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## Comparison of enhanced fluidity liquid chromatography with liquid chromatography for enantioseparation of selected $\gamma$ -lactam derivatives

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1                    **Comparison of enhanced fluidity liquid chromatography with liquid**  
2                    **chromatography for enantioseparation of selected  $\gamma$ -lactam derivatives**

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22 **Key-words:** antibacterial compounds; cellulose *tris*(3-chloro-4-methylphenylcarbamate);  
23 cellulose *tris*(3,5-dimethylphenylcarbamate); elevated percentage of co-solvent; flowrate.

24 **ABSTRACT**

25

26 The present paper describes the study of eight racemic  $\gamma$ -lactam derivatives with the goal of  
27 comparing three modes of liquid chromatography which are the reversed-phase mode, the normal-  
28 phase mode and the polar organic solvent chromatography mode with super/subcritical fluid  
29 chromatography and enhanced fluidity liquid chromatography in extreme conditions *i.e.* when the  
30 CO<sub>2</sub> percentage is practically zero. For this study two CSPs were chosen. Chiralcel OD-H was  
31 used in HPLC conditions and Chiralcel OD-H and Lux Cellulose-2 were used in SFC conditions.  
32 It is reported in this work that the introduction of chlorine substituent on polysaccharide-based  
33 selectors contributes to an improved enantioselectivity in supercritical fluid chromatography  
34 (SFC) compared to the electron-donating substituted phenyl carbamate column, allowing the  
35 complete separation of seven analytes out of the eight.

36 The effect of enhanced fluidity liquid chromatography conditions, described as an intermediate  
37 mode between HPLC and SFC is also explored. In this case, the composition of the mobile phase  
38 starting at 80% CO<sub>2</sub> was replaced with almost 100% ethanol at the end, this assay achieving further  
39 extension of the polarity range of the mobile phase in a single run and successfully enabling the  
40 simultaneous analysis and separation of the racemates. Under a mobile phase constituted of 99%  
41 ethanol, the resolution values of the  $\gamma$ -lactam derivatives on Lux Cellulose-2 CSP, were comprised  
42 between 1.91 and 3.44. High values of flowrates, ranging from 4 mL/min to 10 mL/min were also  
43 evaluated for some analytes on the highly retentive Lux Cellulose-2 CSP, leading to resolution  
44 equal to 6.24 for compound **6** and 4.64 for compound **8**.

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## 48 1. Introduction

49 Separation of enantiomers is a mandatory practice in pharmaceutical industry to launch  
50 enantiopure drugs into the market. One of the most common technique used to obtain pure amounts  
51 of enantiomers in drug discovery and the early phases of drug development, is enantioselective  
52 chromatography [1, 2]. Thus, HPLC or SFC have become regular tools in laboratories to fastly  
53 separate enantiomers in order to further assess the different biological activities that two  
54 enantiomers may have and to understand their mechanism of action *in vivo* [3].

55 The  $\gamma$ -lactam derivatives of interest depicted in Figure 1, contain a pyrrolidin-2-one scaffold which  
56 is well known as a precursor for a broad range of heterocyclic compounds as well as its high  
57 potential to afford small compounds with interesting biological activities. Therefore,  
58 pyrrolidinones are often fundamental parts of the structure of antibacterial or antifungal  
59 compounds [4]. An asymmetrical center is present next to the lactam group in these antibacterial  
60 molecules, thus requiring their resolution.

61 During the last decades, a large number of chiral stationary phases (CSPs) have been developed to  
62 achieve chiral separation of a wide variety of racemic compounds both in industry and academia  
63 [5, 6]. Two of the most widely described and oldest commercially available CSPs are based on the  
64 *tris*(3,5-dimethylphenylcarbamate) of cellulose and amylose. Besides, some halogenated phases  
65 originally developed by Chankvetadze *et al.* [7-9] are commercially available for some years now.  
66 The introduction of chlorine substituents on polysaccharide-based selectors led to significant  
67 different interactions with the analytes with regards to non-chlorinated phases, in specific cases.  
68 This contribution has been studied from a fundamental point of view [10] and allowed an improved  
69 enantioselectivity described in some applicative works [11-13].

70 Until recently, HPLC and SFC were used in a non-linked manner. HPLC can be implemented in  
71 three different modes: in normal phase (NP), reverse phase (RP) or in polar organic solvents  
72 chromatography (POSC). In SFC, mobile phase is constituted of a high amount of supercritical  
73 carbon dioxide (typically above 50%). Its low viscosity and high diffusivity together with a low  
74 pressure drop permit high flow-rate with a reduced influence on efficiency [14]. Carbon dioxide  
75 has an elution strength close to that of hexane, which is its main drawback, therefore a polar  
76 modifier such as a short chain alcohol or acetonitrile must be added to elute the analytes. While  
77 the addition of a liquid modifier moves conditions to subcritical (between 10 and 50% of co-  
78 solvent), the advantages previously stated still exist. Lower proportions of carbon dioxide (below  
79 50%) are employed in enhanced fluidity liquid chromatography (EFLC) [15].

80 The idea of “bridging” different chromatographic methods was aged and was firstly demonstrated  
81 by Ishii *et al.* in 1989, which aimed to use liquid chromatography (LC), supercritical fluid  
82 chromatography (SFC) and gas chromatography (GC) separation, using a single chromatographic  
83 system [16]. It was recently reported that SFC can bridge HPLC through EFLC, in one single  
84 experiment by implementing wide mobile phase gradients with CO<sub>2</sub> proportions varying from  
85 100:0 to 0:100 (v/v). This concept was called unified chromatography (UC) and was firstly  
86 proposed by Bamba and co-workers in 2014 [17]. It was also developed by West’s group for  
87 amino-acids achiral separation [18], flavonoids [19] and very recently to a large set of bioactive  
88 peptides [20]. The work of the Guillarme’s group must also be mentioned [21-23].

89 Thus, the first goal of the current study was to investigate the three modes of liquid  
90 chromatography for resolving a set of antifungal molecules on various polysaccharide CSPs.  
91 Secondly, SFC and EFLC were implemented in isocratic mode, where the mobile phase  
92 composition starting at 80% CO<sub>2</sub> has been replaced by almost 100% ethanol and the influence of

93 these different mobile phase compositions on retention and enantioselectivity was studied. When  
94 run on the same CSP, the results obtained in liquid chromatography were compared to those  
95 obtained on the SFC device. Besides, thanks to the low viscosity of the supercritical CO<sub>2</sub>, its use  
96 in high flow-rates screenings is possible. Therefore, high flowrates which were generally not  
97 reported at analytical scale, varying from 4 up to 10 mL/min were experimented for the  
98 enantioseparation of the selected  $\gamma$ -lactam derivatives.

## 99 **2. Material and methods**

### 100 **2.1. Chemicals**

101 The antifungal molecules used were 5-methoxypyrrolidin-2-one derivatives (racemic compounds)  
102 **1:** 5-((3,4,5-trimethoxybenzyl)oxy)pyrrolidin-2-one, **2:** 5-((1*H*-benzo[*d*][1,2,3]triazol-1-  
103 yl)oxy)pyrrolidin-2-one, **3:** 5-(benzo[*d*][1,3]dioxol-5-ylmethoxy)pyrrolidin-2-one, racemic  
104 compound **4:** 5-(benzylamino)pyrrolidin-2-one, **5:** 5-(4-phenylpiperazin-1-yl)pyrrolidin-2-one,  
105 racemic compound **6:** 5-(4-(4-fluoro-phenyl)piperazin-1-yl)pyrrolidin-2-one, **7:** 5-  
106 ((4chlorophenyl)amino)pyrrolidin-2-one, **8:** 5-((4-bromophenyl)amino)pyrrolidin-2-one, **9:** *N'*-(5-  
107 oxopyrrolidin-2-yl)benzohydrazide, synthesized in the frame of a structure-activity relationship  
108 study, by a chemical pathway reported elsewhere [24]. All molecules presented a single  
109 asymmetric center; thus, two enantiomers were expected.

110 The solvents used in this study (methanol, ethanol, and acetonitrile) were of HPLC grade and were  
111 purchased from VWR (Strasbourg, France). Carbon dioxide (CO<sub>2</sub>) with purity of 99.995% was  
112 purchased from Linde (Saint-Priest, France).

### 113 **2.2. Sample solutions**

114 For analytical screening, solutions of the samples were prepared in methanol at various  
115 concentrations representing 1 mg/mL (**1:** 3.5 mM; **2:** 4.2 mM, **3:** 5.2 mM; **4:** 4.0 mM; **5:** 3.8 mM;

116 **6:** 4.7 mM; **7:** 3.9 mM; **8:** 4.5 mM). The solutions were always degassed by an ultrasonic bath and  
117 filtered on a 0.45µm PTFE syringe-filter (15mm diameter) prior to use.

## 118 **2.3. Supercritical fluid chromatography apparatus**

### 119 **2.3.1. Stationary phases**

120 Two chiral analytical columns were used for this study (Figure 1). Chiralcel OD-H based on  
121 cellulose *tris*(3,5-dimethylphenylcarbamate) was purchased from Chiral Technologies Europe  
122 (Illkirch, France) and Lux Cellulose-2 based on cellulose *tris*(3-chloro-4-methylphenylcarbamate)  
123 was from Phenomenex® (Le Pecq, France). Both columns had dimensions of 250 × 4.6 mm i.d.  
124 with 5 µm particle size and were coated silica-gel phases.

### 125 **2.3.2. Chromatographic systems and conditions**

126 The chromatographic system used was an SFC-PICLAB hybrid 10-20 apparatus (PIC Solution,  
127 Avignon, France) equipped with an autosampler comprising a 48-vial plate (model Alias, Emmen,  
128 Netherlands), three model 40 P pumps (two for CO<sub>2</sub> and a third for the modifier (Knauer, Berlin,  
129 Germany)), a column oven with a Valco ten-position column selection valve, and a Valco six-  
130 position solvent switching valve. The system was also composed of a Smartline 2600 diode array  
131 detector (DAD) (Knauer, Berlin, Germany). Detection wavelength was set at 210 nm. The system  
132 was controlled and the data was acquired using the SFC PicLab Analytic Online v.3.1.2 software  
133 (PIC Solution, Avignon, France). The volume injected was 20 µL. During the separation  
134 optimization, the mobile phase was always a CO<sub>2</sub>-modifier mixture with 20% of MeOH or EtOH  
135 as a modifier at 3 mL/min flow rate. All analyses were run in isocratic mode. The column oven  
136 temperature was 40°C and the outlet pressure was maintained at 150 bar for all experiments.  
137 During the separation screening, the mobile phase was CO<sub>2</sub>-modifier mixtures of 20 to 99% of  
138 either methanol, ethanol under 1 mL/min. During the separation optimization, the flow rate ranged

139 between 1 and 10 mL/min under 20% ethanol. The column oven temperature was 40°C and the  
140 outlet pressure was 150 bar. The column void time ( $t_0$ ) was considered to be equal to the peak of  
141 the solvent front and was taken from each particular run. It was equal to the value obtained by  
142 injection of 1,3,5-tri-*tert*-butylbenzene used as a non-retained sample. Retention times were mean  
143 values of two replicate determinations.

## 144 **2.4. High performance liquid chromatography apparatus**

145 For analytical screening, solutions were prepared at 1.5 mM in either isopropanol or ethanol  
146 depending on the mobile phase used for chromatography. The solutions were always degassed in  
147 an ultrasonic bath and filtered with a 0.45  $\mu\text{m}$  PTFE syringe-filter (15 mm diameter) prior to use.

### 148 **2.4.1. Stationary phases**

149 For normal-phase chromatography, analyses were carried out on a Chiralcel OD-H based on  
150 cellulose *tris*(3,5-dimethylphenylcarbamate) (250  $\times$  4.6 mm i.d; 5 $\mu\text{m}$ ) column.

151 For polar aqueous-organic and polar organic modes, analyses were carried out on Chiralcel OD-  
152 RH based on cellulose *tris*(3,5-dimethylphenylcarbamate) (150  $\times$  4,6 mm; 5 $\mu\text{m}$ ) Both were  
153 purchased from Chiral Technologies Europe (Illkirch, France) and were coated silica-gel phases.

### 154 **2.4.2. Chromatographic system and conditions**

155 The analytical HPLC system used was constituted of a Waters in-line degasser apparatus, a column  
156 oven compartment and a gradient Waters 600 E metering pump model equipped with a Waters  
157 996 photodiode array spectrophotometer. Chromatographic data were collected and processed on  
158 a personal computer running with Millennium 2010 software from Waters. Detection was achieved  
159 at the maximum wavelength 270 nm. The sample loop was 20  $\mu\text{L}$  (Rheodyne 7125 injector). All  
160 separations were carried out at 25°C. All analyses were run in isocratic mode. The column void  
161 time ( $t_0$ ) was considered to be equal to the peak of the solvent front and was taken from each



162 particular run. It was equal to the value obtained by injection of 1,3,5-tri-*tert*-butylbenzene used  
163 as a non-retained sample. Retention times were mean values of two replicate determinations. The  
164 flow-rate varied between 0.4 and 0.8 mL/min.

### 165 **3. Results and discussion**

166 3.1. Comparison of different chromatographic modes for the enantioseparation of  $\gamma$ -lactam  
167 derivatives

168 To separate the enantiomers of eight analytes (Fig. 1), three modes of liquid chromatography and  
169 normal phase in subcritical fluid chromatography were investigated and the resulting  
170 chromatographic parameters were summarized in Table 1.

171 The polysaccharide derivatives CSPs have been extensively used for enantiomer separation of a  
172 wide variety of chiral compounds, particularly the *tris*(3,5-dimethylphenylcarbamate) of cellulose  
173 and amylose, belonging to the “golden-four”. Those four stationary phases are namely amylose  
174 and cellulose *tris*(3,5-dimethylphenylcarbamate), amylose *tris*((S)- $\alpha$ -methylbenzylcarbamate) and  
175 cellulose *tris*(4-methylbenzoate). Previously, five out the six analytical columns available (the  
176 golden four and two chlorinated cellulose-based) were tested towards these compounds without  
177 any satisfactory results (data not shown). The sixth one was the coated-type CSP, *tris*(3,5-  
178 dimethylphenylcarbamate) of cellulose employed to resolve the structurally related  $\gamma$ -lactams  
179 derivatives **1-8**.

180 Hesse and Hagel firstly used esters derivatives of polysaccharide with ethanol as a mobile phase  
181 [25]. Later Okamoto *et al.* recommended the use of alcohol/hydrocarbon mixture as mobile phase  
182 for these polysaccharide-based phases [26]. To date, the normal phase liquid chromatography  
183 (NPLC) mode is the most widely used and reported [27]. Mixtures of heptane/ethanol 70:30 (v/v)  
184 and 90:10 (v/v) were tested on the Chiralcel OD-H column. Under these conditions, resolution was

185 measurable for six (**1**, **3**, **4**, **5**, **6** and **7**) out of the eight compounds and was observed to vary from  
186 1.08 (compound **4**) to 4.29 (compound **1**). Compounds **1**, **3** and **5** were then baseline-resolved.  
187 Meanwhile, for studied analytes **2** and **8**, although enantioresolution was clearly not achieved,  
188 some enantiorecognition appeared to occur under these conditions.

189 POSC mode coupled with polysaccharide-based CSPs is implemented through stationary phase  
190 originally developed by Daicel Corporation to be specifically used with either pure polar organic  
191 solvent, the Chiralcel OD-RH CSP. This CSP can also be used in reversed-phase mod under polar  
192 aqueous-organic mixture of solvents. With the aim of finding conditions for the enantioselective  
193 analysis of all the racemates, they were also screened in POSC. This chromatographic mode  
194 involves only polar organic solvents such as alcohols, acetonitrile and their mixtures as mobile  
195 phase, which can bring some benefits such as a short separation time, a high column efficiency  
196 and commonly a better solubility of solutes in the mobile phase [28, 29]. Pure EtOH and ACN  
197 were used as a mobile phase at 0.5 mL/min, high peak efficiency being achieved at low flowrate  
198 in this mode. Studying the chromatographic results from Table 1, one might see important  
199 differences between EtOH and ACN, in chiral recognition ability of the Chiralcel OD-H towards  
200 the analytes. ACN-based mobile phase allowed better performance compared to EtOH-based, for  
201 enantiomers of compounds **3** and **4** which, in this latter mentioned mobile phase, were baseline  
202 separated (Figure 1, in Supplementary Material). At this stage, regarding the poor results obtained,  
203 the strategy was to proceed with the last mode for the concerned analytes, which is RPLC.

204 Although the use of reversed phase in amylose and cellulose-based as CSPs in enantioselective  
205 liquid chromatography is limited, there are few recently reported studies [30-32]. Besides, this  
206 mode is economic and leads to environmental benefits. In RPLC, the Chiralcel OD-RH CSP  
207 mentioned above, has been tested with EtOH/H<sub>2</sub>O mixtures in 70:30 (v/v) or 80:20 (v/v)

208 proportions, respectively. Under reverse-phase conditions, as shown in Table 1, the results were  
209 less satisfactory compared to previously used POSC mode, except for compound **4** which has been  
210 resolved with a resolution equal to 1.55 in EtOH/H<sub>2</sub>O 70:30 (v/v), all other compounds remaining  
211 partially separated. However, it must be noticed that ACN-based aqueous mobile phase has not  
212 been tested. Regarding the retention, the increase of the volume of water in the EtOH-based mobile  
213 phase, resulted in longer retention times. This observation relies on the fact that hydrogen-bonding  
214 interaction are less favored than hydrophobic ones in this case. This is consistent with a reversed-  
215 phase mode and has been already reported [33].

216 Once tested in NPLC, POSC, RPLC and regarding the partial results obtained, the cellulose based  
217 OD-H was lastly used in SFC with either 20% ethanol or methanol containing CO<sub>2</sub>-based mobile  
218 phases, at 4 mL/min. Indeed, some papers have reported that enantioseparations, obtained in HPLC  
219 for a particular analyte are not necessary observed in SFC and *vice-versa* [34-36]. On Chiralcel  
220 OD-H column, under subcritical conditions, the highest enantioselectivity and shortest elution  
221 times were achieved with four baseline-separated racemates (compounds **1**, **3**, **5** and **6**) out of the  
222 total of eight (Table 1).

223 In summary, compound **1** was the only compound which could be separated in all four  
224 methodologies (NPLC, POSC, RPLC and NPSFC) on Chiralcel OD-H (Figure 2).

225 3.2. Supercritical fluid chromatography on chlorinated CSP for the enantioseparation of  $\gamma$ -lactam  
226 derivatives

227 As previously stated, HPLC and SFC normal-phase methodologies did not lead to similar elution  
228 patterns and resolution toward a given compound, through the same CSP. Thus, a chlorinated  
229 column, a coated silica-gel based on cellulose *tris*(3-chloro-4-methylphenylcarbamate) differing  
230 from the previous *tris*(3,5-dimethylphenylcarbamate) stationary phase by a chlorine atom, was

231 chosen for SFC even it is less efficient in HPLC. The cellulose-based Lux cellulose-2 was tested  
232 with either 20% MeOH or EtOH containing CO<sub>2</sub>-based mobile phases, at 4 mL/min. All the  
233 chromatographic parameters are summarized in Table 2. Two observations can be done. First, the  
234 retention factors of all compounds were higher than those measured on Chiralcel OD-H column  
235 from Table 1. And secondly, under those conditions, all compounds were fully baseline-separated  
236 except for compound **1**, which was, anyway, separated on the Chiralcel OD-H column. Among the  
237 two co-solvents, MeOH and EtOH, the latter led to higher resolution values (e.g. for compound **6**,  
238 8.82 *versus* 3.31). The Lux Cellulose-2 column confirmed past studies carried out in SFC,  
239 reporting that chlorine-containing cellulosic phases would exhibit strong retention and different  
240 enantioselective abilities compared to the polysaccharide-based selectors without chlorine  
241 having otherwise the same similar chemical structure [10]. It is proposed that stronger hydrogen  
242 bonding between the carbamate function of the CSP ligands (R groups on Fig.1) and the amide  
243 group of the lactam derivatives and through halogen bonding between the chlorine atoms on the  
244 CSP ligands and the Cl, F, Br, O or N atoms and aromatic rings as well present on the analytes  
245 [37], originated the elevated retention factors. This strong hydrogen bonding is explained by the  
246 larger portion of free carbamate groups in chlorinated phases, while there are more carbamate  
247 groups involved in intra-molecular hydrogen bonding in the non-chlorinated phases [7-9]. It should  
248 be remembered that these interactions do not necessarily contribute to enantiodiscrimination.  
249 However, in addition to enantioselective interactions, it was reported that in chlorine cellulosic  
250 phase, the strong non-enantioselective interactions contributed to a better enantioseparation [10].  
251 Having in mind these satisfactory results, this CSP was retained for the rest of the study.

252 3.3. Influence of the percentage of co-solvent in mobile phase on chlorinated CSP for the  
253 enantioseparation of  $\gamma$ -lactam derivatives

254 There is an interest in examining the retention and the enantioseparation in more details under very  
255 wide range percentage of co-solvent. Therefore, the separation of the eight derivatives was carried-  
256 out under different percentages of co-solvent, varying from 20 to 99% EtOH in CO<sub>2</sub> on the Lux  
257 Cellulose-2. The flow-rate was reduced to 1 mL/min, to avoid the system from reaching the upper  
258 limit pressure of the pumps. All the chromatographic parameters are gathered in Table 3. SFC  
259 usually employs up to 50% modifier in CO<sub>2</sub>-based mobile phase, while enhanced fluidity liquid  
260 chromatography uses more than 50% co-solvent in CO<sub>2</sub>-based mobile phase (as in normal phase  
261 liquid chromatography) [18]. Near 100% co-solvent, Berger stated that SFC is indistinguishable  
262 from HPLC (as in polar organic solvent mode in liquid chromatography) [38]. Thus, it was  
263 interesting to measure the capacity of our device to operate under SFC, then EFLC and finally  
264 POSC conditions by studying retention, selectivity and resolution parameters along this broad  
265 range of mobile phase compositions. The results obtained on the Lux Cellulose-2 CSP were shown  
266 in Figure 3 and typical chromatograms were presented in Figure 4. As expected, increasing the  
267 proportion of EtOH caused a decrease of the retention factors, due to the increased elution strength  
268 of the mobile phase. It is expected, when plotting the logarithmic values of the retention factor  
269 against the logarithmic values of the proportion of EtOH, that a close to linear relationship will be  
270 found due to the elution strength which varies non-linearly in CO<sub>2</sub> based mobile phase [39]. In the  
271 case of the  $\gamma$ -lactam derivatives, on the Lux Cellulose-2 CSP, linearity was good from 20 to 99%  
272 EtOH. Concerning the enantioselectivity, logarithmic values of enantioselectivity remained almost  
273 constant on the whole range. A final word should be said about the efficiency parameter which  
274 decreased as a function of the percentage increase in EtOH. This should be explained by a decrease  
275 of the mobile phase diffusivity. Thus, referring to the Purnell equation, the decrease in resolution  
276 is mainly explained by this loss of efficiency, but also by the decrease in retention factors.

277 The study was also conducted on the Chiralcel OD-H column, for the resolved compounds (see  
278 section 3.1) which were the derivatives **1**, **5** and **6**, varying the percentage of EtOH from 10 to  
279 50%, except for compound **6** where 10% was not tested as retention was already high at 20%.  
280 Unfortunately, it showed some limitations in separating the compounds at a percentage greater  
281 than 50% of EtOH used as modifier. Again, the results obtained on the Chiralcel OD-H CSP were  
282 presented in Figure 2 (in Supplementary Material) and typical chromatograms have been shown  
283 in Figure 3 (in Supplementary Material). Almost linear curves were obtained over the entire  
284 percentage range, when plotting the logarithmic values of the retention factor against the  
285 logarithmic values of the proportion of EtOH and when plotting the logarithmic values of the  
286 enantioselectivity against the logarithmic values of the proportion of EtOH, indicating that  
287 retention and enantioselectivity (slightly) diminish as the percentage of EtOH increase. As  
288 observed on Lux Cellulose-2, efficiency decreased mainly because of the decrease of the mobile  
289 phase diffusivity.

290 In addition, some observations can be done comparing SFC and NPLC at 1 mL/min, for  
291 compounds **1** and **5** (Table 1 and Table 1 in Supplementary Material). Retention factors, selectivity  
292 and resolution values were, by far, higher for SFC with 10% of EtOH in CO<sub>2</sub> than for NPLC with  
293 10% EtOH in heptane. This was also the case for compound **6** under 30% EtOH. These results  
294 have been previously reported by S. Khater *et al.* [34]. On another hand, it is more surprising that  
295 for compounds **1** and **5**, retention factors, selectivity and resolution values are practically similar  
296 for SFC with 30% of EtOH in CO<sub>2</sub> and for NPLC with 30% EtOH in heptane.

#### 297 3.4. Effect of the flow-rate on chlorinated CSP for the enantioseparation of $\gamma$ -lactam derivatives

298 In this work, the flow-rate effect on retention and enantioselectivity was also explored up to very  
299 high value (10 mL/min). Because of the lower viscosity and higher diffusivity of CO<sub>2</sub> based mobile

300 phase, the flow-rate can be increased, while keeping high efficiency, restoring thus, short retention  
301 times. Flow-rates up to 10 mL/min are no exception in semi-preparative scale-SFC but are less  
302 common in analytical step. Increasing the flow-rate will speed up the analysis significantly,  
303 without compromising the efficiency (thus the resolution) too drastically, as the slope of Van't  
304 Deemter curve in SFC is much less steep than in HPLC. Results obtained for compounds **6** and **8**  
305 (chosen for their higher resolution values under 20% EtOH) were summarized in Table 2 (in  
306 Supplementary Material). When increasing the flow-rate, the retention times drastically decrease,  
307 as expected from the increase in the linear velocity of the mobile phase. On the other hand, the  
308 observed decrease of the retention factors is explained by the compressibility of the fluid in the  
309 column, affecting its density and therefore, affecting its eluting strength. In addition, when  
310 increasing the flow-rate, selectivity (slightly) and resolution diminished. Using 20% EtOH as co-  
311 solvent, when the flow-rate for the enantioseparation of compound **6** was increased from 4 to 10  
312 mL/min, the analysis time was reduced by 62% (from 11.8 to 4.4 min), while the resolution  
313 decreased by 23% only (from 4.04 to 3.12) and  $\alpha$  remained almost unchanged. These variations  
314 have been shown in Figure 4 (in Supplementary Material). The separation at 10 mL/min was still  
315 largely acceptable, with a shorter elution time of 7 minutes compared to the chromatogram under  
316 a flow rate of 4 mL/min. Besides, SFC is known as a high resolution *per* time unit methodology,  
317 thus another parameter can be considered: the  $R_s/tr_2$  ratio. These values were calculated and added  
318 in Table 2 (in Supplementary Material), where it can be seen that the higher the flow-rate, the  
319 higher resolution *per* time unit. However, this observation must be taken with caution as, in this  
320 particular case of  $\gamma$ -lactam derivatives, their resolution values were already high at 4 mL/min.  
321 Things considered, while optimizing analysis times using SFC as a chromatography technique, it  
322 may be recommended to increase the flow rate, since the impact on the decrease in resolution is

323 quite limited. A limiting factor regarding this approach would be the pressure restrictions imposed  
324 by the equipment as well as the chromatographic column.

### 325 **Conclusion**

326 In this paper eight antifungal molecules with various  $\gamma$ -lactam structures, were screened in liquid  
327 chromatography with the goal of comparing three modes: RPLC; NPLC, and POSC on a Chiralcel  
328 OD-H column. The higher rate of full baseline separation was achieved in NPLC with seven pairs  
329 of enantiomers separated out of the eight. SFC in subcritical conditions was also implemented on  
330 the same CSP and only four pairs of enantiomers were fully resolved. In this particular case, it  
331 should be noted that NPLC remains superior to the SFC methodology. However, switching to the  
332 Lux Cellulose-2 CSP was a game-changer under subcritical conditions providing higher overall  
333 resolution values for the separation of seven of these molecules (compound **1** was almost  
334 separated). But the interest of the study was to explore the capacity of the SFC device to deliver  
335 mobile phase composed of 20% to 99% EtOH in carbon dioxide, in isocratic mode, thus bridging  
336 SFC and LC techniques. On this highly retentive and highly enantioselective column, it can be  
337 seen that working in enhanced fluidity liquid chromatography, at large proportion of co-solvent  
338 allows fast and impressive separation of the compounds. This was also the case when working in  
339 POSC mode where only 1% CO<sub>2</sub> was present in the mobile phase. Implemented on the Chiralcel  
340 OD-H column, this study shows that retention factors, selectivity and resolution values are, by far,  
341 higher for EFLC than for NPLC or at least, in the same range, depending on the nature of the  
342 compound. This opens a way to practice different chromatographic modes with only one device.  
343 To the best of our knowledge, this possibility has not been studied for chiral separation, except by  
344 West *et al.* on chlorinated polysaccharide stationary phases [12] and by Wolrab *et al.* on chiral  
345 zwitterionic ion-exchanger [40].



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## Figure captions

486

487 **Figure 1** Structure of the  $\gamma$ -lactam derivatives **1-8** and polysaccharide-based stationary  
488 phases used in this study.

489 **Figure 2**

490 2a: Polar organic solvent chromatography for compound **1** on Chiralcel OD-RH, under 100% ACN  
491 at 0.5 mL/min (dark blue), 100% EtOH at 0.5 mL/min (orange) and 100% EtOH at 0.3 mL/min  
492 (red).

493 2b: Reversed-phase liquid chromatography for compound **1** on Chiralcel OD-RH, under  
494 (EtOH/H<sub>2</sub>O) 80/20 (v/v) (brown) and (EtOH/H<sub>2</sub>O) 70/30 (v/v) (green) at 0.4 mL/min.

495 c: Normal-phase liquid chromatography for compound **1** on Chiralcel OD-H under (Hept/EtOH)  
496 90/10 (v/v) (burgundy) and (Hept/EtOH) 70/30 (v/v) (pink), at 1.0 mL/min.

497 2d: Normal-phase supercritical fluid chromatography for compound **1** on Chiralcel OD-H under  
498 (CO<sub>2</sub>/EtOH) 80/20 (v/v) at 4.0 mL/min (light blue) and under (CO<sub>2</sub>/EtOH) 70/30 (v/v) at 1.0  
499 mL/min (dark blue).

500 **Figure 3** Enantioseparation results of compound **6** on Lux Cellulose-2 CSP. Decimal  
501 logarithm of the retention factor as a function of the decimal logarithm of the percentage of ethanol  
502 (higher part); Decimal logarithm of the selectivity as a function of the decimal logarithm of the  
503 percentage of ethanol (lower part)

504 **Figure 4** Overlay of the obtained chromatograms of compound **6** on Lux Cellulose-2 CSP,  
505 under 20 to 99% of EtOH in CO<sub>2</sub> based mobile phase, at 1.0 mL/min, 150 bar, 40 °C.

506



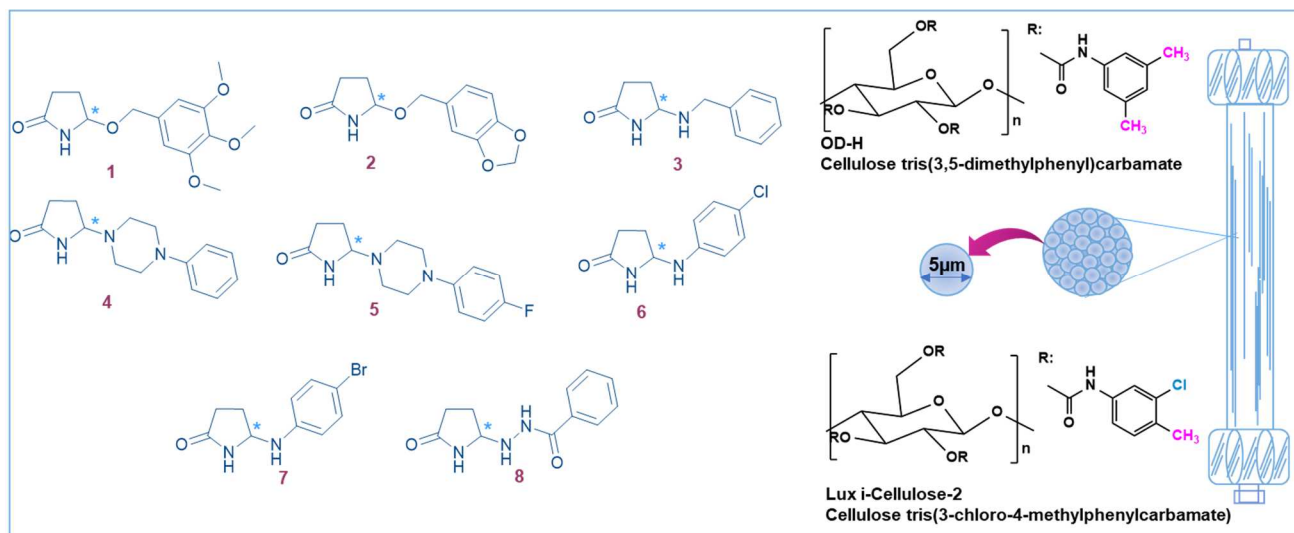


Figure 1

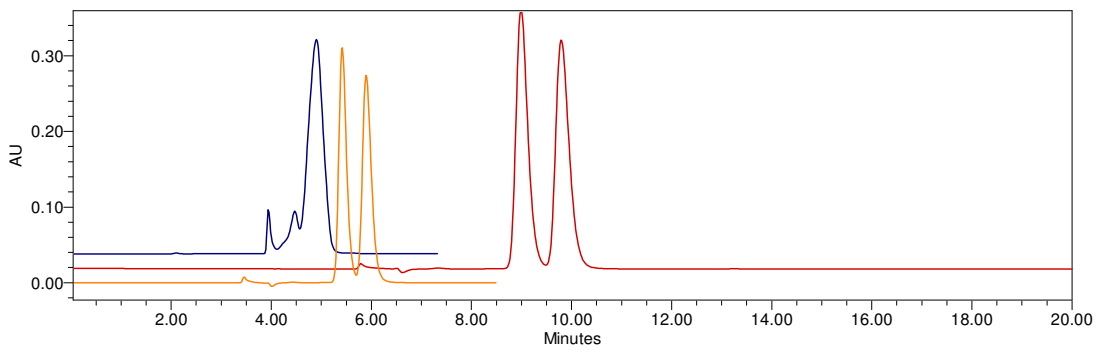


Figure 2a

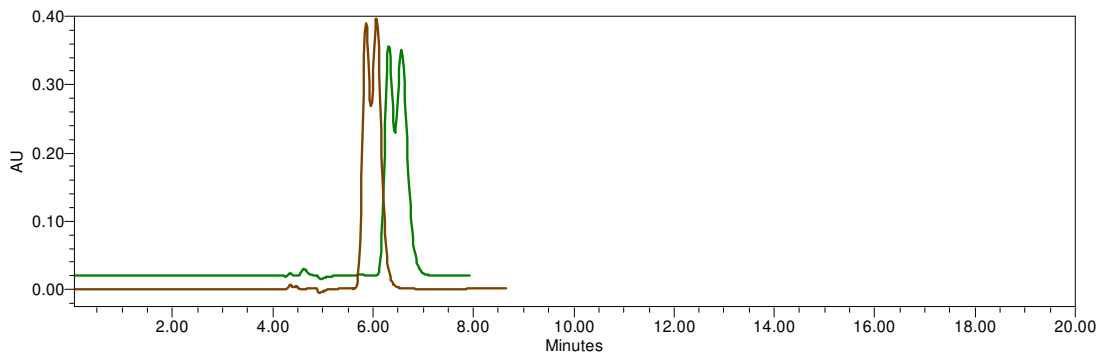


Figure 2b

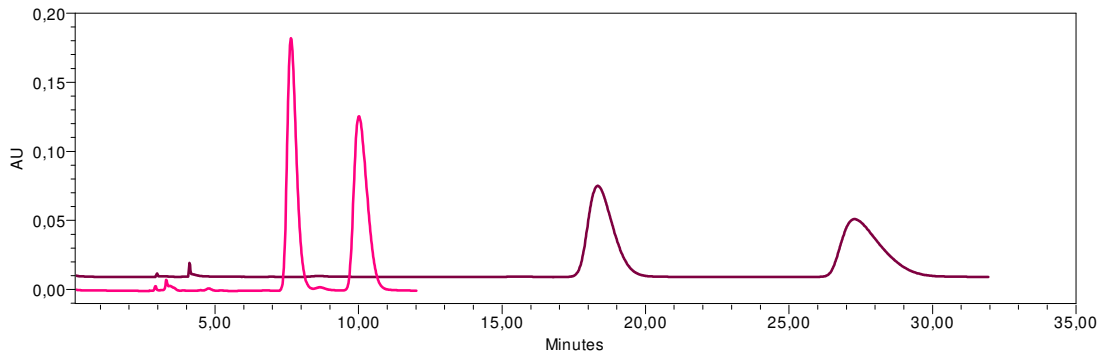


Figure 2c

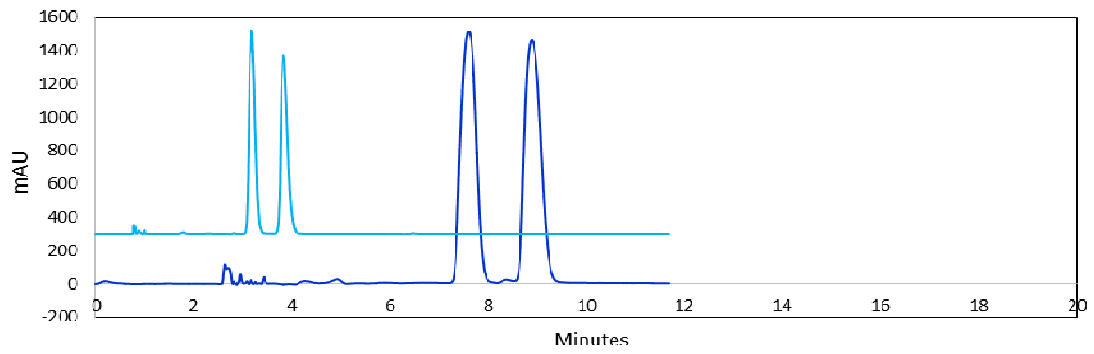


Figure 2d

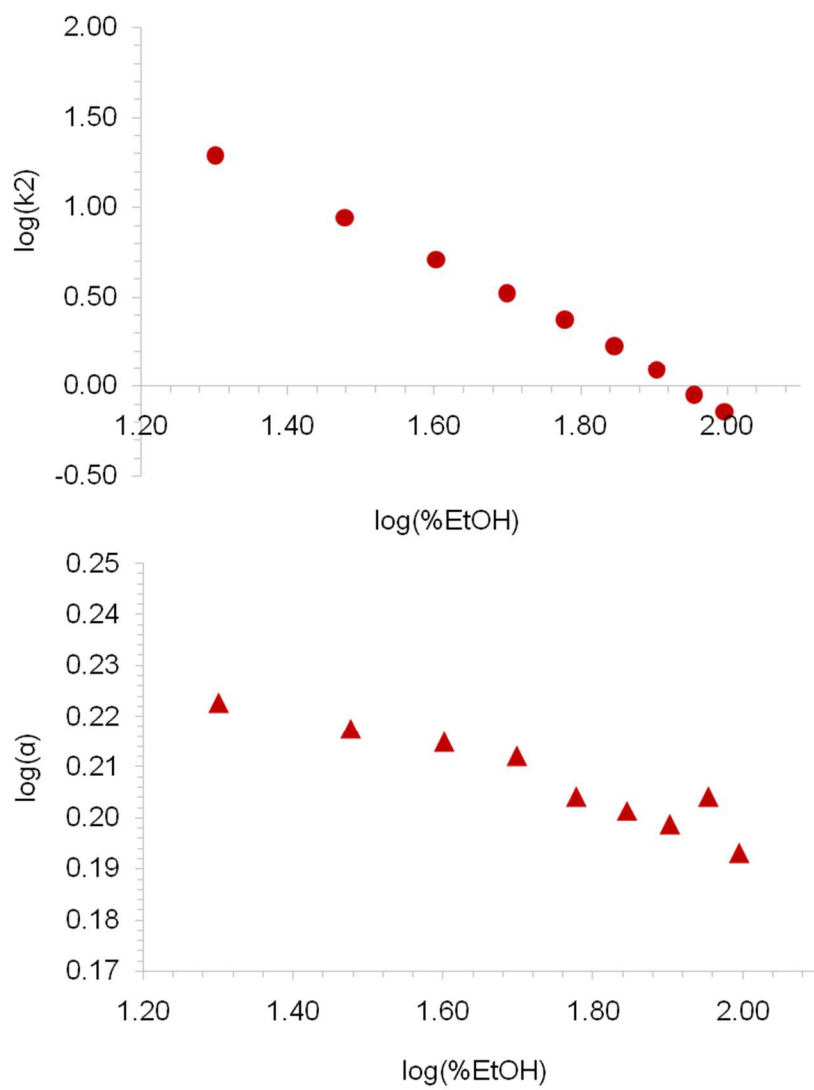


Figure 3

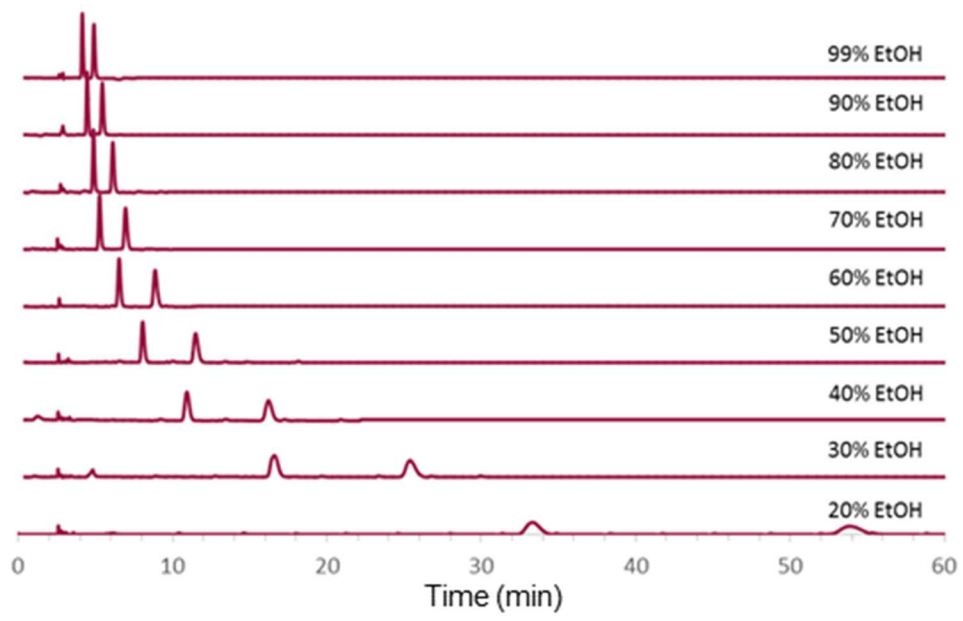


Figure 4

Table 1 Chromatographic parameters of compounds **1-8** in HPLC and SFC on Chiralcel OD-H CSP

| Compound                                | Mode | Conditions                              | tr <sub>1</sub> (min) | tr <sub>2</sub> (min) | k <sub>1</sub> | k <sub>2</sub> | Rs   | $\alpha$ |
|---|------|---|-----------------------|-----------------------|----------------|----------------|------|----------|
| <b>1</b>                                | POSC | 100% EtOH, 0.3 mL/min                   | 9.01                  | 9.8                   | 0.49           | 0.62           | 1.44 | 1.27     |
|   |      | 100% EtOH, 0.5 mL/min                   | 5.42                  | 5.89                  | 0.49           | 0.62           | 1.35 | 1.26     |
|   |      | 100% ACN, 0.5 mL/min                    | 4.90                  | -                     | 0.35           | -              | -    | -        |
|   | RPLC | 70:30 EtOH/H <sub>2</sub> O, 0.4 mL/min | 6.31                  | 6.56                  | 0.39           | 0.44           | <0.5 | 1.14     |
|   |      | 80:20 EtOH/H <sub>2</sub> O, 0.4 mL/min | 5.42                  | 5.89                  | 0.19           | 0.30           | 1.35 | 1.54     |
|   | NPLC | 30:70 EtOH /Hept, 1.0 mL/min            | 7.64                  | 10.04                 | 1.52           | 2.30           | 2.96 | 1.51     |
|   |      | 10:90 EtOH /Hept, 1.0 mL/min            | 18.33                 | 27.28                 | 5.05           | 8.00           | 4.29 | 1.58     |
|   | SFC  | 20:80 EtOH/CO <sub>2</sub> , 4.0 mL/min | 2.98                  | 3.58                  | 2.92           | 3.71           | 2.68 | 1.27     |
| 20:80 MeOH/CO <sub>2</sub> , 4.0 mL/min |      | 2.67                                    | 3.10                  | 2.51                  | 3.08           | 1.91           | 1.23 |          |
| <b>2</b>                                | POSC | 100% EtOH, 0.3 mL/min                   | 8.98                  | -                     | 0.48           | -              | -    | -        |
|   |      | 100% EtOH, 0.5 mL/min                   | 5.43                  | -                     | 0.49           | -              | <0.5 | -        |
|   |      | 100% ACN, 0.5 mL/min                    | 5.36                  | -                     | 0.47           | -              | -    | -        |
|   | RPLC | 30:70 EtOH/H <sub>2</sub> O, 0.4 mL/min | 7.06                  | 7.33                  | 0.55           | 0.61           | <0.5 | 1.11     |
|   |      | 20:80 EtOH/H <sub>2</sub> O, 0.4 mL/min | 5.40                  | -                     | 0.19           | -              | <0.5 | -        |
|   | NPLC | 30:70 EtOH /Hept, 1.0 mL/min            | 6.51                  | 6.71                  | 1.15           | 1.21           | <0.5 | 1.06     |
|   |      | 10:90 EtOH /Hept, 1.0 mL/min            | 12.58                 | 13.41                 | 3.15           | 3.43           | <0.5 | 1.09     |
|   | SFC  | 20:80 EtOH/CO <sub>2</sub> , 4.0 mL/min | 2.34                  | 2.54                  | 2.08           | 2.34           | 0.63 | 1.13     |
| 20:80 MeOH/CO <sub>2</sub> , 4.0 mL/min |      | 2.32                                    | -                     | 2.05                  | -              | <0.5           | -    |          |
| <b>3</b>                                | POSC | 100% EtOH, 0.3 mL/min                   | 8.41                  | -                     | 0.39           | -              | -    | -        |
|   |      | 100% EtOH, 0.5 mL/min                   | 6.02                  | -                     | 0.65           | -              | <0.5 | -        |
|   |      | 100% ACN, 0.5 mL/min                    | 7.09                  | 8.03                  | 0.95           | 1.21           | 1.77 | 1.27     |
|   | RPLC | 70:30 EtOH/H <sub>2</sub> O, 0.4 mL/min | 6.52                  | -                     | 0.43           | -              | <0.5 | -        |
|   |      | 80:20 EtOH/H <sub>2</sub> O, 0.4 mL/min | 6.01                  | -                     | 0.32           | -              | <0.5 | -        |
|   | NPLC | 30:70 EtOH /Hept, 1.0 mL/min            | 8.06                  | 8.97                  | 1.66           | 1.96           | 1.51 | 1.18     |
|   |      | 10:90 EtOH /Hept, 1.0 mL/min            | 15.00                 | 17.47                 | 3.95           | 4.77           | 2.19 | 1.21     |
|   | SFC  | 20:80 EtOH/CO <sub>2</sub> , 4.0 mL/min | 3.31                  | 3.80                  | 3.36           | 4.00           | 1.31 | 1.19     |
| 20:80 MeOH/CO <sub>2</sub> , 4.0 mL/min |      | 3.05                                    | 3.37                  | 3.01                  | 3.43           | 1.55           | 1.14 |          |
| <b>4</b>                                | POSC | 100% EtOH, 0.3 mL/min                   | 11.09                 | 11.43                 | 0.83           | 0.89           | <0.5 | 1.07     |
|   |      | 100% EtOH, 0.5 mL/min                   | 6.69                  | 6.87                  | 0.84           | 0.89           | <0.5 | 1.06     |
|   |      | 100% ACN, 0.5 mL/min                    | 7.40                  | 8.18                  | 1.04           | 1.25           | 1.54 | 1.21     |
|   | RPLC | 70:30 EtOH/H <sub>2</sub> O, 0.4 mL/min | 7.78                  | 8.65                  | 0.71           | 0.90           | 1.55 | 1.27     |
|   |      | 80:20 EtOH/H <sub>2</sub> O, 0.4 mL/min | 6.99                  | 7.58                  | 0.54           | 0.67           | 1.37 | 1.24     |
|   | NPLC | 30:70 EtOH /Hept, 1.0 mL/min            | 7.86                  | 8.51                  | 1.59           | 1.81           | 1.08 | 1.13     |
|   |      | 10:90 EtOH /Hept, 1.0 mL/min            | 15.58                 | 17.43                 | 4.14           | 4.75           | 1.41 | 1.15     |
|   | SFC  | 20:80 EtOH/CO <sub>2</sub> , 4.0 mL/min | 4.17                  | 4.33                  | 4.49           | 4.70           | <0.5 | 1.05     |
| 20:80 MeOH/CO <sub>2</sub> , 4.0 mL/min |      | 4.37                                    | -                     | 4.75                  | -              | -              | -    |          |
| <b>5</b>                                | POSC | 100% EtOH, 0.3 mL/min                   | 9.01                  | 9.52                  | 0.49           | 0.57           | 1.16 | 1.17     |
|   |      | 100% EtOH, 0.5 mL/min                   | 5.48                  | 5.78                  | 0.51           | 0.59           | 1.04 | 1.16     |
|   |      | 100% ACN, 0.5 mL/min                    | 6.29                  | 6.86                  | 0.73           | 0.89           | 1.08 | 1.21     |
|   | RPLC | 70:30 EtOH/H <sub>2</sub> O, 0.4 mL/min | 6.57                  | -                     | 0.45           | -              | <0.5 | -        |
|   |      | 80:20 EtOH/H <sub>2</sub> O, 0.4 mL/min | 6.08                  | -                     | 0.34           | -              | <0.5 | -        |
|   | NPLC | 30:70 EtOH /Hept, 1.0 mL/min            | 6.47                  | 7.53                  | 1.14           | 1.49           | 1.89 | 1.31     |
|   |      | 10:90 EtOH /Hept, 1.0 mL/min            | 12.29                 | 14.84                 | 3.06           | 3.90           | 1.54 | 1.28     |
|   | SFC  | 20:80 EtOH/CO <sub>2</sub> , 4.0 mL/min | 2.76                  | 3.12                  | 2.63           | 3.11           | 1.68 | 1.18     |

|                       |      |   |                       |       |      |       |      |      |
|-----------------------|------|---|-----------------------|-------|------|-------|------|------|
| 6                     | POSC | 20:80 MeOH/CO <sub>2</sub> , 4.0 mL/min | 2.73                  | 3.05  | 2.59 | 3.01  | 2.01 | 1.16 |
|                       |      | 100% EtOH, 0.3 mL/min                   | 8.94                  | 9.20  | 0.48 | 0.52  | <0.5 | 1.09 |
|                       |      | 100% EtOH, 0.5 mL/min                   | 5.49                  | 5.66  | 0.51 | 0.56  | <0.5 | 1.09 |
|                       |      | 100% ACN, 0.5 mL/min                    | 6.19                  | -     | 0.70 | -     | <0.5 | -    |
|                       | RPLC | 70:30 EtOH/H <sub>2</sub> O, 0.4 mL/min | 7.43                  | 7.86  | 0.63 | 0.73  | <0.5 | 1.15 |
|                       |      | 80:20 EtOH/H <sub>2</sub> O, 0.4 mL/min | 6.84                  | 7.09  | 0.50 | 0.56  | <0.5 | 1.11 |
|                       | NPLC | 30:70 EtOH /Hept, 1.0 mL/min            | 7.87                  | 8.54  | 1.60 | 1.82  | 1.18 | 1.14 |
|                       |      | 10:90 EtOH /Hept, 1.0 mL/min            | 33.09                 | 35.07 | 9.92 | 10.57 | 1.41 | 1.07 |
|                       | SFC  | 20:80 EtOH/CO <sub>2</sub> , 4.0 mL/min | 5.59                  | 6.39  | 6.36 | 7.41  | 2.30 | 1.17 |
|                       |      | 20:80 MeOH/CO <sub>2</sub> , 4.0 mL/min | 4.73                  | 5.61  | 5.22 | 6.38  | 3.23 | 1.22 |
| 7                     | POSC | 100% EtOH, 0.3 mL/min                   | 8.85                  | 9.40  | 0.46 | 0.55  | 1.34 | 1.20 |
|                       |      | 100% EtOH, 0.5 mL/min                   | 5.51                  | 5.90  | 0.52 | 0.62  | 1.22 | 1.21 |
|                       |      | 100% ACN, 0.5 mL/min                    | 3.95                  | -     | 0.09 | -     | -    | -    |
|                       | RPLC | 70:30 EtOH/H <sub>2</sub> O, 0.4 mL/min | 6.83                  | 7.00  | 0.50 | 0.54  | <0.5 | 1.07 |
|                       |      | 80:20 EtOH/H <sub>2</sub> O, 0.4 mL/min | 5.51                  | 5.90  | 0.21 | 0.30  | 1.17 | 1.40 |
|                       | NPLC | 30:70 EtOH /Hept, 1.0 mL/min            | 7.66                  | 8.09  | 1.53 | 1.67  | 0.90 | 1.09 |
|                       |      | 10:90 EtOH /Hept, 1.0 mL/min            | 11.93                 | 13.08 | 2.94 | 3.32  | 1.19 | 1.13 |
|                       | SFC  | 20:80 EtOH/CO <sub>2</sub> , 4.0 mL/min | 2.87                  | 3.31  | 2.78 | 3.36  | 1.26 | 1.21 |
|                       |      | 20:80 MeOH/CO <sub>2</sub> , 4.0 mL/min | 2.60                  | 3.05  | 2.42 | 3.01  | 1.36 | 1.24 |
|                       | 8    | POSC                                    | 100% EtOH, 0.3 mL/min | 5.19  | -    | 0.14  | -    | -    |
| 100% EtOH, 0.5 mL/min |      |   | 4.91                  | -     | 0.35 | -     | -    | -    |
| 100% ACN, 0.5 mL/min  |      |   | 6.17                  | -     | 0.70 | -     | -    | -    |
| RPLC                  |      | 70:30 EtOH/H <sub>2</sub> O, 0.4 mL/min | 5.33                  | -     | 0.17 | -     | -    | -    |
|                       |      | 80:20 EtOH/H <sub>2</sub> O, 0.4 mL/min | 4.90                  | -     | 0.08 | -     | -    | -    |
| NPLC                  |      | 30:70 EtOH /Hept, 1.0 mL/min            | 5.81                  | 6.16  | 0.92 | 1.03  | <0.5 | 1.13 |
|                       |      | 10:90 EtOH /Hept, 1.0 mL/min            | 13.20                 | 14.27 | 3.36 | 3.71  | <0.5 | 1.11 |
| SFC                   |      | 20:80 EtOH/CO <sub>2</sub> , 4.0 mL/min | 2.91                  | 3.02  | 2.83 | 2.97  | <0.5 | 1.05 |
|                       |      | 20:80 MeOH/CO <sub>2</sub> , 4.0 mL/min | 2.66                  | -     | 2.50 | -     | -    | -    |

$tr_1$  parameter corresponds to retention time of the 1<sup>st</sup> enantiomer

$tr_2$  parameter corresponds to retention time of the 2<sup>nd</sup> enantiomer

$k_1$  parameter corresponds to the retention factor of the 1<sup>st</sup> enantiomer calculated as follow  $k_1 = (tr_1 - t_0/t_0)$

$k_2$  parameter corresponds to the retention factor of the 2<sup>nd</sup> enantiomer calculated as follow  $k_2 = (tr_2 - t_0/t_0)$

$R_s$  and  $\alpha$  parameters correspond to respectively resolution and selectivity between enantiomers 1 and 2 calculated as follow  $R_s = 2 (tr_2 - tr_1) / (\omega_2 + \omega_1)$  and  $\alpha = k_2/k_1$

Table 3 Chromatographic parameters for compounds **4**, **5**, **6** and **8** under various percentages of co-solvent on Lux Cellulose-2 CSP

| Compound | EtOH (%) | tr <sub>1</sub> (min) | tr <sub>2</sub> (min) | k <sub>1</sub> | k <sub>2</sub> | Rs   | α    | N <sub>1</sub> | N <sub>2</sub> |
|----------|----------|-----------------------|-----------------------|----------------|----------------|------|------|----------------|----------------|
| <b>4</b> | 20       | 30.65                 | 42.77                 | 10.65          | 15.26          | 8.69 | 1.43 | 8058           | 11023          |
|          | 30       | 15.69                 | 21.23                 | 4.96           | 7.07           | 8.69 | 1.42 | 9940           | 12617          |
|          | 40       | 10.49                 | 13.75                 | 2.98           | 4.22           | 6.16 | 1.41 | 8748           | 8810           |
|          | 50       | 8.03                  | 10.22                 | 2.05           | 2.88           | 5.28 | 1.41 | 7491           | 7575           |
|          | 60       | 6.69                  | 8.23                  | 1.54           | 2.12           | 4.42 | 1.38 | 7545           | 7188           |
|          | 70       | 5.85                  | 7.03                  | 1.19           | 1.63           | 3.82 | 1.37 | 7271           | 6626           |
|          | 80       | 5.39                  | 6.30                  | 0.96           | 1.29           | 3.43 | 1.34 | 5941           | 6414           |
|          | 90       | 5.13                  | 5.87                  | 0.80           | 1.06           | 2.37 | 1.32 | 4619           | 5480           |
| 99       | 4.91     | 5.42                  | 0.72                  | 0.90           | 1.91           | 1.25 | 5940 | 6000           |                |
| Compound | EtOH (%) | tr <sub>1</sub> (min) | tr <sub>2</sub> (min) | k <sub>1</sub> | k <sub>2</sub> | Rs   | α    | N <sub>1</sub> | N <sub>2</sub> |
| <b>5</b> | 20       | 23.38                 | 33.29                 | 7.88           | 11.65          | 9.82 | 1.47 | 6512           | 9966           |
|          | 30       | 12.07                 | 17.02                 | 3.58           | 5.47           | 8.60 | 1.52 | 7613           | 8975           |
|          | 40       | 8.35                  | 11.31                 | 2.17           | 3.30           | 6.91 | 1.51 | 8685           | 8606           |
|          | 50       | 6.63                  | 8.62                  | 1.52           | 2.27           | 6.14 | 1.49 | 9550           | 8374           |
|          | 60       | 5.65                  | 7.11                  | 1.14           | 1.70           | 4.97 | 1.48 | 7234           | 7843           |
|          | 70       | 5.09                  | 6.23                  | 0.90           | 1.33           | 4.34 | 1.47 | 7584           | 7940           |
|          | 80       | 4.79                  | 5.70                  | 0.74           | 1.08           | 3.82 | 1.44 | 7870           | 8093           |
|          | 90       | 4.58                  | 5.33                  | 0.60           | 0.87           | 3.08 | 1.43 | 6688           | 6617           |
| 99       | 4.46     | 5.10                  | 0.56                  | 0.77           | 2.50           | 1.40 | 6445 | 5852           |                |
| Compound | EtOH (%) | tr <sub>1</sub> (min) | tr <sub>2</sub> (min) | k <sub>1</sub> | k <sub>2</sub> | Rs   | α    | N <sub>1</sub> | N <sub>2</sub> |
| <b>6</b> | 20       | 33.31                 | 53.87                 | 11.66          | 19.48          | 9.77 | 1.67 | 6445           | 7091           |
|          | 30       | 16.64                 | 25.76                 | 5.32           | 8.79           | 8.65 | 1.65 | 6140           | 7563           |
|          | 40       | 10.88                 | 16.16                 | 3.13           | 5.14           | 8.46 | 1.64 | 6895           | 7835           |
|          | 50       | 8.04                  | 11.45                 | 2.05           | 3.35           | 7.55 | 1.63 | 7338           | 7460           |
|          | 60       | 6.52                  | 8.87                  | 1.47           | 2.37           | 6.21 | 1.60 | 6471           | 6815           |
|          | 70       | 5.53                  | 7.23                  | 1.07           | 1.70           | 5.49 | 1.59 | 6948           | 6758           |
|          | 80       | 4.89                  | 6.15                  | 0.78           | 1.24           | 4.75 | 1.58 | 7221           | 6834           |
|          | 90       | 4.45                  | 5.42                  | 0.56           | 0.90           | 4.15 | 1.60 | 7159           | 6474           |
| 99       | 4.17     | 4.92                  | 0.46                  | 0.72           | 3.44           | 1.56 | 7408 | 6544           |                |
| Compound | EtOH (%) | tr <sub>1</sub> (min) | tr <sub>2</sub> (min) | k <sub>1</sub> | k <sub>2</sub> | Rs   | α    | N <sub>1</sub> | N <sub>2</sub> |
| <b>8</b> | 20       | 30.24                 | 45.56                 | 10.49          | 16.32          | 7.69 | 1.55 | 5287           | 6230           |
|          | 30       | 14.25                 | 20.65                 | 4.41           | 6.85           | 6.76 | 1.55 | 4702           | 5948           |
|          | 40       | 9.03                  | 12.63                 | 2.43           | 3.80           | 5.71 | 1.56 | 4623           | 4713           |
|          | 50       | 6.74                  | 9.06                  | 1.56           | 2.44           | 4.61 | 1.56 | 3597           | 4146           |
|          | 60       | 5.55                  | 7.20                  | 1.11           | 1.73           | 4.05 | 1.56 | 2619           | 3179           |
|          | 70       | 4.83                  | 6.09                  | 0.80           | 1.28           | 3.32 | 1.58 | 3020           | 3530           |
|          | 80       | 4.43                  | 5.43                  | 0.61           | 0.98           | 3.16 | 1.59 | 3022           | 3244           |
|          | 90       | 4.19                  | 5.06                  | 0.47           | 0.77           | 2.49 | 1.64 | 2603           | 2876           |
| 99       | 4.04     | 4.78                  | 0.41                  | 0.67           | 2.16           | 1.62 | 2495 | 2641           |                |

Conditions: 150 bar, 40°C, 1 mL/min, λ=220 nm.

Chromatographic parameters definitions as Table 1.

$N_1$  parameter correspond to efficiency of the stationary phase regarding enantiomer 1 calculated as follow  $N_1 = 16 (tr_1/\omega_1)^2$

$N_2$  parameter correspond to efficiency of the stationary phase regarding enantiomer 2 calculated as follow  $N_2 = 16 (tr_2/\omega_2)^2$



Table 2 Chromatographic parameters for compounds **1-8** with two different types of co-solvent on Lux Cellulose-2 CSP

| Compound | Co-solvent | tr <sub>1</sub> (min) | tr <sub>2</sub> (min) | k <sub>1</sub> | k <sub>2</sub> | $\alpha$ | Rs   |
|----------|------------|-----------------------|-----------------------|----------------|----------------|----------|------|
| <b>1</b> | EtOH       | 5.86                  | 6.11                  | 7.14           | 7.49           | 1.05     | 0.99 |
|          | MeOH       | 5.38                  | 5.66                  | 6.37           | 6.75           | 1.06     | 1.01 |
| <b>2</b> | EtOH       | 3.05                  | 3.88                  | 3.23           | 4.38           | 1.35     | 1.52 |
|          | MeOH       | 3.14                  | 3.79                  | 3.36           | 4.19           | 1.25     | 1.21 |
| <b>3</b> | EtOH       | 4.55                  | 5.61                  | 5.32           | 6.79           | 1.28     | 2.83 |
|          | MeOH       | 4.35                  | 5.09                  | 4.96           | 5.97           | 1.20     | 2.66 |
| <b>4</b> | EtOH       | 6.59                  | 9.13                  | 8.15           | 11.68          | 1.43     | 5.98 |
|          | MeOH       | 7.09                  | 9.17                  | 8.71           | 11.56          | 1.33     | 5.09 |
| <b>5</b> | EtOH       | 4.91                  | 7.17                  | 5.82           | 8.96           | 1.54     | 5.95 |
|          | MeOH       | 5.44                  | 7.16                  | 6.45           | 8.81           | 1.37     | 5.41 |
| <b>6</b> | EtOH       | 7.21                  | 11.42                 | 9.01           | 14.86          | 1.65     | 8.82 |
|          | MeOH       | 7.09                  | 9.50                  | 8.59           | 12.01          | 1.40     | 3.31 |
| <b>7</b> | EtOH       | 4.35                  | 4.74                  | 5.04           | 5.58           | 1.11     | 1.40 |
|          | MeOH       | 4.15                  | 4.58                  | 4.76           | 5.36           | 1.12     | 1.53 |
| <b>8</b> | EtOH       | 6.43                  | 9.69                  | 7.93           | 12.46          | 1.57     | 5.92 |
|          | MeOH       | 6.00                  | 8.10                  | 7.34           | 10.26          | 1.40     | 4.76 |

Conditions: 150 bar, 40°C, 4 mL/min, 20% co-solvent,  $\lambda=220$  nm.

Chromatographic parameters definitions as Table 1.

