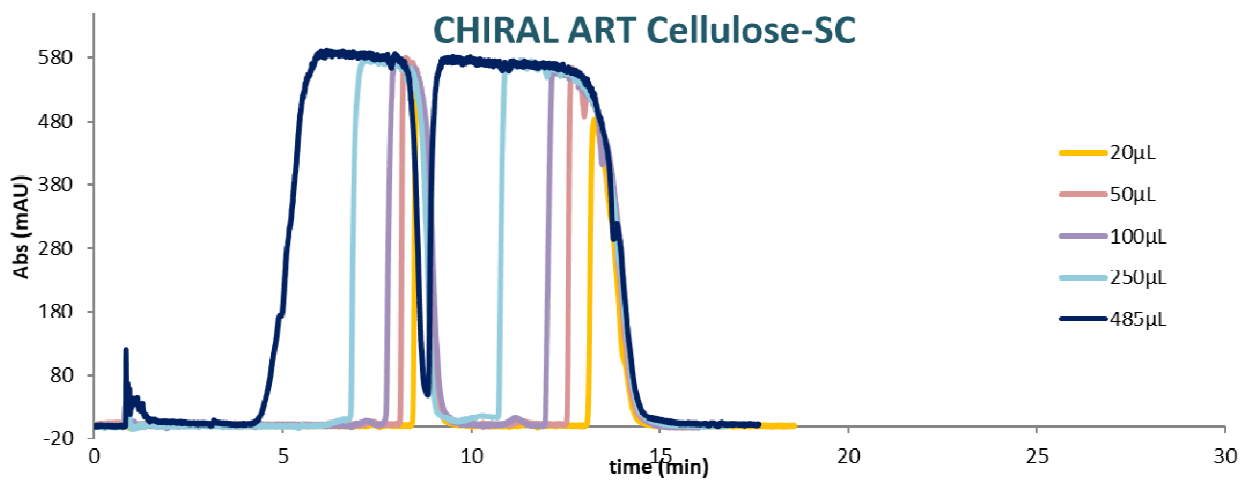
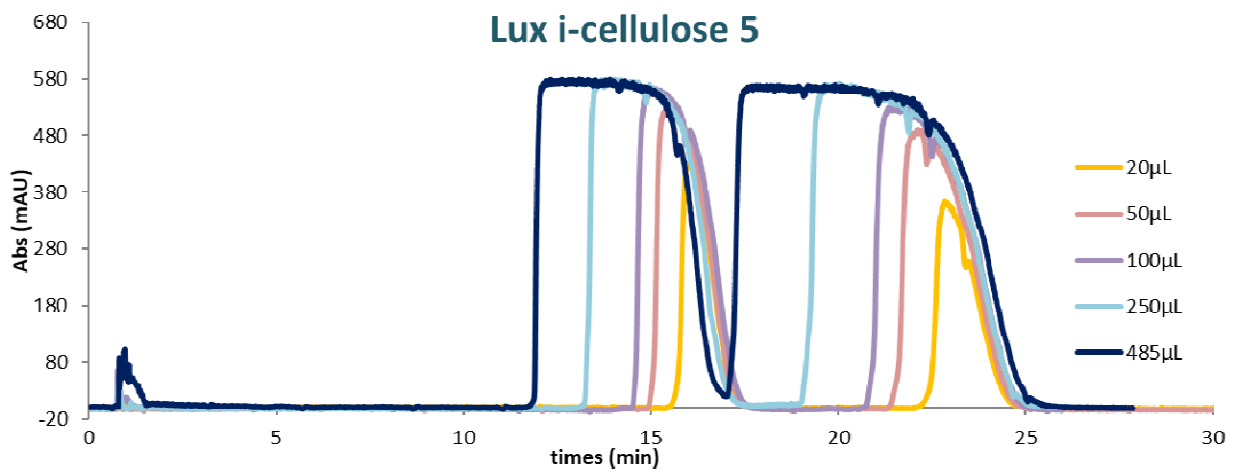
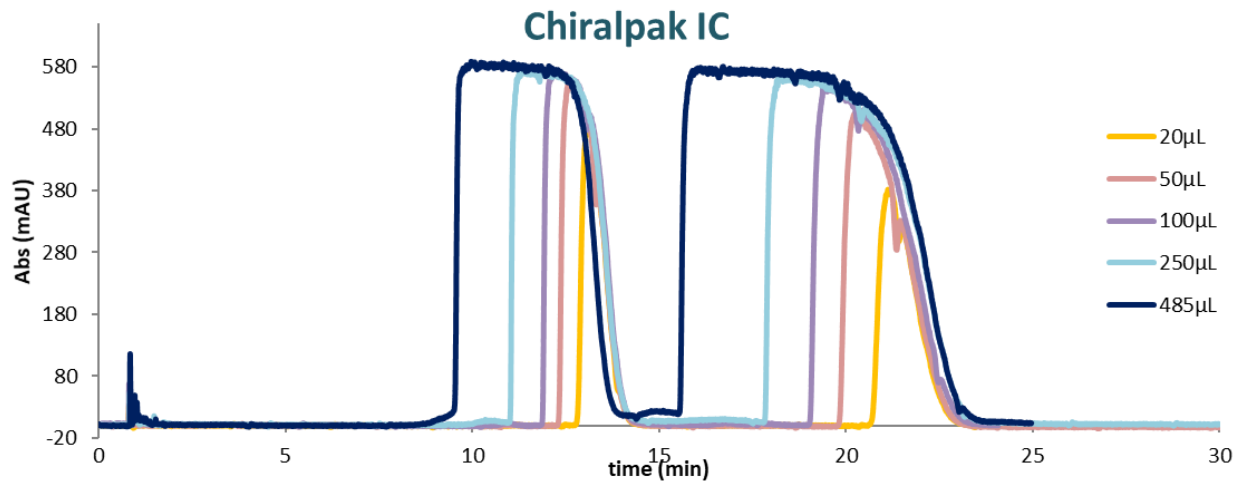


Figure 5



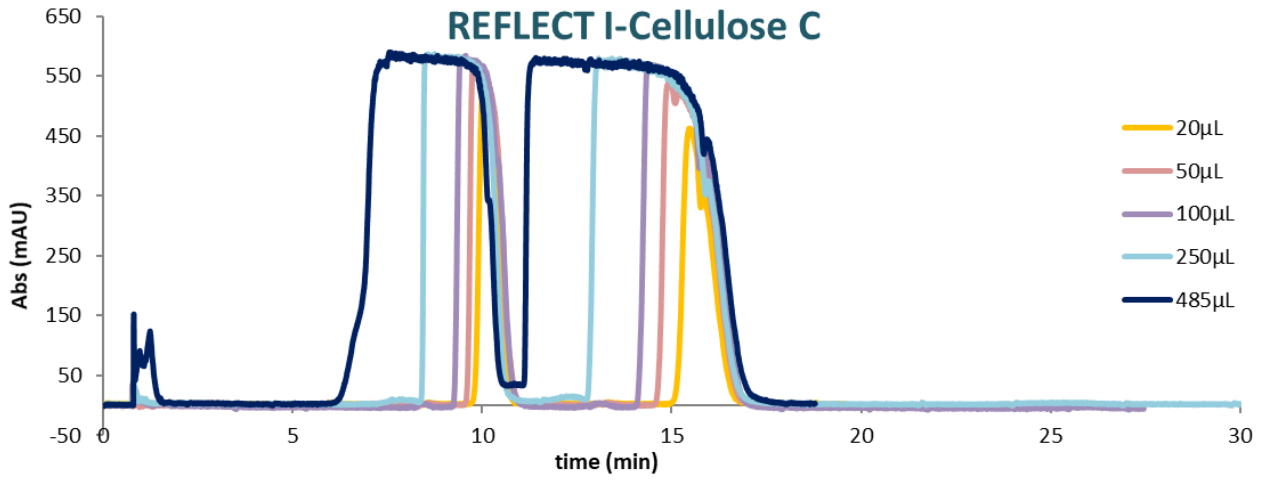


Figure 6

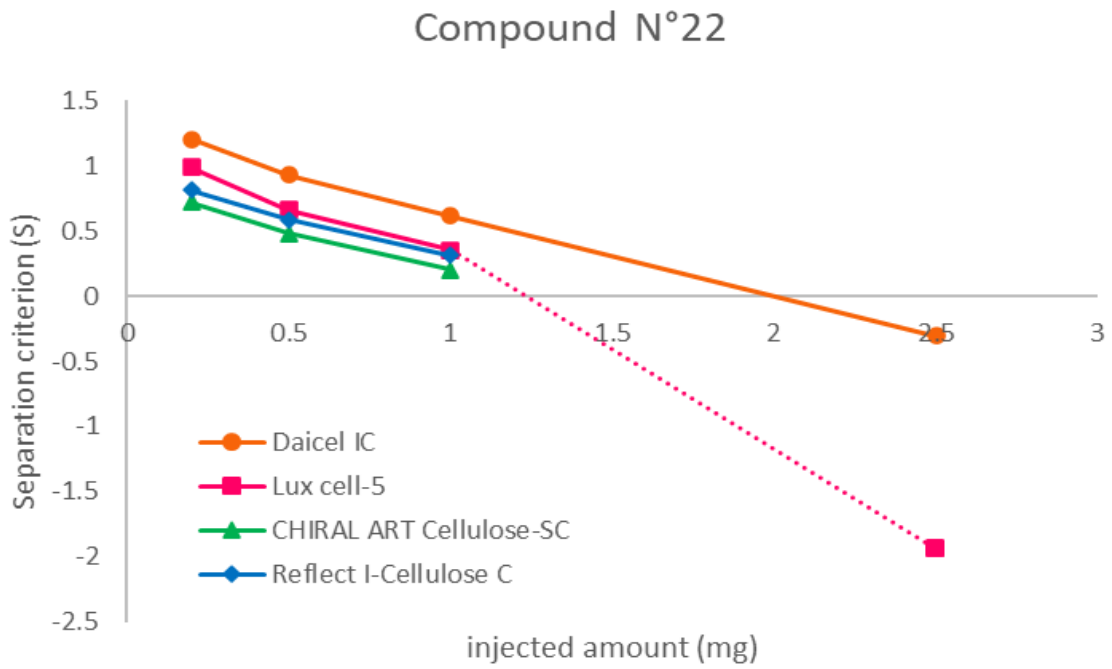
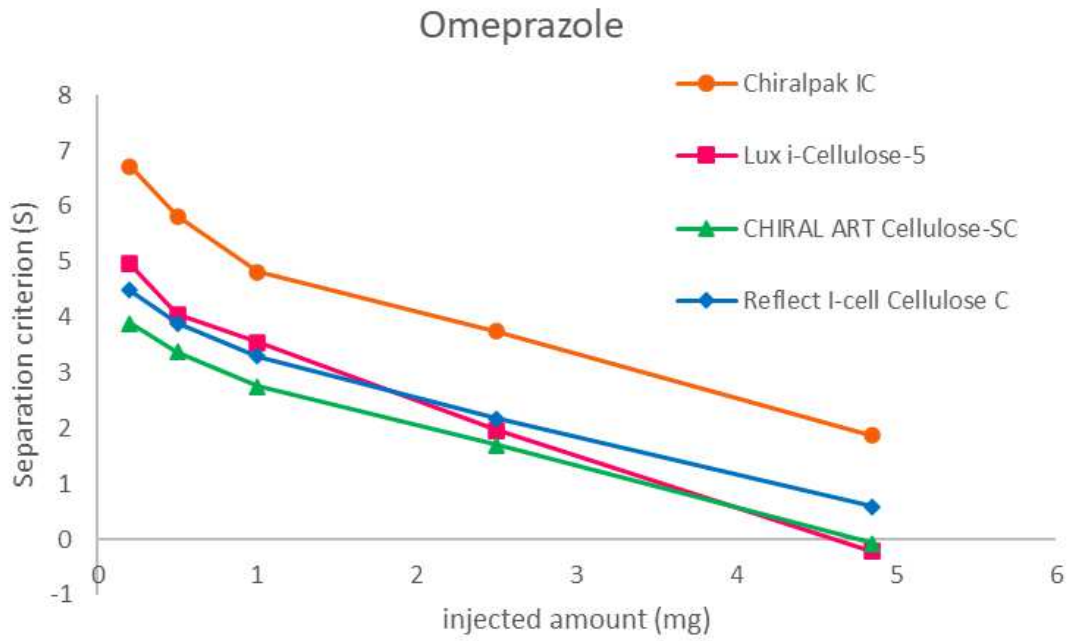


Figure 7