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Supercritical fluid chromatography and liquid chromatography for isomeric separation of a multiple chiral centers analyte

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► **To cite this version:**

Zuo La, Julie Charton, Lucile Etienne, Julie Bourey, Emmanuelle Lipka. Supercritical fluid chromatography and liquid chromatography for isomeric separation of a multiple chiral centers analyte. *Journal of Chromatography A*, 2021, 1651, pp.462270. 10.1016/j.chroma.2021.462270 . hal-04403353

HAL Id: hal-04403353

<https://hal.science/hal-04403353>

Submitted on 22 Jul 2024

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26 ABSTRACT

27 The development of a chiral separation strategy has always been a challenge of crucial
28 importance, particularly in the pharmaceutical field. Chromatographic methods have become
29 popular, particularly High Performance Liquid Chromatography and Supercritical Fluid
30 Chromatography from a preparative scale point of view. A bioactive compound bearing three
31 stereogenic centers was entrusted in our laboratory and the aim of this work was to obtain the
32 complete resolution of the eight stereoisomers. Nine different polysaccharide-based columns
33 were tested in SFC under various carbon dioxide-based mobile phases. The use of a single
34 chiral column Lux Cellulose-2 under 30% 2-PrOH in carbon dioxide, at a flow-rate of 1
35 mL/min, column temperature of 40°C, 120 bar outlet pressure allowed the obtention of eight
36 peaks. To further improve the resolution of the two last isomers, two columns were serially
37 coupled ~~thanks to the properties of supercritical fluid~~. The results obtained with the six
38 different combinations are discussed. The tandem column supercritical fluid chromatography
39 has demonstrated to be a useful technique to resolve the eight stereoisomers on Lux
40 Cellulose-2//Cellulose-2 tandem of coupled columns with 30% 2-PrOH in carbon dioxide, at a
41 flow-rate of 1 mL/min, column temperature of 40°C and 120 bar outlet pressure, despite a
42 long analysis time. **In order to compare the two methods (i.e supercritical and liquid)**, chiral
43 liquid chromatography under polar aqueous-organic mode, polar organic mode and normal-
44 phase mode, was implemented. The last mode allowed the full baseline resolution of the eight
45 isomers on Cellulose-5 CSP, with 20% 2-PrOH in *n*-heptane at a flow-rate of 0.8 mL/min, at
46 25°C, $\lambda = 220$ nm. The limits of detection and of quantification were determined for this
47 method and the **best** values obtained for isomer 8 were equal to 2.84 and 9.37 nM
48 respectively. Finally, a small-scale preparative separation of the multiple chiral centers
49 compound was implemented on Cellulose-5 CSP within 10% 2-PrOH in *n*-heptane in order to
50 study the stereoisomer elution order on Cellulose-2, Cellulose-5 and Chiralpak AD-H, under

51 EtOH or 2-PrOH in *n*-heptane mobile phases, and partial reversal elution orders were
52 observed.

53 **1. Introduction**

54 ~~Chirality is a key feature of amino acids and polysaccharides and evolution exclusively~~
55 ~~selected L-amino acids and D-sugars as homochiral building block of proteins and nucleic~~
56 ~~acids in all living species. Even if the origin of chirality in life is still obscure, Nowadays, it is~~
57 ~~widely known that chirality profoundly influences most biological mechanisms.~~ Because of
58 the interest in the consequences of chirality on the physical and biological properties of
59 molecules, the separation of enantiomers (and stereoisomers in a larger frame) has been
60 extensively studied. Among the chromatographic methods giving simultaneously access to
61 both enantiomers, high performance liquid chromatography (HPLC) and supercritical fluid
62 chromatography (SFC) are the preferred ones because of the preparative upscale possibility
63 among other reasons. Polysaccharide-based chiral stationary phases (CSP) are applicable
64 under SFC and HPLC conditions and are today very popular owing to their broad range of
65 applications, their availability of numerous different chemical moieties and their high loading
66 capacity which is useful at the preparative scale [1; 2]. ~~Okamoto *et al.* developed the tris(3,5-~~
67 ~~dimethylphenylcarbamate) of cellulose and amylose stationary phases, together with the~~
68 ~~cellulose tris(4-methylbenzoate) and the amylose tris((S)- α -methylbenzylcarbamate) phases,~~
69 ~~the so-called “golden four”, then, chlorinated polysaccharides have also come to market.~~
70 ~~Immobilized versions of these coated phases were also recently commercialized. Different~~
71 ~~strategies are using those CSPs in liquid chromatography and have been defined as polar~~
72 ~~aqueous-organic solvent chromatography (or reversed phase chromatography) [3; 4], polar~~
73 ~~organic solvent chromatography [5; 6] and alcohol hydrocarbon solvent chromatography (or~~
74 ~~normal phase) chromatography [7].~~

75 Besides, the intrinsic properties of supercritical CO₂ (low viscosity and high diffusivity of the
76 mobile phase, together with a lower pressure drop) are particularly suitable for use with
77 multiple columns in series. ~~In addition, there is no distinction in SFC between normal and~~
78 ~~reverse phase separations.~~ This feature enables the coupling of two (or more) columns in
79 series, as well as combinations of columns with different polarities, in order to achieve high
80 separation efficiency with greater column length and targeted selectivity. Berger and Wilson,
81 for example, coupled 11 columns in series and reported only a 160 bar pressure drop [3]. The
82 number of possible combinations, comprising matched chiral-chiral similar phases [4], chiral-
83 chiral different phases [5-7] and achiral-chiral tandems [8; 9] is large, allowing high
84 efficiency and giving birth to many novel and unique chiral selectivity properties. It is
85 noteworthy that some former papers have highlighted that tandem columns separation may
86 depend on the order in which the columns are coupled [10; 11].

87 The dihydropyridone derivative **1** bearing three chiral carbons was synthesized in a project of
88 discovery of Takeda G-protein-coupled receptor-5 (TGR5) agonists for the treatment of
89 metabolic and inflammatory diseases (Figure 1) [12]. ~~The compound was first synthesized~~
90 ~~and tested as a mixture of isomers. A synthesis starting from chiral commercially available~~
91 ~~reactants is now planned to obtain the eight isomers individually in order to evaluate their~~
92 ~~pharmacological activity. Thus, a method allowing the full chiral separation in a short time is~~
93 ~~highly desired, in order to check the chiral purity of each synthesized isomer. However, the~~
94 ~~approach is more complex when three asymmetric centers are presents giving birth to eight~~
95 ~~stereoisomers, with an enantiomeric relationship two by two, requiring both chiral recognition~~
96 ~~and achiral interaction with the stationary phase for successful separation. The difficulty of~~
97 ~~this task is demonstrated by the scarce number of publications summarized in a review paper~~
98 ~~by Al-Othman *et al.* in 2016 [13] and devoted to compounds containing two chiral centers~~
99 ~~[14-16]. However, the challenge represented by the separation of multiple chiral centers~~

100 (MCC) compounds remains a hot topic and is coming in the spotlight. In 2018, a paper from
101 Amgen Corp depicted the separation of a three chiral centers compound which was fully
102 separated under normal-phase liquid chromatography on Chiralcel OD-H [17]. Very recently
103 Genentech Corp reported HPLC and SFC screening strategies and a two-dimensional
104 multicolumn liquid chromatography approaches for compounds with MCC [18; 19]. In the
105 first paper, whose goal was to select CSPs offering the greatest complementarity, 13 MCC
106 compounds were chosen, among them 8 have two chiral centers, 4 have three chiral centers
107 and 1 has five chiral centers and none of the five last MCC compounds were fully separated
108 neither in SFC nor in HPLC. In the second paper, 8 isomers of a proprietary compound were
109 fully separated in 24 minutes, but it required a 2D-LC approach.

110 Thus, in the present paper the different possibilities of chromatography with polysaccharide-
111 based CSP, were explored in order to separate all the isomers of this dihydropyridone
112 compound in one step, as the synthesis of the pure stereoisomers will be undertaken.

113 The first goal of this work was to fully separate the eight isomers of the dihydropyridone
114 derivative in the best conditions. To that aim, we investigated chiral supercritical fluid
115 chromatography, either through a single column screening or thanks to tandems of columns
116 and compared it with chiral liquid chromatography under three modes: polar aqueous-organic
117 mode, polar organic mode and normal-phase mode. The second step was to evaluate the limit
118 of detection and the limit of quantification of the method. An additional step was to study the
119 elution order of the eight isomers under different conditions of either mobile phase or chiral
120 stationary phase. To date, successful separation of MCC compound has not been reported in
121 SFC neither on a single column, nor thanks to coupled-columns.

122 **2. Material and methods**

123 **2.1 Chemicals**

124 Compound **1** was synthesized according to procedures previously described [12]. The
125 methanol (MeOH), ethanol (EtOH), propan-2-ol (iPrOH) and acetonitrile (ACN) were HPLC
126 grade and were purchased from Carlo-Erba Reagents (Val-de-Reuil, France). Carbon dioxide
127 (CO₂, 99.995% purity) was purchased from Linde (Saint Priest, France). Deionized (DI) water
128 was obtained from Milli-Q system (Millipore, Saint Quentin-en-Yvelines, France).

129 **2.2 Supercritical Fluid Chromatography Apparatus**

130 **2.2.1 Solution samples**

131 For analytical screening, solutions were prepared at 1.5 mM in EtOH. The solutions were
132 always degassed in an ultrasonic bath and filtered with a 0.45 m PTFE syringe-filter (15 mm
133 diameter) prior to use.

134 **2.2.2 Stationary phases**

135 Nine chiral analytical columns were used for this study (~~Figure 1b~~). Chiralpak AS-H based on
136 amylose *tris*((*S*)- α -methylbenzylcarbamate), Chiralcel OJ-H based on cellulose *tris*(4-
137 methylbenzoate), Chiralcel OD-H based on cellulose *tris*(3,5-dimethylphenylcarbamate) and
138 Chiralpak AD-H based on amylose *tris*(3,5-dimethylphenylcarbamate), Chiralpak IA based on
139 amylose *tris*(3,5-dimethylphenylcarbamate) and Chiralpak IB based on cellulose *tris*(3,5-
140 dimethylphenylcarbamate) were purchased from Chiral Technologies Europe (Illkirch,
141 France) and Lux Cellulose-2 based on cellulose *tris*(3-chloro-4-methylphenylcarbamate), Lux
142 Amylose-2 based on amylose *tris*(3-chloro-4-methylphenylcarbamate) and Lux i-Cellulose-5
143 based on cellulose *tris*(3,5-dichlorophenylcarbamate) from Phenomenex[®] (Le Pecq, France).
144 All columns had dimensions of 250 \times 4.6 mm i.d. with 5 μ m particle size and were coated
145 silica-gel phases excepted for the Lux i-Cellulose-5, Chiralpak IA and Chiralpak IB which
146 were immobilized silica-gel phases. The tubing used to couple columns was a 1/32" Inox
147 flexible tube of 0.170 \times 400 mm purchased from PIC Solution (Avignon, France) with a
148 negligible influence on pressure.

149 **2.2.3. Chromatographic system and conditions**

150 The chromatographic system used was an SFC-PICLAB hybrid 10-20 apparatus (PIC
151 Solution, Avignon, France) equipped with an autosampler comprising a 48-vial plate (model
152 Alias, from Knauer, Berlin, Germany), three model 40 P pumps (two for CO₂ and a third for
153 the modifier (Knauer, Berlin, Germany)), a column oven with a Valco ten-position column
154 selection valve, and a Valco six-position solvent switching valve. The system was also
155 composed of a Smartline 2600 diode array detector (DAD) (Knauer, Berlin, Germany).
156 Detection wavelength was set at 210 nm. The system was controlled and the data was
157 acquired using the SFC PicLab Analytic Online v.3.1.2 software (PIC Solution, Avignon,
158 France). The volume injected was 20 µL. During the separation optimization, the mobile
159 phase was always a CO₂-modifier mixture with 20% of MeOH or EtOH as a modifier at 3
160 mL/min flow rate. All analyses were run in isocratic mode. The column oven temperature was
161 40°C and the outlet pressure was maintained at 120 bar for all experiments.

162 **2.3 High Performance Liquid Chromatography Apparatus**

163 For analytical screening, solutions were prepared at 1.5 mM in either isopropanol or ethanol
164 depending on the mobile phase used for chromatography. The solutions were always degassed
165 in an ultrasonic bath and filtered with a 0.45 µm PTFE syringe-filter (15 mm diameter) prior to
166 use.

167 **2.3.1 Stationary phases**

168 For normal-phase chromatography, analyses were carried out on Chiralpak AD-H based on
169 amylose *tris*(3,5-dimethylphenylcarbamate) and Chiralcel OD-H based on cellulose *tris*(3,5-
170 dimethylphenylcarbamate) (250 × 4.6 mm i.d; 5 µm) columns, Chiralpak AS based on
171 amylose *tris*((*S*)- α -methylbenzylcarbamate) and Chiralcel OJ based on cellulose *tris*(4-
172 methylbenzoate) (250 × 4.6 mm i.d; 10 µm) columns. They were purchased from Chiral
173 Technologies Europe (Illkirch, France) and were coated silica-gel phases. Lux Cellulose-2

174 based on cellulose *tris*(3-chloro-4-methylphenylcarbamate) and Lux i-Cellulose-5 based on
175 cellulose *tris*(3,5-dichlorophenylcarbamate) (250 × 4.6 mm i.d; 5 μm) were from
176 Phenomenex® (Le Pecq, France). Lux Cellulose-2 was coated silica-gel phase and the Lux i-
177 Cellulose-5 was immobilized silica-gel phase.

178 For polar aqueous-organic and polar organic modes, analyses were carried out on Chiralcel®
179 OD-RH based on cellulose *tris*(3,5-dimethylphenylcarbamate (150 × 4,6 mm ; 5 μm) et
180 Chiralcel® OJ-R based on cellulose *tris*(4-methylbenzoate) (150 × 4,6 mm ; 10 μm).
181 They were purchased from Chiral Technologies Europe (Illkirch, France) and were coated
182 silica-gel phases.

183 2.3.2. Chromatographic system and conditions

184 The analytical HPLC system used was constituted of a Waters in-line degasser apparatus, a
185 column oven compartment and a gradient Waters 600 E metering pump model equipped with
186 a Waters 996 photodiode array spectrophotometer. Chromatographic data were collected and
187 processed on a personal computer running with Millennium 2010 software from Waters. The
188 sample loop was 20 μL (Rheodyne 7125 injector). All the instrumentation was from Waters
189 Corporation (Millford, MA, USA). Detection was achieved at the maximum wavelength 210
190 nm. All separations were carried out at 25°C. All analyses were run in isocratic mode. The
191 column void time (t_0) was considered to be equal to the peak of the solvent front and was
192 taken from each particular run. It was equal to the value obtained by injection of 1,3,5-tri-*tert*-
193 butylbenzene used as a non-retained sample. Retention times were mean values of two
194 replicate determinations. The flow-rate varied between 0.4 and 0.8 mL/min.

195 3. Results and discussion

196 In order to separate the eight stereoisomers of the dihydropyridone derivative **1** containing
197 three chiral centers, the three modes of liquid chromatography and individual *versus* tandem
198 columns in supercritical fluid chromatography were explored. Considering the CSPs, eleven

199 chiral phases differing in the polysaccharide backbone (cellulose or amylose), the derivative
200 type (carbamate or ester), the nature of the CSP binding (coated or immobilized), the nature of
201 the substituents and the utilization mode (reversed-phase, POSC or normal-phase), were
202 chosen. ~~(Figure 1b)~~ Most of them are used in both HPLC and SFC methodologies [20; 21].
203 Besides, SFC is known to be the most effective for chiral separation as the resolution *per* time
204 unit is most of the time better than in HPLC [22] and is unequivocally greener than normal
205 phase HPLC.

206 3.1 Supercritical fluid chromatography

207 3.1.1 Individual column screening

208 A screening of the nine different CSPs ~~represented in Figure 1b~~, was carried-out. This
209 screening was run under the following conditions: 20% of co-solvent (either EtOH or MeOH)
210 in a carbon dioxide-based mobile phase; 3 mL/min flow-rate; 40°C and 120 bar outlet
211 pressure. Stacked chromatograms of compound **1**, related to the four amylose-based CSPs
212 under 20% EtOH are represented on Figure 1a (Supplementary Information). From this figure
213 some remarks can be done, i) the elution profile is very dissimilar from one CSP to another
214 under the same co-solvent, ii) conversely the chromatograms obtained on same CSP but in
215 MeOH (data not shown), are very similar to those observed under EtOH lastly iii) no
216 amylose-based CSP is able to fully resolve the eight isomers in SFC. To complete the
217 screening, cellulose-based CSPs were tested and stacked chromatograms of compound **1**,
218 related to the four first cellulose-based CSPs under 20% EtOH are given on Figure 1b
219 (Supplementary Information). Here again, it is worth noting that i) the elution profile is very
220 dissimilar from one CSP to another under the same co-solvent, ii) on the contrary the
221 chromatograms obtained on same CSP but in MeOH (data not shown), are very similar to
222 those observed under EtOH and iii) among the four first cellulose-based CSPs none is able to
223 fully resolve the eight isomers in SFC, at the best, seven peaks were observed on Cellulose-5

224 CSP. However, the result obtained with the Cellulose-2 column is presented on the Figure 2
225 (upper part), within 20% EtOH in carbon dioxide-based mobile phase, where it can be seen
226 that eight peaks are eluted. Cellulose-5 (Fig. 1b, SI) and Cellulose-2 (Fig. 2) columns confirm
227 past studies carried out in SFC, indicating that chlorine-containing phases would show
228 additional enantiorecognition abilities compared to the polysaccharide-based selectors without
229 chlorine [23]. Thus, the Cellulose-2 column was chosen and three types of different co-
230 solvent (*i.e* MeOH, 2-PrOH, ACN) were tested at 20%. Chromatograms are illustrated on the
231 Figure 2, all four solvents leading to eight peaks. In addition, 20% of ACN and 2-PrOH
232 mixture in various proportions, were investigated, but were found to allow systematically six
233 peaks (data not shown). In chiral SFC, the parameters which deeply affect the resolution are
234 the nature and the percentage of co-solvent and the flow-rate [22; 24]. With watching the
235 Figure 2, ACN and 2-PrOH were the most promising co-solvents. **However, the former one**
236 **conducts to broad peaks and long analysis time because of its low hydrogen-bonding**
237 **capabilities due to its aprotic character.** Further experiments were then carried-out with 2-
238 PrOH in order to get a full baseline resolution. The percentage of co-solvent was moved from
239 20 to 15 to 10% (Figure 2a in Supplementary Information and Table 1). The most affected
240 parameter was the retention which increases drastically and, to a lesser extent, the resolution
241 which was improved. However, the analysis time was not acceptable (around 80 minutes
242 under the lower percentage).

243 **The second parameter which can be investigated is the flow-rate, usually, to reduce analysis**
244 **time. But in our case, the flow-rate was reduced to enhance the resolution through a higher**
245 **number of plates (in spite of expected longer analysis time).** Thus, moving from 3 to 1
246 mL/min within 20% 2-PrOH, the overall resolution values were improved, leading to at least
247 1.50 for the lower value (Figure 2b, in Supplementary Information and Table 1). As expected
248 the analysis time was longer (around 70 minutes). In a last effort to reduce analysis time

249 without affecting the resolution too much, a flow-rate of 1 mL/min was kept but 30% 2-PrOH
250 was tested. Analysis time was indeed divided by 2 and resolution values were found to get
251 from 1.50 between peak 1 and 2, between 3 and 4 and between peak 7 and 8, to 5.85 between
252 peak 4 and 5 and 10.10 between peak 6 and 7. The chromatogram obtained under those
253 conditions, giving satisfactory results in around 25 min, is presented in the Figure 3 and
254 chromatographic parameters values in Table 1.

255 Besides, as it is known that the low pressure drop generated by the mobile phase in SFC
256 enables to couple columns, this approach was also explored to further improve resolution
257 values. **However, one must keep in mind that contrary to LC, this pressure drop has an**
258 **important effect on retention in SFC.** In addition, as chiral columns usually display limited
259 achiral selectivity and a relatively low efficiency, serially coupling different CSPs can then be
260 interesting because it alters selectivity and efficiency [25].

261 **3.1.2 Tandem coupled-columns screening**

262 In this section and in Table 2, Chiralcel OJ-H will be denoted OJ-H, Cellulose-2, denoted LC-
263 2 and Cellulose-5 denoted LC-5 for a sake of clarity.

264 Serially coupling two columns increases both the separation efficiency and the working
265 pressure by a factor of two, thus allowing an improved resolution through: i) the
266 complementarity of two different stationary phases, ii) increased separation efficiency and, iii)
267 a change in the selectivity as a result of the pressure increase. Obviously, the contrary can also
268 occur and a total loss of enantioseparation may result when coupling columns with competing
269 separation mechanisms. In the case of n available columns, $n \times (n - 1)$ tandem combinations
270 are possible. In our case, nine different chiral stationary phases (CSPs) were available, thus
271 the 72 possible combinations corresponded to 36 possible tandems able to adopt two
272 configurations each (CSP_A upstream//CSP_B downstream) and (CSP_B upstream//CSP_A
273 downstream).

274 In a previous work [26] it has been shown that some combinations of stationary phases with
275 opposing selectivity resulted, when coupled in tandem, in a loss of resolution. Thus, in order
276 to save time, only the three-best cellulose-based columns (*i.e* OJ-H; LC-5 and LC-2) were
277 explored. The screening was undertaken on the 6 tandems by injection of compound **1** within
278 EtOH or 2-PrOH in carbon dioxide-based mobile phase, at 2 mL/min flow-rate, 40°C and 120
279 bar outlet pressure. Besides, as the LC-2 CSP gave the best resolution, a tandem consisting in
280 serially coupled LC-2 columns was also evaluated for separation of the eight isomers of
281 compound **1**. All the chromatographic parameters were summarized in Table 2.

282 *3.1.2.1 Tandem of two LC-2 columns*

283 This experiment can be seen as multiplying by two the length of the LC2 column to enhance
284 the efficiency. Maintaining other parameters (type and percentage of co-solvent, flow-rate,
285 temperature and outlet pressure) equal, the separation with LC2//LC2 tandem (Figure 4a) did
286 not enable any progress on the resolution values compared to the last results (Figure 3),
287 however it costed almost twice the analysis time. Thus, subsequent combinations of different
288 columns were tested.

289 *3.1.2.2 Tandem of LC-2 and LC-5 columns*

290 In this particular case whatever the column upstream, less than four peaks was observed (data
291 not shown).

292 *3.1.2.3 Tandem of LC-2 and OJ-H columns*

293 OJ-H column had a good performance on the separation of compound **1** in the screening of
294 single column in the previous research. Therefore, the combination of LC-2 and OJ-H was
295 tested. Due to the fact that the sequence of columns has an effect on the separation, the
296 tandem of columns was tested in different order, with 20% 2-PrOH and 20% EtOH, at 2
297 mL/min. The four chromatograms are depicted in Figure 3a (Supplementary Information). As
298 we can see on the chromatograms, whatever the order of the two columns, the elution profiles

299 were very different from 2-PrOH to EtOH under same tandem of columns. In addition, when
300 swapping the columns from OJ-H/LC-2 to LC-2//OJ-H, chromatograms of compound 1 were
301 very different. This has been reported by Wang *et al.* [6] explaining that if a CSP_A is placed to
302 the front of CSP_B, the upstream column CSP_A experiences higher back pressure (P_u) than the
303 downstream column CSP_B (P_d). The difference between those two pressures is the pressure
304 drop over the downstream column CSP_B. The pressure difference between (P_u) and (P_d) is the
305 pressure drop over the downstream column CSP_B. The overall retention time t_{AB} is the
306 addition of the retention times on both columns at corresponding outlet pressure: $t_{AB} =$
307 $t_{A,P_u} + t_{B,P_d}$, where t_{A,P_u} is the retention time on the upstream column CSP_A at higher back
308 pressure (P_u), and t_{B,P_d} is the retention time on the downstream column CSP_B at lower outlet
309 pressure (P_d). When columns CSP_A and CSP_B are swapped, the overall retention time t_{BA}
310 changes to $t_{BA} = t_{B,P_u} + t_{A,P_d}$. When moving the column CSP_A from upstream to
311 downstream of the CSP_B column, the pressure induced a retention gain on column CSP_A,
312 ($t_{A,P_d} - t_{A,P_u}$), which rarely compensates exactly the retention loss on column CSP_B, ($t_{B,P_u} -$
313 t_{B,P_d}). As previously stated, retention strongly depends on the pressure drop, consequently,
314 tandem column retention times of a compound usually change when column coupling order is
315 reversed, leading to very different separations. In our case, seven peaks were separated most
316 of the time however eight peaks were obtained when LC2 is on the upstream, tested with 20%
317 EtOH as co-solvent and the flow rate at 2 mL/min.

318 3.1.2.4 Tandem of LC-5 and OJ-H columns

319 Subsequently, the tandem of OJ-H and LC-5 columns was explored and the different
320 corresponding chromatograms are depicted in Figure 3b, in Supplementary Information. In
321 the first group of experiments, 2-PrOH at 20% as co-solvent in different order of the columns
322 was tested. The flow rate was set at 2mL/min. Whatever the sequence the two obtained
323 chromatograms (upper part of Fig.3b, SI) were quite identical. This result is surprising but

324 was already reported [26]. Different experiments have been conducted with various
325 percentages of EtOH (middle and lower part of Fig. 3b, SI). Among these chromatograms,
326 one can see that the experiments with EtOH as co-solvent, induced higher separation than
327 using 2-PrOH. Meanwhile the analysis time was shorter when using EtOH (comparing the
328 same conditions). In addition, the selectivity was better when LC-5 is on the upstream. Either
329 with EtOH or 2-PrOH, this LC-5//OJ-H tandem separated more isomers with higher
330 resolutions. The last condition, under 10% EtOH at 3 mL/min flow-rate was experimented to
331 both keep efficiency and improve selectivity as reported by Asberg *et al.* [27]. It leads to a
332 loss of resolution giving seven isomers rather than the eight obtained at 20% EtOH, 2 mL/min
333 on LC-5//OJ-H tandem. Finally, 10% EtOH at 2 mL/min flow-rate was experimented in spite
334 of a high expected analysis time. The eight peaks corresponding to the eight isomers were
335 recovered in 45 min (Figure 4b, Table 2).

336 Different conditions were able to fully separate the eight isomers either on a single LC-2
337 column as seen on Figure 3 or in tandem LC-5//OJ-H columns as seen on Figure 4b.

338 Liquid chromatography was investigated, in order to further improve the resolution values and
339 to compare the two methods.

340 In an effort to greening chromatography, polar aqueous-organic and polar organic modes were
341 firstly explored.

342 **3.2 Liquid chromatography**

343 **3.2.1 Polar aqueous-organic and polar organic eluents**

344 As previously said in the Introduction section, ester and carbamate derivatives of cellulose are
345 reported as the most efficient selectors, in particular cellulose *tris*(3, 5-
346 dimethylphenylcarbamate) and cellulose *tris*(4-methylbenzoate). They were developed
347 originally by Daicel Corporation, to be used both in polar aqueous-organic and polar organic
348 modes under the names Chiralcel[®] OD-RH and Chiralcel[®] OJ-R. Thus, those two columns

349 were chosen for chiral separation of present MCC compound's eight isomers. In polar
350 aqueous-organic mode, high peak efficiency is achieved under low flow-rate. The later was
351 fixed at 0.5 mL/min, according to the Instruction Manual for Chiralcel[®] OD-RH and
352 Chiralcel[®] OJ-R columns. Two mobile phases were tested H₂O/ACN and H₂O/EtOH 40:60
353 (v/v) on the two CSPs. Interesting results were observed on the OD-RH CSP, with 5 peaks
354 under H₂O/EtOH 40:60 (v/v) but the analysis time was above 85 minutes and with 4 peaks
355 under H₂O/ACN 40:60 (v/v) but the analysis time was even longer, above 140 minutes. In
356 order to explore if this mode really reflects a reversed-phase mode, H₂O/ACN 20:80 (v/v) on
357 the OD-RH CSP was also tested and retention times were found to decrease (and resolution as
358 well). This result will be discussed later.

359 In polar organic mode, pure ACN, EtOH and 2-PrOH were tested as mobile phase, at 0.5
360 mL/min. This mode is known to have the benefit of short analysis times. On the OJ-R CSP
361 the chiral separation was modest. Under ACN one peak at 4.48 min was observed, whereas
362 with EtOH and 2-PrOH four isomers were separated at 6.46; 7.66; 9.58 and 14.56 min and
363 three isomers separated at 7.01; 7.92 and 10.09 min respectively. On the OD-RH CSP, the
364 results were more interesting. Under ACN mobile phase four isomers were separated at 4.45;
365 4.58; 5.34; and 5.82 min, under EtOH five isomers were separated at 6.19; 6.64; 7.02; 8.59
366 and 9.22 min, finally under 2-PrOH six isomers out of the eight expected, were separated at
367 7.12; 7.81; 8.27; 10.48; 11.32 and 12.22 min (Figure 4a in Supplementary Information).
368 Those result highlight two facts i) polar aqueous-organic and polar organic chromatographic
369 modes do not allow the complete resolution of the eight isomers of this MCC compound and
370 ii) with increasing the amount of water in the mobile phase (consisted of ACN and EtOH),
371 elution times increase as expected from a reversed-phase mode. Those results are consistent
372 with many works [28-32] and rely on the fact that hydrophobic interactions are more favored
373 than hydrogen-bonding in this particular case. The increased amount of water enhances

374 hydrophobic interactions thus leads to higher retention factors. This behavior is typical of a
375 reversed-phase separation system. ~~It is worth mentioning that an opposite behavior can~~
376 ~~sometimes occur: when increasing the amount of water, retention factors decrease then~~
377 ~~reflecting an hydrophilic interaction liquid chromatography (HILIC) like behavior [37-40].~~ In
378 addition, comparing the results obtained under polar organic mode and polar aqueous-organic
379 mode, especially on the OD-RH column, it can be seen that the chiral separation ability was
380 better under polar organic mode, observing five isomers under pure 2-PrOH. This has been
381 already reported by B. Chankvetadze's group [28]. Indeed, even if applicability of aqueous-
382 alcohol mixture as mobile phase is possible, the reason for discouraging the use of this type of
383 mobile phase is based on the knowledge that hydrogen-bonding and/or dipole-dipole
384 interactions between chiral analytes and polysaccharide-based chiral selectors are essential to
385 chiral discrimination. These interactions may be significantly diminished in aqueous phase, as
386 water can compete for hydrogen interaction sites with both analyte and chiral selector.
387 Okamoto *et al.* have used pure alcohol in the very early step of development of the
388 polysaccharide-based CSPs [33], but later have recommended alcohol-hydrocarbon mobile
389 phases. This mode was called normal phase and is, to date, the most reported.

390 **3.2.2 Normal-phase eluents**

391 Some papers have reported that enantioseparations for a certain analyte, obtained in SFC are
392 not necessary observed in HPLC and *vice-versa* [34]. Thus, a screening on six different
393 columns was undertaken.

394 The analyses were run at 0.8 mL/min flow-rate, with a mobile phase constituted of EtOH/*n*-
395 heptane 40:60 (v/v) on the so-called "golden-four" CSPs, namely amylose and cellulose
396 tris(3,5-dimethylphenylcarbamate), amylose tris((*S*)- α -methylbenzylcarbamate) and cellulose
397 tris(4-methylbenzoate) and on chlorinated CSPs namely cellulose tris(3-chloro-4-
398 methylphenylcarbamate) and cellulose tris(3,5-dichlorophenylcarbamate). The six

399 chromatograms obtained on those different columns are presented in Figure 4b in
400 Supplementary Information. On the Chiralpak AS and on the Chiralcel OJ CSPs one and four
401 peaks were respectively observed. On amylose and cellulose tris(3,5-
402 dimethylphenylcarbamate) the separations were more interesting: six peaks were separated on
403 the amylose phase (at 6.64; 7.24; 7.58; 8.88; 10.84 and 13.73 min) with unsatisfactory
404 resolution and six peaks were separated on the cellulose phase (at 5.65; 6.14; 6.50; 8.38; 9.24
405 and 9.80 min). Results obtained on Cellulose-5 and Cellulose-2 columns showed their ability
406 to separate respectively seven peaks and the eight isomers and confirmed past studies carried
407 out in HPLC indicating that chlorine-containing phases would show additional
408 enantioselectivity abilities compared to the polysaccharide-based selectors without chlorine
409 [35].

410 Comparing SFC and HPLC methodologies under normal phase mode, it is noteworthy that, as
411 already reported, the elution profile obtained on the AD-H CSP under EtOH mobile phase is
412 very different between SFC (Fig. 1a, SI) and HPLC (Fig. 4b, SI). This is also the case for the
413 OD-H CSP under EtOH mobile phase between SFC (Fig. 1b, SI) and HPLC (Fig. 4b, SI) [34].
414 Whereas, on the contrary, the behavior of chlorinated phases against this multiple chiral
415 centers compound under EtOH mobile phase, was similar for both techniques.

416 Thus, the LC-2 CSP was chosen to optimize the separation of the eight isomers.

417 3.2.2.1 Resolution optimization on LC-2 column

418 The resolution values obtained between peaks 1 and 2 and between peaks 3 and 4 were
419 insufficient under a mobile phase constituted of *n*-heptane/EtOH 60:40 (v/v) (Fig. 4b, SI). The
420 analyses were carried-out with a mobile phase constituted of *n*-heptane/2-PrOH 60:40 (v/v).
421 In chiral normal-phase chromatography, it is known that isopropanol leads to improved
422 enantioselectivity as this long alcohol competes less efficiently for the chiral cavity than short
423 alcohols such as methanol and ethanol. Eight isomers were observed on the Figure 5 (upper

424 part) but without baseline return. The percentage of isopropanol was further decreased to 30,
425 20 and 10% in *n*-heptane. The separations are represented in Figure 5. Resolution values were
426 improved from 40 to 10% (chromatographic parameters are summarized in Table 3), but were
427 insufficient between peak 1 and 2 and 3 and 4. Thus the Cellulose-5 was experimented.

428 3.2.2.2 Resolution optimization on LC-5 column

429 Only seven peaks were obtained under a mobile phase constituted of *n*-heptane/EtOH 60:40
430 (v/v) (Fig. 4b, SI). In addition, resolution values between peaks 1 and 2 and between peaks 3
431 and 4 were insufficient. The analyses were subsequently implemented with a mobile phase
432 constituted of *n*-heptane/2-PrOH 60:40 (v/v) and eight isomers were separated despite a Rs_{1-2}
433 = 1.46 (Figure 6, first chromatogram). The percentage of isopropanol was further decreased to
434 30, 20 and 10% in *n*-heptane at 0.8 mL/min. The chromatograms are given on Figure 6.
435 Resolution values, summarized in Table 3, were improved but at the cost of a higher analysis
436 time. A flow-rate of 1 mL/min was implemented and was found to shorten the analysis time
437 from 94 to 74 minutes while keeping resolution values higher than 1.69 (Table 3).

438 3.2.3 Instrument limits of detection and quantification determination

439 In the following conditions of *n*-heptane/2-PrOH 80:20 (v/v) at 0.8 mL/min, the **IDL** and **IQL**
440 of each stereoisomer were determined through a study of a series of dilutions of the isomers
441 mixture in order to obtain signal to noise ratios of 3 and 10 respectively [36]. The lowest
442 amount of analyte which could be detected **by the instrument** but not necessarily quantitated
443 as an exact value (**IDL**) was equal to 2.74 nM. The smallest amount of analyte which could be
444 quantitatively determined with a reasonable reliability of the given procedure (**IQL**) was 9.37
445 nM for isomer 8. The **IDLs** and **IQLs** of the eight isomers are given in the Table 4. These **IDL**
446 values permit the determination of 0.5% of one isomer as the other one. The evaluated *criteria*
447 were in agreement with the common acceptance rules of the ICH.

448 3.2.4 Isomers elution order study on LC-5, LC-2 and AD-H columns

449 The study of the elution order is a major step of chiral separation. Under different conditions
450 reversal elution order can be noted. Those phenomena have been deeply studied by the
451 Chankvetadze's group [37-39]. It is expected that reversal elution order is closely linked to
452 the chiral recognition mechanism at the molecular level, which is not fully understood.
453 Besides, to assess the enantiomeric excess of a preparative separated isomer, the minor
454 enantiomer should elute first. This is also the case for achiral analysis. An impurity will be
455 quantified with better precision when it elutes before the major peak. Hence the knowledge of
456 elution order under particular conditions could be of interest.

457 A small collect of each of the eight isomers was undertaken by injecting a 3.2 mM
458 concentration isomers mixture solution on the Cellulose-5 (LC-5) column with a mobile phase
459 constituted of 2-PrOH/*n*-heptane 10:90 (v/v), at 0.8 mL/min, in order to evaluate the elution
460 order under different conditions. The eight isomers were named P1(LC-5/2-PrOH), P2(LC-
461 5/2-PrOH), P3(LC-5/2-PrOH), P4(LC-5/2-PrOH), P5(LC-5/2-PrOH), P6(LC-5/2-PrOH),
462 P7(LC-5/2-PrOH) and P8(LC-5/2-PrOH) following their elution order respectively (Figure 5,
463 SI and Table 5). It is known that isomer elution order is influenced by several parameters
464 which are the following: the polysaccharide backbone (secondary structure due to α -1, 4-
465 linked glucopyranose units for amylose or β -1, 4-linked glucopyranose units for cellulose),
466 the derivative type (carbamate or benzoate), the nature and the position of the carbamate
467 moiety substituent(s), the type and percentage of alcoholic modifier and the temperature. The
468 use of different additives either basic or acidic can also impact the elution order of some
469 enantiomers [46]. In this work, to have a better insight of the elution order, the eight isomers
470 were injected on the same type of CSP (*i.e.* LC-5) under *n*-heptane/EtOH 95:5 (v/v), at 0.8
471 mL/min (mobile phase for which a total baseline is obtained within around 50 minutes). It can
472 be seen, that the same elution order than under *n*-heptane/2-PrOH 90:0 (v/v) mobile phase, is

473 observed (Figure 6, SI and Table 5). The elution order was also evaluated on two other
474 columns: the Cellulose-2 and the Chiralpak AD-H.

475 The eight isomers were injected on the Cellulose-2 (LC-2) column under *n*-heptane/2-PrOH
476 90:10 (v/v) and *n*-heptane/EtOH 95:5 (v/v), mobile phases at 0.8 mL/min (Figures 7 and 8, SI
477 and Table 5) respectively. Under those two conditions the stereoisomers elute with following
478 the same order. As explained above, the nature and position of the substituents on a phenyl
479 moiety may play a role in the elution order. Thus, comparing the Cellulose-5 with the
480 Cellulose-2 where the first contains two chlorine substituents in position 3 and 5 on the
481 phenyl group, and the second a chlorine and a methyl substituent in position 3 and 4 of the
482 phenyl moiety, some differences are observed. Stereoisomers P1(LC5/2-PrOH) to P4(LC5/2-
483 PrOH) elute following the same order between the two CSPs (*i.e* first eluted to fourth eluted)
484 but P5(LC5/2-PrOH) and P6(LC5/2-PrOH) *versus* P7(LC5/2-PrOH) and P8(LC5/2-PrOH)
485 elute in the opposite order (*i.e* seventh and eighth eluted) *versus* (*i.e* fifth and sixth eluted).
486 This effect is observed in both 2-PrOH-based (Figures 5 and 7, SI) and EtOH-based (Figures
487 6 and 8, SI) eluents. Nevertheless, this remark should be taken with caution as the CSP
488 binding is not the same (coated for the first one and immobilized for the second).

489 Finally, the Chiralpak AD-H CSP was also tested. The analyses were carried-out with a
490 mobile phase constituted of *n*-heptane/2-PrOH 90:10 (v/v) and under *n*-heptane/EtOH 95:5
491 (v/v), at 0.8 mL/min (Figures 9 and 10, SI and Table 5) respectively and allow us to know the
492 exact elution order of each isomers. It is practically equal between the two eluents (which was
493 not expected as type and percentage of alcohol are not the same). The difference remains in
494 the elution of P1(LC5/2-PrOH) and P5(LC5/2-PrOH) fractions which elute in first and third
495 respectively under 2-PrOH eluent and in third and first respectively under EtOH-based mobile
496 phase, with keeping in mind that percentages of alcohol are different.

497 **4. Conclusion**

498 To perform the pharmacological assay of individual stereoisomers from a TGR5 agonist
499 compound, their synthesis as pure isomers is planned. Thus, a chromatographic method
500 allowing the chiral separation of those isomers is required to assess their chiral purity. In this
501 paper the successful separation of eight stereoisomers is reported, appearing as a challenging
502 task. This work highlights the extreme versatility of liquid and supercritical fluid
503 chromatography which appears as powerful tools for complex separation of compound
504 bearing multiple chiral centers. Indeed, following a screening of CSPs, several conditions
505 were found to fully resolve the whole isomers. In supercritical fluid chromatography on a
506 single column, the Lux Cellulose-2 was successful with 2-PrOH as a co-solvent, in 25
507 minutes (Figure 3). Coupling two Lux Cellulose-2 columns, thus doubling the separation
508 efficiency has shown its utility in fully resolve all the isomers but in a rather long but
509 expected time, around 50 minutes (Figure 4a). Coupling two columns of different selectivity
510 has been also explored and very different separations of compound **1** were observed by
511 swapping the upstream and downstream columns (Figures 3a and 3b in Supplementary
512 Information). The tandem consisting of Lux Cellulose-5 upstream and the Chiralcel OJ-H
513 downstream, under 10% EtOH as a co-solvent was the most promising (Figure 4b). In liquid
514 chromatography, again the Lux Cellulose-5 column outperformed all other polysaccharide-
515 based CSPs. The method developed on the Lux Cellulose-5 within 20% 2-PrOH was used to
516 calculate the limit of detection and of quantification of each isomers and was found to meet
517 the ICH requirements in order to evaluate the chiral purity of each synthesized isomer. The
518 last point highlighted by this work, is the importance of studying the stereoisomers elution
519 order. In this part it is noteworthy that the Lux Cellulose-5 followed by the Lux Cellulose-2
520 and then the Chiralpak AD-H have all the ability to separate the eight isomers in various
521 conditions.

522 It can be seen that finally both methodologies are able to fully separate the eight isomers. But
523 comparing them from a time point of view, the shorter chromatogram is obtained under SFC
524 conditions. From a practical point of view, even if liquid chromatography can be implemented
525 into three modes, only the normal-phase was successful whereas supercritical fluid
526 chromatography was a useful tool with single and tandem columns modes for the baseline
527 separation of the present MCC compound. The last point of comparison is the environmental
528 impact of each technique. Here, carbon dioxide-based mobile phase is undoubtedly greener
529 than *n*-heptane-based mobile phase used in normal phase liquid chromatography.

530 ~~In a next future a comparison will be undertaken, from an economical point of view, to~~
531 ~~evaluate the cost of the planned synthesis of pure isomers versus a chiral separation at the~~
532 ~~preparative scale.~~

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Figure captions

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Figure 1 Structure of the dihydropyridone derivative **1**.

~~**Figure 1b** — Structure of the polysaccharide based stationary phases used in this study.~~

Figure 2 Staked chromatograms obtained for compound **1** stereoisomers on LC-2 CSP, with 20% EtOH, 20% 2-PrOH, 20% MeOH and 20% ACN in carbon dioxide, at a flow-rate of 3 mL/min, column temperature of 40°C, 120 bar outlet pressure, $\lambda = 220$ nm.

Figure 3 Chromatogram obtained for compound **1** stereoisomers on LC-2 CSP with 30% 2-PrOH in carbon dioxide, at a flow-rate of 1 mL/min, column temperature of 40°C, 120 bar outlet pressure, $\lambda = 220$ nm.

Figure 4a Chromatograms obtained for compound **1** stereoisomers on LC-2//LC-2 tandem of coupling columns with 30% 2-PrOH in carbon dioxide, at a flow-rate of 1 mL/min, column temperature of 40°C and 120 bar outlet pressure, $\lambda = 220$ nm.

Figure 4b Chromatograms obtained for compound **1** stereoisomers on LC-5//OJ-H tandem of coupling columns with 10% EtOH in carbon dioxide, at a flow-rate of 2 mL/min, column temperature of 40°C and 120 bar outlet pressure, $\lambda = 220$ nm.

Figure 5 Staked chromatograms obtained for compound **1** stereoisomers on Cellulose-2 CSP, with 40 to 10% 2-PrOH in *n*-heptane at a flow-rate of 0.8 mL/min, column temperature of 25°C, $\lambda = 220$ nm.

726 **Figure 6** Staked chromatograms obtained for compound **1** stereoisomers on Cellulose-5
727 CSP, with 40 to 10% 2-PrOH in *n*-heptane at a flow-rate of 0.8 mL/min and with 10% 2-
728 PrOH in *n*-heptane at a flow-rate of 1.0 mL/min; column temperature of 25°C, $\lambda = 220$ nm.

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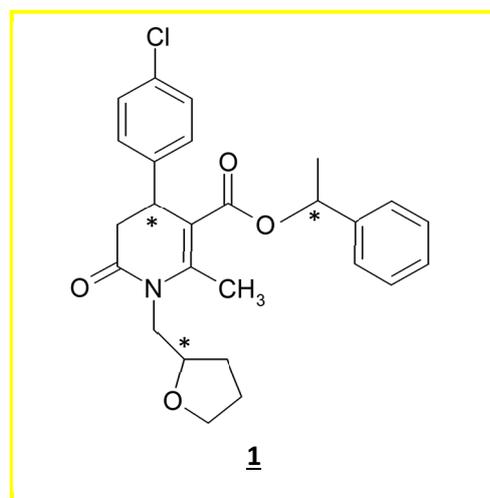
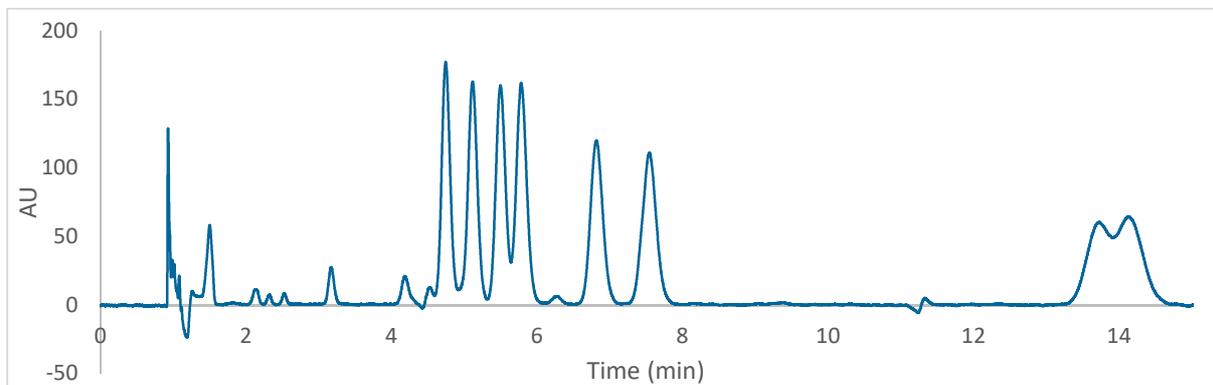
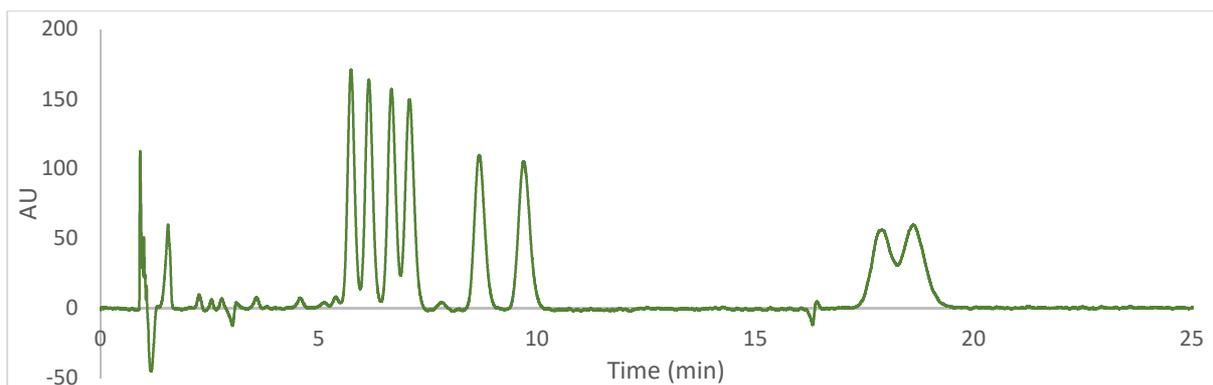


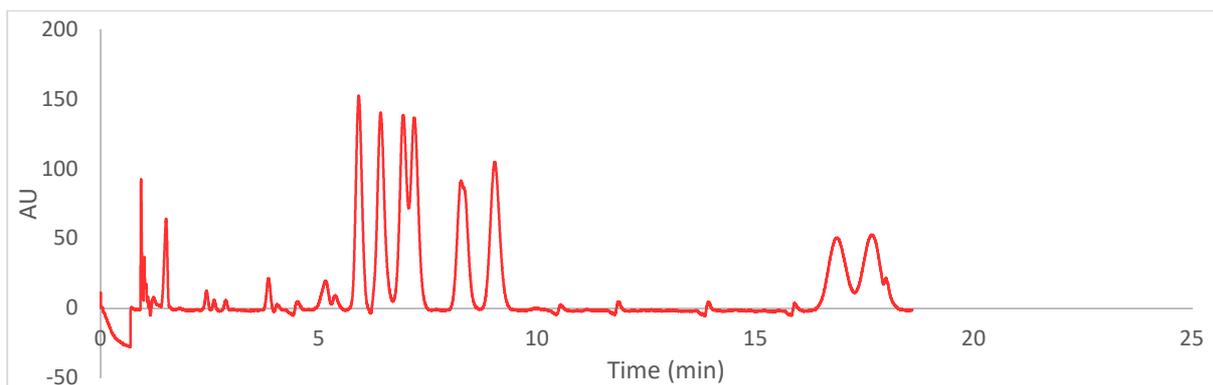
Figure 1



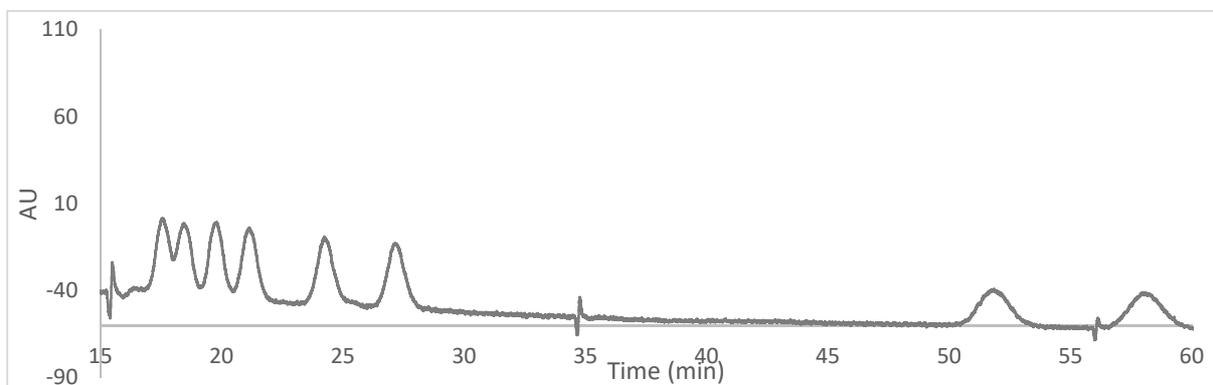
Compound **1**, on the Cellulose LC-2 CSP, 20% EtOH in CO₂, 3 mL/min, 40°C, 120 bar



Compound **1**, on the Cellulose LC-2 CSP, 20% 2-PrOH in CO₂, 3 mL/min, 40°C, 120 bar



Compound **1**, on the Cellulose LC-2 CSP, 20% MeOH in CO₂, 3 mL/min, 40°C, 120 bar



Compound **1**, on the Cellulose LC-2 CSP, 20% ACN in CO₂, 3 mL/min, 40°C, 120 bar

Figure 2

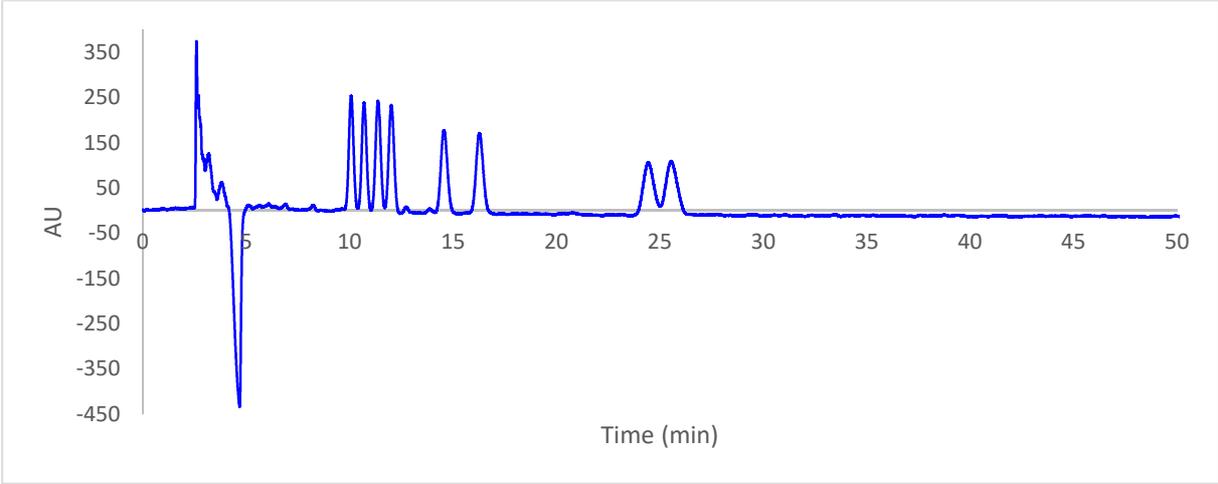


Figure 3

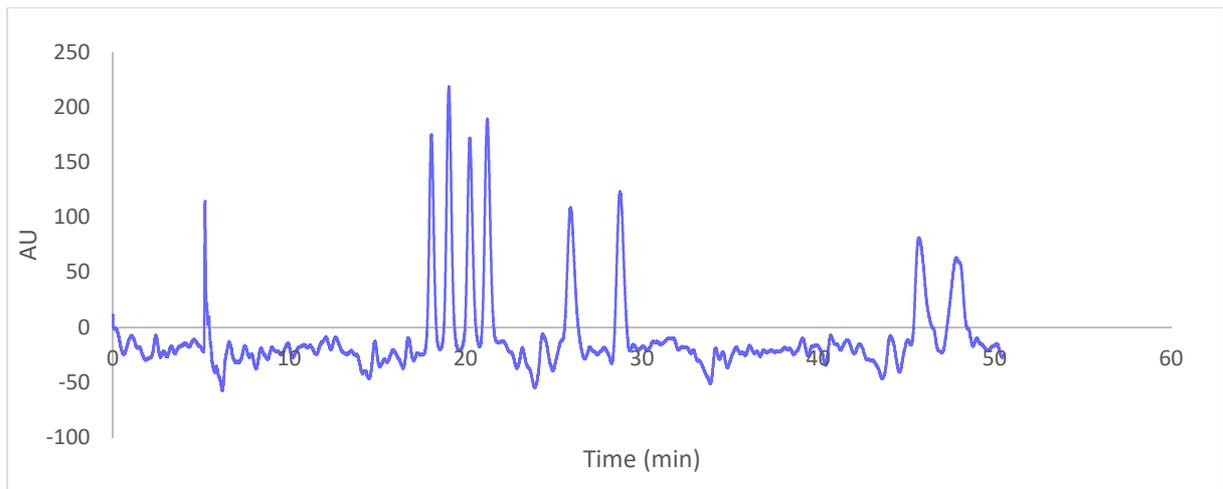


Figure 4a

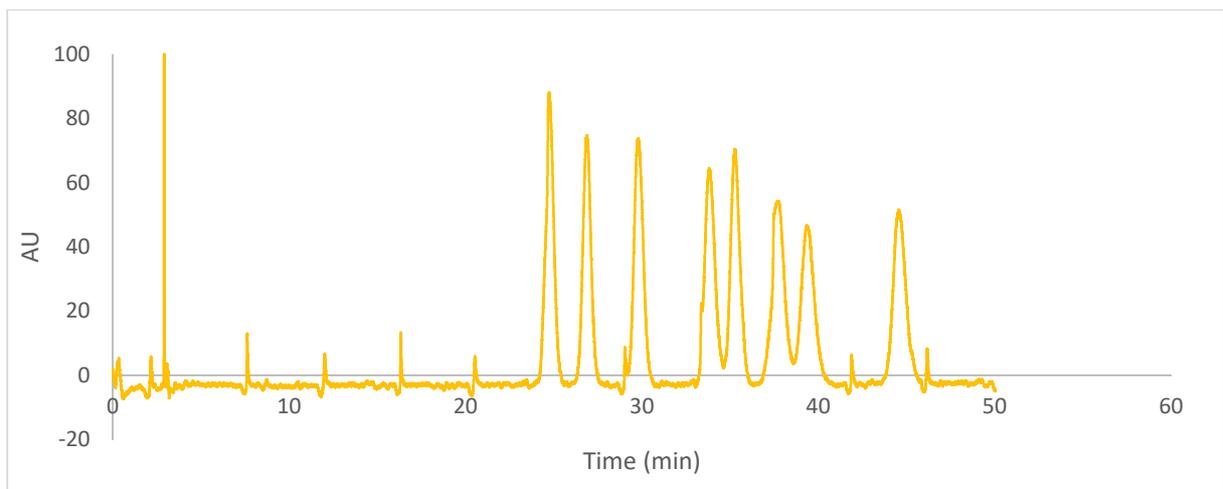


Figure 4b

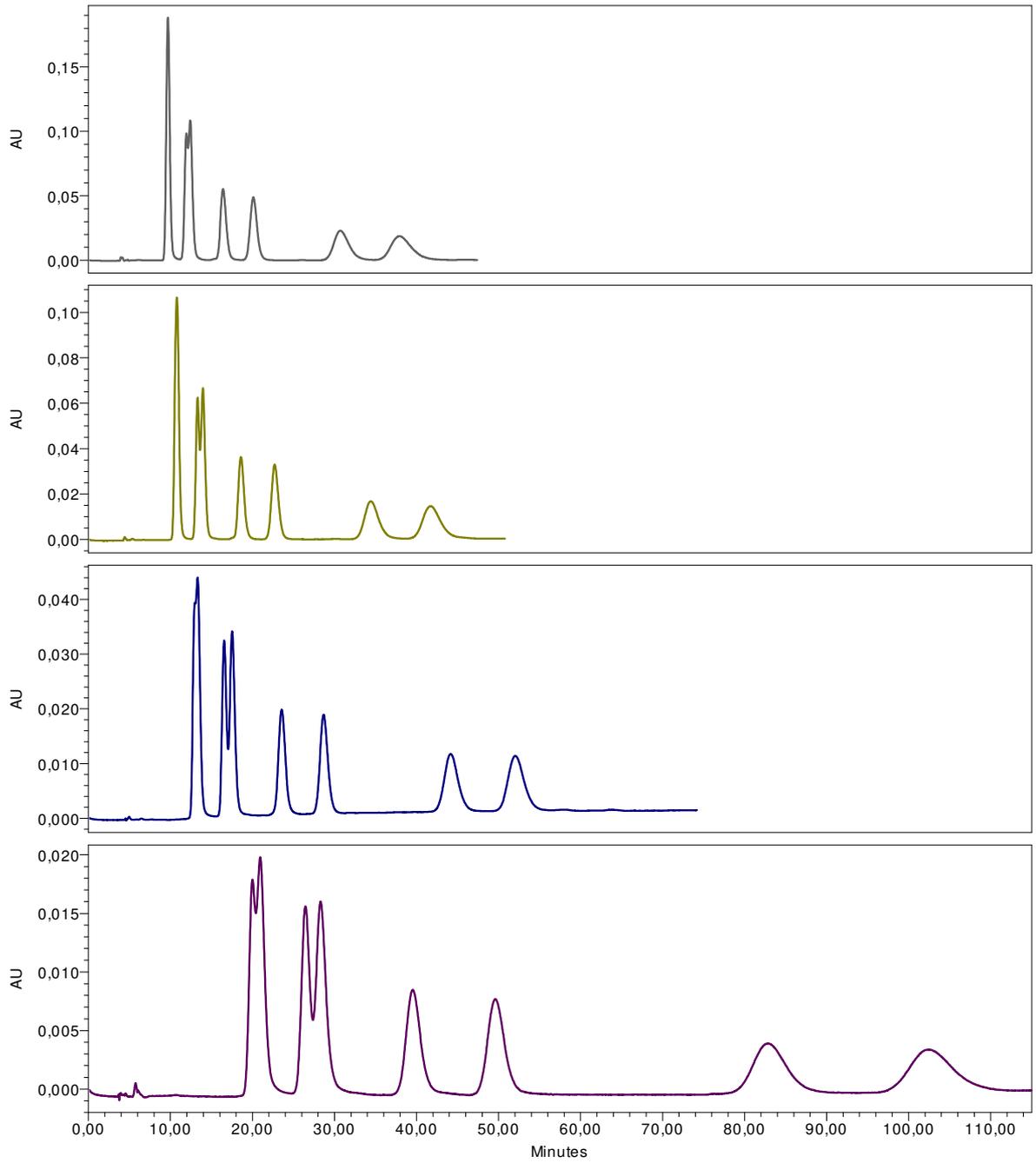


Figure 5

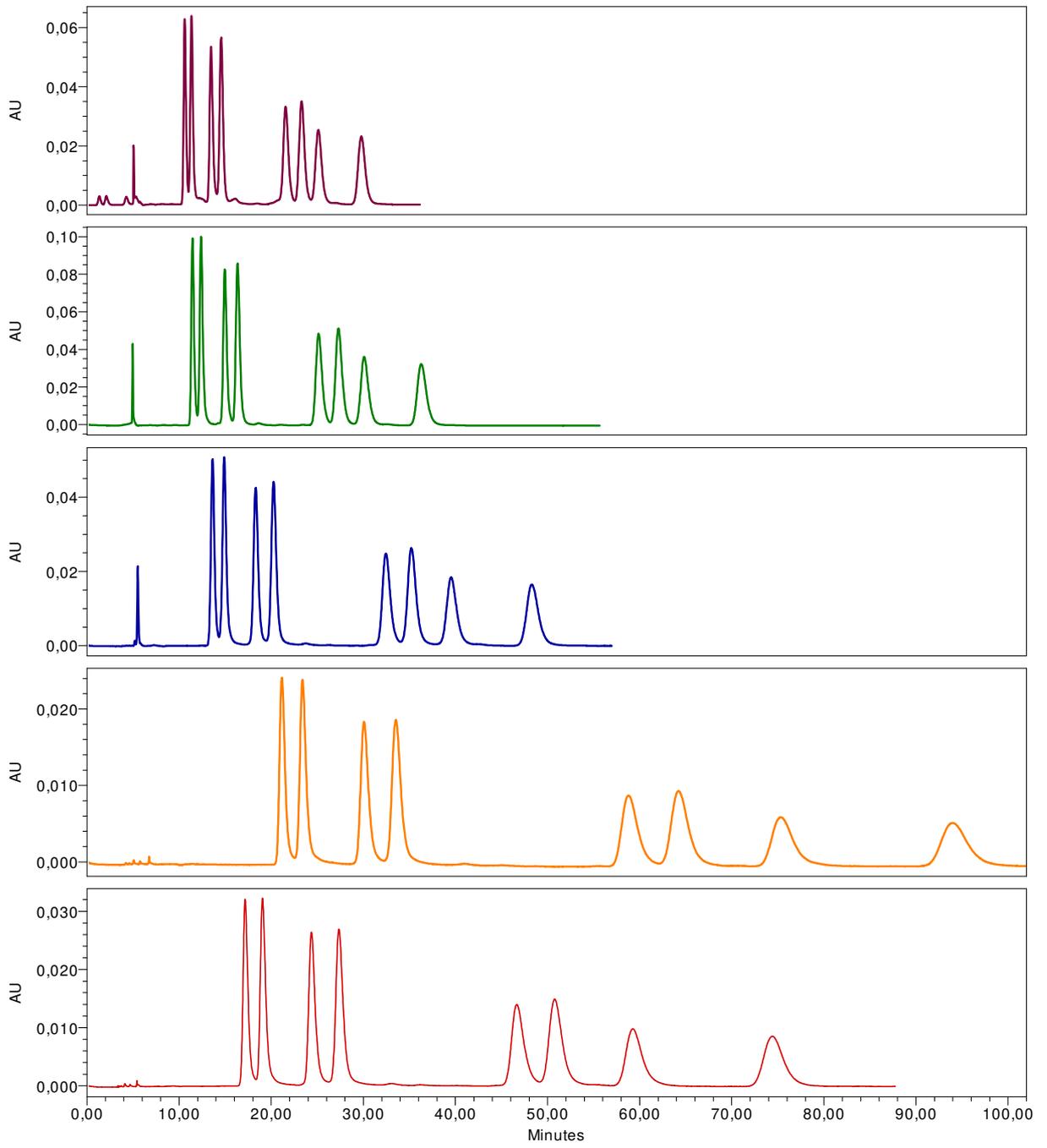


Figure 6

Table 1 Chromatographic parameters for compound **1** separation in SFC on LC-2 CSP

		1	2	3	4	5	6	7	8
LC2	$t_r(\text{min})$	5.73	6.14	6.66	7.07	8.67	9.68	17.83	18.60
20% 2-PrOH	α		1.06	1.07	1.06	1.21	1.11	1.56	1.04
3 mL/min	R_s		0.87	1.13	0.82	2.38	1.58	14.43	0.68
LC2	$t_r(\text{min})$	9.31	10.12	11.08	11.93	14.78	16.27	30.28	32.25
15% 2-PrOH	α		1.10	1.11	1.09	1.24	1.12	1.52	1.08
3 mL/min	R_s		1.48	1.50	1.32	8.31	5.03	18.71	0.89
LC2	$t_r(\text{min})$	19.80	21.32	23.68	25.38	31.72	36.27	70.77	74.67
10% 2-PrOH	α		1.09	1.11	1.07	1.25	1.15	1.97	1.05
3 mL/min	R_s		2.22	3.02	2.90	9.85	6.28	19.41	1.28
LC2	$t_r(\text{min})$	20.5	21.88	23.78	25.22	31.40	35.75	59.83	62.69
20% 2-PrOH	α		1.07	1.09	1.06	1.27	1.13	1.68	1.06
1 mL/min	R_s		1.50	1.88	1.85	12.5	6.39	26.8	3.11
LC2	$t_r(\text{min})$	10.05	10.68	11.36	12.00	14.55	16.28	24.42	25.55
30% 2-PrOH	α		1.06	1.07	1.05	1.22	1.12	1.52	1.06
1 mL/min	R_s		1.50	1.51	1.54	5.85	3.20	10.10	1.49

Other conditions : $P_{\text{outlet}} = 120 \text{ bar}$; $T = 40^\circ\text{C}$

Table 2 Chromatographic parameters for compound **1** separation on columns tandem in SFC

		1	2	3	4	5	6	7	8
LC2	t_r (min)	10.05	10.68	11.36	12.00	14.55	16.28	24.42	25.55
30% 2PrOH	α		1.06	1.07	1.05	1.22	1.12	1.52	1.06
1mL/min	R_s		1.50	1.51	1.54	5.85	3.20	10.10	1.49
LC2//LC2	t_r (min)	18.08	19.06	20.22	21.21	25.97	28.76	45.74	48.08
30% 2-PrOH	α		1.06	1.06	1.05	1.22	1.11	1.59	1.05
1mL/min	R_s		1.91	1.90	2.01	7.60	4.16	14.83	1.93
		1	2	3	4	5	6	7	8
OJ-H//LC2	t_r (min)	7.07	7.68	7.94	8.89	9.83	13.28	13.96	-
20% 2PrOH	α		1.09	1.03	1.12	1.11	1.35	1.05	-
2mL/min	R_s		1.34	0.68	1.96	1.47	4.89	0.94	-
OJ-H//LC2	t_r (min)	10.74	11.88	13.03	14.10	15.60	23.08	24.11	-
20% EtOH	α		1.11	1.10	1.08	1.11	1.48	1.04	-
2mL/min	R_s		1.42	1.46	1.48	1.95	8.00	0.98	-
		1	2	3	4	5	6	7	8
LC2//OJ-H	t_r (min)	11.32	11.56	12.70	13.72	15.32	17.34	25.37	26.98
20% 2PrOH	α		1.02	1.10	1.08	1.12	1.13	1.46	1.06
2mL/min	R_s		0.45	1.85	1.51	2.04	2.12	6.37	1.01
LC2//OJ-H	t_r (min)	10.28	10.79	11.39	13.01	13.41	14.92	22.18	23.46
20% EtOH	α		1.05	1.06	1.14	1.03	1.11	1.49	1.06
2mL/min	R_s		0.94	0.90	2.36	0.70	2.24	7.19	1.01
		1	2	3	4	5	6	7	8
OJ-H//LC5	t_r (min)	12.63	14.99	15.49	19.02	20.47	24.32	-	-
20% 2PrOH	α		1.20	1.04	1.24	1.08	1.20	-	-
2mL/min	R_s		3.69	0.60	3.58	1.69	4.48	-	-
OJ-H//LC5	t_r (min)	11.05	11.48	12.64	13.99	15.22	16.04	16.76	-
20% EtOH	α		1.04	1.10	1.11	1.09	1.06	1.05	-
2mL/min	R_s		1.05	3.06	2.81	2.58	1.83	1.39	-
OJ-H//LC5	t_r (min)	15.36	15.76	18.56	20.21	20.67	23.00	23.98	25.47
10% EtOH	α		1.03	1.18	1.09	1.02	1.12	1.04	1.06
3mL/min	R_s		0.32	2.24	2.71	0.78	2.81	1.19	2.18
		1	2	3	4	5	6	7	8
LC5//OJ-H	t_r (min)	11.96	12.20	14.02	14.83	17.53	18.93	22.30	-
20% 2PrOH	α		1.02	1.16	1.06	1.19	1.08	1.18	-
2mL/min	R_s		0.14	3.05	1.37	5.47	2.14	5.22	-
LC5//OJ-H	t_r (min)	9.62	10.23	10.93	12.33	12.70	13.36	13.74	15.04
20% EtOH	α		1.07	1.07	1.14	1.03	1.05	1.03	1.10
2mL/min	R_s		2.12	2.03	2.15	<0.5	1.48	0.52	3.14
LC5//OJ-H	t_r (min)	13.10	14.17	15.63	17.07	18.27	19.04	20.33	21.78
10% EtOH	α		1.09	1.11	1.10	1.07	1.04	1.07	1.07
3mL/min	R_s		2.43	2.95	2.62	1.85	1.11	2.07	2.72
LC5//OJ-H	t_r (min)	24.68	26.89	29.79	33.76	34.91	37.70	39.48	44.58
10% EtOH	α		1.09	1.11	1.14	1.04	1.07	1.04	1.14
2mL/min	R_s		2.61	3.25	4.18	1.40	2.13	1.28	3.94

P_{outlet} = 120 bar ; T = 40°C

Table 3 Chromatographic parameters for compound **1** separation in normal-phase LC

		1	2	3	4	5	6	7	8
LC-2	t _r (min)	7.99	8.43	10.14	10.67	12.61	14.43	30.19	32.71
40% EtOH	α		1.14	1.49	1.10	1.34	1.24	2.66	1.10
0.8mL/min	R _s		1.04	3.48	0.96	3.03	2.44	12.04	1.32
LC-2	tr(min)	9.69	11.94	12.41	16.42	20.10	30.72	37.91	-
40% 2PrOH	α		1.47	1.06	1.53	1.32	1.70	1.28	-
0.8mL/min	R _s		2.20	<0.50	2.71	2.62	4.44	1.91	-
LC-2	t _r (min)	10.78	13.31	13.95	18.59	22.71	34.43	41.69	-
30% 2PrOH	α		1.43	1.08	1.51	1.30	1.66	1.25	-
0.8mL/min	R _s		2.15	<0.5	2.83	2.90	5.18	2.18	-
LC-2	t _r (min)	13.26	16.58	17.45	23.60	28.70	44.17	52.09	-
20% 2PrOH	α		1.38	1.08	1.48	1.27	1.65	1.20	-
0.8mL/min	R _s		2.55	0.89	4.54	3.15	6.72	2.54	-
LC-2	t _r (min)	19.99	20.95	26.46	28.31	39.54	49.58	82.84	102.38
10% 2-PrOH	α		1.06	1.34	1.08	1.48	1.29	1.74	1.25
0.8mL/min	R _s		<0.50	1.73	0.88	4.41	3.30	6.35	2.32

		1	2	3	4	5	6	7	8
LC-5	t _r (min)	8.13	8.53	9.59	9.93	12.97	14.12	15.33	-
40% EtOH	α		1.12	1.30	1.07	1.61	1.14	1.13	-
0.8mL/min	R _s		1.21	2.95	0.84	6.57	1.57	1.53	-
LC-5	tr(min)	10.61	11.34	13.46	14.56	21.56	23.29	25.11	29.77
40% 2PrOH	α		1.13	1.33	1.13	1.73	1.10	1.10	1.23
0.8mL/min	R _s		1.46	3.75	1.70	8.33	1.68	1.60	3.55
LC-5	t _r (min)	11.45	12.38	14.95	16.34	25.14	27.29	30.08	36.28
30% 2PrOH	α		1.14	1.35	1.14	1.77	1.11	1.12	1.25
0.8mL/min	R _s		1.61	3.92	1.86	8.62	1.67	1.96	3.73
LC-5	t _r (min)	13.62	14.88	18.31	20.25	32.44	35.22	39.53	48.29
20% 2PrOH	α		1.14	1.34	1.15	1.80	1.10	1.14	1.25
0.8mL/min	R _s		1.74	4.15	2.08	9.58	1.73	2.42	4.18
LC-5	t _r (min)	21.16	23.40	30.07	33.53	58.79	64.21	75.29	94.03
10% 2PrOH	α		1.14	1.36	1.14	1.88	1.11	1.19	1.27
0.8mL/min	R _s		1.99	4.90	2.13	10.54	1.69	3.03	4.14
LC-5	t _r (min)	17.17	19.06	24.37	27.35	46.66	50.77	59.26	74.40
10% 2-PrOH	α		1.15	1.38	1.15	1.86	1.10	1.18	1.28
1mL/min	R _s		1.90	4.63	2.24	10.19	1.69	3.12	4.64

Other conditions : temperature 25°C ; $\lambda = 220$ nm

Table 4 Limit of detection and limit of quantification in HPLC for compound **1**

	1	2	3	4	5	6	7	8
LOD (nM)	2.74	2.75	2.75	2.74	2.79	2.81	2.83	2.84
LOQ (nM)	9.11	9.16	9.15	9.12	9.21	9.26	9.35	9.37

Conditions: C=0.015 mM; 0.8 mL/min; 25°C; λ : 210 nm; Cellulose-5; *n*-Hept/2-PrOH 80:20

v:v.

Table 5 Elution order of the eight stereoisomers of compound **1** in HPLC

CSP	MP	1 st eluted	2 nd eluted	3 rd eluted	4 th eluted	5 th eluted	6 th eluted	7 th eluted	8 th eluted
LC-5	2-PrOH 10%	P1(LC-5/2-PrOH)	P2(LC-5/2-PrOH)	P3(LC-5/2-PrOH)	P4(LC-5/2-PrOH)	P5(LC-5/2-PrOH)	P6(LC-5/2-PrOH)	P7(LC-5/2-PrOH)	P8(LC-5/2-PrOH)
	EtOH 5%	P1(LC-5/2-PrOH)	P2(LC-5/2-PrOH)	P3(LC-5/2-PrOH)	P4(LC-5/2-PrOH)	P7(LC-5/2-PrOH)	P6(LC-5/2-PrOH)	P7(LC-5/2-PrOH)	P8(LC-5/2-PrOH)
LC-2	2-PrOH 10%	P1(LC-5/2-PrOH)	P2(LC-5/2-PrOH)	P3(LC-5/2-PrOH)	P4(LC-5/2-PrOH)	P7(LC-5/2-PrOH)	P8(LC-5/2-PrOH)	P5(LC-5/2-PrOH)	P6(LC-5/2-PrOH)
	EtOH 5%	P1(LC-5/2-PrOH)	P2(LC-5/2-PrOH)	P3(LC-5/2-PrOH)	P4(LC-5/2-PrOH)	P7(LC-5/2-PrOH)	P8(LC-5/2-PrOH)	P5(LC-5/2-PrOH)	P6(LC-5/2-PrOH)
AD-H	2-PrOH 10%	P1(LC-5/2-PrOH)	P2(LC-5/2-PrOH)	P5(LC-5/2-PrOH)	P7(LC-5/2-PrOH)	P3(LC-5/2-PrOH)	P4(LC-5/2-PrOH)	P6(LC-5/2-PrOH)	P8(LC-5/2-PrOH)
	EtOH 5%	P5(LC-5/2-PrOH)	P2(LC-5/2-PrOH)	P1(LC-5/2-PrOH)	P7(LC-5/2-PrOH)	P3(LC-5/2-PrOH)	P4(LC-5/2-PrOH)	P6(LC-5/2-PrOH)	P8(LC-5/2-PrOH)

Other conditions: flow-rate 0.8 mL/min; temperature 25°C ; $\lambda = 220$ nm.