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1 **Comparison between centrifugal partition chromatography and preparative liquid**
2 **chromatography as first dimensions in off-line two-dimensional separation: application to**
3 **the isolation of multi-targeted compounds from Edelweiss plant**

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7

8 **Keywords**

9 Preparative chromatography, comprehensive two-dimensional chromatography, centrifugal partition
10 chromatography, countercurrent chromatography

11 **Abstract**

12 Preparative two-dimensional chromatography is gaining interest in the elucidation of complex
13 samples as it allows the recovery of a large number of molecules without the risks inherent to
14 tedious multi-step sample preparation. While the second dimension is often selected to be liquid
15 chromatography, it may be of interest to compare the specificities of two different techniques,
16 namely liquid chromatography and centrifugal partition chromatography, to be used as first
17 dimension.

18 A fair comparison between off-line CPCxLC and prepLCxLC in selective comprehensive mode for
19 preparative purposes is carried out in this study, illustrated by the isolation of five compounds from
20 high-value Edelweiss plant. The method development of each configuration is achieved on laboratory
21 scale instruments. The quality of separation is compared using 2D-contour plots.

22 The prepLCxLC exhibits a large separation space that leads to an overall large peak capacity, which is
23 of great interest for complex samples. But its limited loading capacity involves a large number of 2D
24 runs increasing the running costs for preparative purposes. On the other hand, CPCxLC provides a
25 low peak capacity due to the poor efficiency provided by CPC. However, this liquid-liquid technique
26 can be finely tuned to generate a high selectivity, decreasing the number of runs necessary to
27 produce a limited number of target solutes.

28 **1. Introduction**

29 In recent years, two-dimensional separations are becoming increasingly implemented in a wide range
30 of applications as complex samples are more and more appealing. These 2D separations can be set
31 up for analytical purposes in order to detect and identify new molecules [1, 2]. They can also be used
32 for preparative purposes, to produce unknown target to be sent to further analysis such as NMR, IR
33 or mass quantification, or to generate small amounts of reference substances from high value
34 samples. In these preparative issues, it is crucial that the entire sample is subjected to the two
35 dimensions without any loss. Thus, the implementation of 2D separation for preparative purposes
36 has different constraints and requirements than the ones for analytical purposes.

37 In the literature, 2D preparative separations have just emerged, mostly in the natural product field
38 [3-11] where the discovery of new active compounds is intrinsically related to the unraveling of the
39 complex matrix. The techniques involved in the 2D setups are liquid chromatography (LC) and
40 countercurrent chromatography (CCC), the later one using a liquid stationary phase. All combinations
41 exist (LC/LC, LC/CCC, CCC/CCC), but the separations involving only countercurrent apparatus are very
42 rarely implemented due to the low efficiency of the technique [9-12]. Amongst countercurrent
43 technologies, centrifugal partition chromatography technique (CPC) is of particular interest as it can
44 strongly hold liquid stationary phase at fast flow rate and high sample concentration.

45 In the literature, CPC and LC have already been compared in one-dimensional separation for
46 preparative objectives [13, 14]. The purpose of this publication is to fairly compare the advantages
47 and drawbacks of each technique in the 2D configurations CPCxLC and prepLCxLC, keeping the
48 second dimension strictly identical throughout the study.

49 The 2D preparative separation was applied to the isolation of multi-targeted compounds from a rare
50 sample with high yield and purity requirements. Edelweiss is a protected plant from European Alps
51 which produces a wide diversity of secondary plant metabolites, especially some with anti-oxidative
52 properties due to its growing at high altitude [15-17]. While the plant is just started to get cultivated
53 mainly in Austria and Switzerland, it is essential to qualify the biological activity and chemical

54 structures of its compounds of interest. Recently two main compounds, namely leontopodic acid A
55 and 3,5-dicaffeoylquinic acid, have been isolated by the combination of CPC and LC in heart-cut
56 mode, both at lab-scale and pilote scale [18]. In the present study, the two-dimensional preparative
57 strategy is considered in a selective comprehensive mode [19] to recover five targeted compounds at
58 laboratory scale (mg-scale).

59

60 **2. Materials and methods**

61 *2.1. Chemicals and materials*

62 All solvents were of analytical grade. Methyl tert-butyl ether was purchased from Acros Organics
63 (Fisher Scientific, Illkirch, France). Formic acid, ammonium acetate and ethanol were purchased from
64 Fisher Scientific. Acetonitrile was purchased from Biosolve Chimie (Dieuze, France). The dried aerial
65 part of Edelweiss plant was provided by Extrasynthese (Genay, France).

66 *2.2. Instrumentation*

67 The SpotPrep II system from Gilson (Saint-Avé, France) was set up as first dimension instrument, with
68 a 5.2 ml sample loop and detection set at 330 nm. The CPC instrument was the FCPC-A frame from
69 Kromaton Rousselet-Robatel (Annonay, France) equipped with a 35.8 ml rotor and thermostated at
70 30°C. The HPLC system used as second dimension was an Alliance 2690 system from Waters (Saint-
71 Quentin-en-Yvelines, France), set up at 330 nm and with a 100 µl sample loop for the analytical step
72 or 2.6 ml sample loop for the loading step. The delay volume is 1 ml without sample loop.

73 *2.3. Preparation of crude extract*

74 The crude extract was prepared from the dried aerial part of Edelweiss plant by macerating 4 g of the
75 plant in 200 ml of water/ethanol 70/30 (v/v) in acidic conditions for 16 hours. The mixture was
76 centrifuged and the supernatant was passed through filter paper. Then the obtained sample was
77 concentrated 10 times with the rotary evaporator to get 20 ml of the crude extract in water. The final
78 crude extract was filtrated through Nylon membrane 0.45 µm.

79 *2.4. First-dimension separations*

80 The CPC method was developed in our previous study [18]. A two-phase solvent system composed of
81 methyl tert-butyl ether and water pH 3 was prepared. The upper phase was equilibrated as
82 stationary phase at 1720 rpm, while the lower phase as mobile phase was pumped into the column
83 at a flow-rate of 5 ml/min in descending mode. When the equilibrium was established, 1 ml of the
84 crude extract was injected and the total run time was 50 min. The fractions were collected every
85 minute which corresponds to a recovery of 50 fractions of 5 ml.

86 For the prepLC method, a reversed-phase XSelect CSH Phenyl-Hexyl column (150 mm x 10 mm i.d.
87 5 μ m, column dead volume 7.7 ml) was used as the stationary phase, thermostated at 30°C. The
88 mobile phase was composed of water at 10 mM of ammonium acetate (pH 7) (A) and ACN (B) in
89 linear gradient: 1% to 25% B for 19.2 minutes, 25% to 95% B for 0.8 minute, 95% to 1% B for
90 0.8 minute and 1% B for 4.2 minutes. The flow-rate was 9.5 ml/min. After equilibration of the
91 column, 1 ml of the crude extract was injected for a total run time of 25 minutes. The fractions were
92 collected every 0.5 minute which corresponds to a recovery of 50 fractions of 4.75 ml each.

93 *2.5. Second-dimension LC separation*

94 A reversed-phase XSelect CSH Phenyl-Hexyl column (150 mm x 4.6 mm i.d. 5 μ m, column dead
95 volume 1.6 ml) was used as the second dimension HPLC column. For ¹D fraction analysis, an injection
96 volume of 20 μ l (1% of the column volume) was selected. For the loading step, 65% of the ²D column
97 volume was injected meaning a fraction injection volume of 1 ml. The mobile phase was water with
98 0.1% of formic acid (pH 3) (A) and acetonitrile with 0.1% of formic acid (B) in a gradient mode as
99 following: 12% B for 1.1 minutes, 12% to 35% B for 18.4 minutes, 35% to 95% B for 0.8 minute, 95%
100 to 12% B for 0.8 minute and 12% B for 3.9 minutes. The total elution time was 25 minutes. The flow-
101 rate of the mobile phase was 2 ml/min and the effluents were monitored at 330 nm.

102

103 **3. Results and discussion**

104 *3.1. Comprehensive strategy implementation and resulting 2D-contour plots*

105 The HPLC chromatogram of the Edelweiss extract is shown in Figure 1. In this crude sample, the two
106 compounds of interest from our previous study [18], namely 3,5-dicaffeoylquinic acid (compound A)
107 and leontopodic acid A (compound D), are present in high quantity. Three other compounds, B, C and
108 E, are unknown compounds that we suspect to be isomers and products of degradation of compound
109 D. As shown on the chromatogram and in our previous study, the isolation of the five compounds of
110 interest with high purity level cannot be considered by one-dimensional preparative LC or CPC due to
111 insufficient resolution. Hence a two-dimensional preparative strategy is pursued.

112 While many two-dimensional (2D) configurations exist for preparative purpose, it appears from our
113 recent review [12] that using the highly resolutive LC technique in the second and last dimension
114 provides better resolution and hence purification quality. Hence in this comparison, two preparative
115 2D configurations are compared (Figure 2). The first one is an off-line CPCxLC, using our previously
116 developed CPC method, which has shown some interesting orthogonality degrees with RPLC
117 methods. The second configuration is an off-line LCxLC configuration from which the orthogonality is
118 obtained by changing the mobile phase pH while using the very same stationary phase. These
119 configurations have been selected to be as cost-effective as possible, i.e. limiting column investment.

120 For an understandable simplification, the first dimension is noted ¹D and the second is noted ²D.

121 To get an estimation of the pros and cons of each first-dimension technology, the second dimension
122 has been optimized and is similar in both configurations, i.e. based on phenyl hexyl stationary phase
123 and acidic gradient mobile phase, as in Figure 1. This separation can be carried out on a semi-
124 preparative column (150 mm x 10 mm i.d. 5 μm, column dead volume 7.7 ml) for the production
125 step, but can also be scaled down to a laboratory-scale column (150 mm x 4.6 mm i.d. 5 μm, column
126 dead volume 1.6 ml) for the development of our 2D strategy (Figure 2).

127 The off-line 2D separations are carried out, during production, in a selective comprehensive mode
128 where only the fractions of interest from first dimension are sent to the second dimension (Figure 2,
129 production step). This imposes that the ¹D and ²D fractions are finely selected and that the fraction
130 transfer between ¹D and ²D columns is total without any split or loss. The method development of

131 such a separation requires two steps in which the 2D separations are performed in a full
132 comprehensive mode with the second dimension implemented at smaller scale. In the first step, the
133 orthogonality of the two dimensions is validated and/or optimized by monitoring the position of each
134 compound of interest (Figure 2, analytical step). It requires only aliquots of the ¹D fractions to be
135 analyzed, while in the second stage, the ²D lab-scale column is loaded in such a way to simulate the
136 full transfer of ¹D fractions and hence ensures that the purity of the targeted compounds will not be
137 compromised during the transfer process (Figure 2, loading step). It is important to notice here that
138 the full transfer is processed without any treatment of the ¹D fractions and this specification will
139 remain during the study.

140 In order to conduct a fair comparison of the first-dimension technology, it is decided that the 2D
141 configurations should initially treat the same number of fractions, as the overall cost of a 2D
142 separation is mainly governed by the number of runs on the second dimension. In our previous study
143 [18], the CPC eluent in first dimension was collected every minute in order to produce fractions
144 volume of 5 ml. In the present study, this criterion is maintained which leads to the collection of 50
145 fractions from first dimension, and hence, 50 ²D runs for comprehensive separations. Moreover, the
146 same sample load of 1 ml is injected in ¹D CPC and prepLC to allow an equitable comparison. Of
147 course, this initial injection could be further optimized but this is not the purpose of this publication.

148

149 *3.2. Quality evaluation of the 2D separations at the analytical step*

150 With the objective to isolate as much targeted compounds as possible at the lowest cost, it is
151 important to visualize the distribution of the targeted peaks regarding the others in the given
152 separation space, because the distances between peaks usually relate to the loading capacity of a
153 preparative method [20], providing that practical transfer conditions are favorable. Hence, the ¹D
154 separations, either CPC or prepLC, were performed and only 20- μ l aliquots of the 50 recovered
155 fractions were transferred in the ²D HPLC separation. The resulting 2D-contour plots are shown in
156 Figure 3. This visualization allows the evaluation of the peak distribution through two criteria: the

157 theoretical sample peak capacity which defines the theoretical separation space of the 2D
158 separation, and the selectivity which describes the distribution of the peaks in the separation space.

159 3.2.1. Peak distribution of prepLCxLC separation

160 The sample peak capacity, noted n_c , was introduced in 1967 by Giddings [21] in order to describe the
161 maximal number of peaks that could theoretically be separated by a given column. This notion was
162 established for isocratic and gradient elution modes in one-dimensional separations [21-23]. In our
163 prepLCxLC separation, the first and the second dimensions were conducted in gradient mode. The
164 calculation of the sample peak capacity in gradient mode is provided by the following equation [22]:

$$165 \quad n_c = 1 + \frac{2.3S\Delta C}{2.3Ss + 1} \cdot \frac{\sqrt{N}}{4}$$

166 With S the slope of the retention model of the compound (usually equal to 4 for small molecules), ΔC
167 the elution composition range of the compounds, s the normalized gradient slope, and N the number
168 of theoretical plates during the separation.

169 By implementing the ¹D prepLC in one-dimensional separation, the resulting sample peak capacity is
170 30 (with an efficiency of 4000 plates and an elution composition range of 0.24). Similarly, the
171 resulting sample peak capacity of the ²D LC implemented in one-dimensional separation is 30 due to
172 the same efficiency and elution composition range.

173 The 2D theoretical sample peak capacity being the product of sample peak capacities in each
174 dimension [24], its value for prepLCxLC is here 900. This may allow the separation of around 300
175 compounds, as stated by Davis [25] who claim that the effective peak number that can be separated
176 is 37% of the theoretical peak capacity. On the Figure 3a corresponding to the 2D-contour plot of the
177 prepLCxLC separation, about seventy compounds are separated.

178 The space occupation of the separation space is high with a value of 69% (calculated using Convex
179 Hull method [26]). Despite this large space occupation, no peak elutes at bottom right corner and top
180 left corner of the 2D-contour plot. Indeed, reversed phase chromatography mechanisms involving
181 hydrophobic interactions between compounds and stationary phase are implemented in both
182 dimensions. Consequently, the same type of interactions is settled in the two dimensions and thus,

183 despite the pH switch (from pH 7 to pH 3) between dimensions, the retention data are correlated
184 leading to a peak distribution along a regression line with a certain degree of dispersion around this
185 line. Indeed, to reduce the costs of investment, we experimented here an orthogonality
186 implementation based only on the change of ionization state of solutes towards the very same
187 reversed phase stationary phase. The selectivity based on the electronic properties of the solutes is
188 of interest for natural products, where a large number of organic acids and bases can be found.
189 Compounds that remain in the same ionization state during the pH switch are to be found on the
190 regression line $x=y$ on the 2D-contour plot. This is the case for compound B, which provides an extra
191 information on this unknown compound. Because hydrophobic interactions are decreased when the
192 solute is ionized, tracking the solutes during this pH switch through their position on the 2D plot
193 provides an insight of their pKa. For example, known compound A (3,5-dicaffeoylquinic acid) is less
194 retained in the second dimension than in the first one, indicating that it is more ionized at pH 3. This
195 is coherent with its known pKa of 3.3. Conversely, leontopodic acid A (compound D) and unknown
196 compounds C and E are less retained in the first dimension than in the second one thus are more
197 ionized at pH 7 than at pH 3.
198 The prepLCxLC separation with a high theoretical sample peak capacity allows the spread of the
199 whole matrix in the separation space, and an homogeneous distribution of compounds, at the
200 detriment of selectivity.

201

202 *3.2.2. Peak distribution of CPCxLC separation*

203 In our application, the CPC elution was conducted in isocratic mode. This has a consequence on the
204 sample peak capacity, as in isocratic mode, peaks become broader as they are more retained. The
205 sample peak capacity in isocratic mode is provided by the following equation [21, 24]:

$$206 \quad n_c = \frac{\sqrt{N}}{4} \ln \left(\frac{Vr_n}{Vr_1} \right)$$

207 With N the number of theoretical plates during the separation, V_{r_1} and V_{r_n} the retention volume of
208 the first and the last retained compound respectively.

209 With an efficiency less than 500 plates and a retention volume window from 20 to 220 ml (Figure 3b),
210 the sample peak capacity in ¹D CPC is around 10 peaks. The low efficiency of the CPC can be observed
211 on the Figure 3b where peaks are spread out along the CPC axis. This well-known lower efficiency is
212 due to the use of a liquid stationary phase and its consequent slow mass transfer. As calculated
213 previously, the sample peak capacity of the ²D LC is 30. Thus, the theoretical sample peak capacity of
214 the CPCxLC separation is around 300 meaning that the number of peaks that can be separated in the
215 resulting separation space is three times lower than with the previous LCxLC method. Of course, this
216 theoretical comparison does not take non-ideal transfer due to injection effects into account (see
217 below practical condition assessment).

218 As shown on the Figure 3b corresponding to the 2D-contour plot of the CPCxLC separation, the
219 important baseline width of the peaks does not allow the separation of the whole matrix in the
220 separation space. So the strategy to separate the targeted compounds from the rest of the matrix
221 must rely on an important selectivity between them. The CPC separation is based on the partition of
222 the solute between two liquid phases and hence can rely on several interactions such as Van der
223 Waals, hydrogen or hydrophobic bonds depending on the solvent system. Thus, the choice of the
224 solvents (nature and composition) that composed the stationary and mobile phases is important in
225 CPC in order to involve the expected interactions. In our example, methyl tert-butyl ether was
226 chosen as stationary phase with acidified water as mobile phase. This reversed-phase column can
227 involve hydrophobic interactions, hydrogen bonds as well as Van der Waals bonds with the targeted
228 compounds (K values over 3). As the ²D LC relies only on hydrophobic interactions, the retention data
229 from the first and second dimension separations can be non correlated offering a high selectivity
230 between peaks and a high occupation of the separation space as observed on the 2D-contour plot.
231 One of the advantages of CPC is the large choice of the solvent systems allowing a refined selection
232 of the solvent system specifically adapted to the desired separation. This high selectivity is an

233 obvious requirement in order to compensate for the low efficiency of CPC. The implementation of LC
234 as second dimension brings more efficiency to finally isolate targeted compounds.

235 3.2.3. Practical condition assessment

236 As explained in Stoll et al. and Dugo et al. reviews in 2007 and 2008 [1, 27], the implementation of
237 comprehensive 2D separations allows an improvement in resolving power by increasing the effective
238 sample peak capacity. They resumed the work of Murphy et al. [28] in which the resulting 2D
239 resolution depends on the ¹D and ²D resolutions. To maintain the ¹D and ²D resolutions during the 2D
240 separation, the fractionation of the ¹D eluent and the transfer of the fractions to the second
241 dimension are of great concern [1, 24, 27].

242 The ¹D eluent has to be fractionated in a strategic way in order to preserve the ¹D separation during
243 the transfer to the second dimension. This fractionation can be evaluated through the ¹D
244 undersampling factor noted α [29, 30]:

$$245 \quad \alpha = \frac{1}{\sqrt{1 + 0.21 \left(\frac{t_s}{\sigma_{t,obs}} \right)^2}}$$

246 With t_s the sampling time of the first-dimension eluent and $\sigma_{t,obs}$ the ¹D peak width.

247 To preserve the ¹D separation to the second dimension, the factor α must be at least 0.8 meaning
248 that each peak from first dimension is cut at least in three fractions. If this criterion is not respected,
249 separated peaks in first dimension may re-mix during transfer leading to a decrease of the sample
250 peak capacity and a doubt concerning the purity of the compounds [28, 31]. For the prepLCxLC
251 separation, the baseline width of the ¹D peaks is around 1.5 min. Thus, with a sampling time of 0.5
252 min, the peaks are adequately cut in three fractions. For the CPCxLC separation, as the CPC was
253 implemented in isocratic elution, the width of the peaks becomes broader with the retention. For the
254 targeted compounds, the smallest baseline width is 15 min (compound E). Thus, with a sampling time
255 of 1 min, the undersampling is not an issue with an α value of 0.98. For a larger fractionation of peaks
256 in three cuts ($\alpha = 0.8$), the sampling time should not exceed 5 min. Thus, with the implementation of

257 CPC in first dimension, the sampling time can be 10 times higher than for prepLC, which induces less
 258 fractions to inject on second dimension for a comprehensive strategy.
 259 As explained in François et al. review in 2009 [24], the solvents compatibility between the ¹D eluent
 260 contained in transferred fractions and the ²D mobile phase is another major issue. In order to
 261 maintain the ²D resolution, it is of importance to avoid peaks widening during the ²D injection. These
 262 negative effects can be due to the absence of focusing effect [32] or due to viscous fingering [33, 34].
 263 In off-line process, an intermediate treatment of the fractions can avoid these effects. This is
 264 however really tedious and time consuming when many fractions are to be transferred, which is
 265 usually the case in any 2D preparative strategy. In analytical 2D LC, the issue is avoided by
 266 transferring small amounts or by using trapping columns [35, 36]. With 2D preparative aim, however,
 267 the volumes to be treated are usually not compatible with this kind of solution. If no fraction
 268 treatment is carried out, these peak band broadening due to injection effects can be evaluated
 269 through the correction factor noted β [29]:

$$270 \quad \beta = \frac{1}{\sqrt{1 + \frac{1}{\delta i^2} \cdot \frac{{}^2V_i^2}{2\sigma_{v,col}^2} \cdot \frac{1}{C_F^2}}}$$

271 With δi^2 a parameter related to the shape of the injection band (equal to 4 in a practical case), ²V_i the
 272 ²D injection volume, ² $\sigma_{v,col}^2$ the standard deviation resulting from the column dispersion and C_F the
 273 compression factor.

274 The injection effects on second dimension are favorable when the factor β is close to 1. In our
 275 comparison of CPCxLC and prepLCxLC separations, with a same transferred volume ²V_i on the same
 276 ²D column (² $\sigma_{v,col}^2$), the ²D injection effect factor β only depends on the compression factor C_F [37,
 277 38]. This one is related to the solute retention factors on the second dimension, and defined as:

$$278 \quad C_F = \frac{1 + {}^2k_{e,1}}{1 + {}^2k_{e,2}}$$

279 With ²k_{e,1} the solute retention factor on the ²D column, in ¹D mobile phase at elution (fraction
 280 solvent) and ²k_{e,2} the solute retention factor on the ²D column in ²D mobile phase at elution.

281 The compression factor is favorable (>1) if the injection solvent in the transferred fraction has a
282 weaker eluent strength than the mobile phase at peak elution in second dimension. As the second
283 dimension shows a reverse-phase mechanism and runs with a mobile phase composed of 88% water
284 to 65% water during the elution (with acetonitrile as organic solvent), the solvent in fractions must
285 contain more water than the mobile phase at elution. For the prepLC implemented as first
286 dimension, the solvent in fractions was composed from 99% water for the first fraction to 75% water
287 for the last fraction. In this case, the compression factor is higher than 1 for only a part of the peaks.
288 The part where the compression factor is lower than 1 can be visualized on the top left corner of the
289 2D-contour plot on the Figure 3a where the eluent strength of the injection solvent (solvent in
290 fraction) is stronger than the eluent strength of the mobile phase. Thus, for this part, there is no
291 focusing effect on the peaks and some deformation can occur. On the 2D-contour plot, no negative
292 effects are observed for the analytical step due to low 2D injection volume but these effects are
293 visible during the loading step with higher 2D injection volume (Figure 3c). When CPC was
294 implemented as first dimension, and run in isocratic mode, all 1D fractions are composed of 93.5%
295 water (with MtBE as organic solvent), which is obviously favorable going into a 88%-65% 2D gradient
296 elution. These conditions give a compression factor much higher than 1 for all fractions separations
297 allowing a focusing effect on all peaks. We found that the compatibility of solvent between both
298 dimensions is easier to deal with when an isocratic tunable mobile phase is used in first dimension,
299 and CPC technique may offer that opportunity.

300 It is important to notice here that the dilution factor D_f is higher for the CPCxLC separation which can
301 lead to some detection problems. As described in the following equation [39, 40], the dilution factor
302 depends on the peak width observed on first-dimension and the compression factor. For CPCxLC,
303 despite a high compression factor during transfer, the dramatically low efficiency of the CPC
304 technique leads to a large peak width hence a high dilution factor in the order of 75, in opposition to
305 a dilution factor of 17 in prepLCxLC.

306
$$D_F = \frac{\sqrt{2\pi}}{^1V_i} \cdot ^1\sigma_{v,obs} \cdot \frac{\sqrt{2\pi}}{\delta i \sqrt{1-\beta^2}} \cdot \frac{1}{C_F}$$

307 Where δ^2i is a parameter dependent on the injection process, $^1\sigma_{v, col}$ is the standard deviation
 308 resulting of band broadening in the 1D column and 1V_i is the injected volume in $1D$.

309

310 3.3. Quality evaluation of the 2D separations when loading

311 Since scale-up is easily achieved on both techniques, the requirement before moving to production is
 312 the overloading study. As the objective is the full recovery of compounds of interest, the injection
 313 volume in second dimension has to be equal to the fraction volume obtained from first dimension. In
 314 our study, the fraction volume from each first dimensions is initially 5 ml. Transferring this amount to
 315 a 10 mm internal diameter prepLC column represents an injection volume of 65% of its dead volume.
 316 This loading study can be evaluated on the second dimension with a smaller 4.6 mm internal
 317 diameter column (specifications in material and methods section). So, the study of such a volume
 318 load on a 4.6 mm internal diameter column can be achieved by injecting 65% of its volume, i.e. 1 ml.
 319 The 2D-contour plots resulting from the loading step are shown in Figures 3c and 3d.

320

321 3.3.1. Resulting peaks resolution on second dimension

322 Some peaks deformations can be observed on the top left corner of the 2D contour plot of the
 323 prepLCxLC separation (Figure 3c). As already mentioned, this is due to the decrease of the 2D
 324 injection effect factor β . As the 2D injection volume increases from 1% to 65% of the 2D column
 325 volume, the ratio $^2V_i^2 / ^2\sigma_{v,col}^2$ is 2500 times higher than during the analytical step. These effects do not
 326 appear with the use of 1D CPC due to the much higher compression factor that compensates for the
 327 larger transferred fraction volume.

328 The resolution loss after the 65% load can be visualized on critical pair of targeted compounds (blue
 329 circles, Figure 3). For prepLCxLC, the critical pair is compound D with its impurity i_D contained in
 330 fraction #30 (from 14.5 to 15 minutes) and for CPCxLC, the critical pair is compounds A and B

331 contained in fraction #30 (from 29 to 30 minutes). The chromatograms of both fractions #30 from
332 prepLC and CPC are shown on Figure 4.

333 It can be seen that the resolution is totally lost between peaks D and i_D from ¹D prepLC #30 when the
334 ²D LC column is loaded in preparative conditions (Figure 4a and c). Hence with these loading
335 specifications, corresponding to 5 ml fractions being fully transferred to a 10 mm internal diameter
336 ²D column, only four compounds can be isolated as shown on Figure 3c. The compound D has
337 coeluted with an impurity. Conversely, peaks A and B from CPC #30 keep the same resolution during
338 loading and the ²D LC column is not saturated (Figure 4b and d). This is due to the high dilution of the
339 compounds during CPC and the high compression factor of the peaks during the transfer to the
340 second dimension, as mentioned before. Because the solutes coming from CPC are highly diluted in a
341 low strength solvent, a 65% injection volume is easily handled by the ²D column. The overall 2D plot
342 (Figure 3d) shows that the five targeted compounds can be fully transferred and isolated with
343 baseline return.

344 It is clear through this study that the solvent compatibility issue is even more problematic when
345 injection large volumes of fractions on the second dimension. The selections of the first dimension
346 and its elution mobile phase composition are crucial in the resulting preparative performances.
347 Because ¹D prepLC and CPC have here very different elution phases, it is interesting to optimize the
348 transferred volume for each configuration.

349

350 *3.3.2. Charge capacity on second dimension for prepLCxLC separation*

351 The injection volume of the critical fraction #30 from ¹D prepLC was incrementally increased to
352 achieve baseline return between peak D and its impurity. For the quantitative evaluation of the
353 return to baseline [41], the free-space between peaks, noted ΔV (expressed in ml), was calculated
354 with the following equation:

$$355 \quad \Delta V = (V_{r,2} - V_{r,1}) - 3(\sigma_{v,1} + \sigma_{v,2})$$

356 With the retention volumes of peaks 1 and 2 and their corresponding standard deviation in volume
357 unit. As the peaks are not symmetric with the load, the standard deviations were calculating from the
358 right peak width for peak 1 and left peak width for peak 2. Statistically, 99.7% of the solute molecules
359 can be found within the 6- σ baseline width which was a satisfactory criterion for the evaluation of
360 the baseline return. Thus, using the ¹D prepLC #30 fraction as sample, the ΔV was calculated for the
361 injection volume of 1%, 6%, 13%, 19%, 25%, 31% and 65% ²D LC column. In our study, the maximum
362 load is observed for an injection volume of 19%.

363 If the specifications require that the fraction coming from the first dimension is fully injected in the
364 second one, the use of a 150 x 10 mm i.d. prepLC as ²D imposes that the ¹D fractions need to be
365 reduced down to 1.5 ml each. It then represents a sampling time of 0.16 min on the ¹D prepLC and
366 the collection of 157 fractions. In this case, as the sampling time is much lower than 0.5 minute, the
367 peaks from first dimension are cut in more than three fractions leading to the ¹D undersampling
368 factor α closer to 1 and thus favorable for the separation. Concerning the ²D injection effect factor β ,
369 as the injection volume on the second dimension decreases from 65% to 19% of the column dead
370 volume, the ratio $^2V_i^2/\sigma_{v,col}^2$ will be 11 times minimized leading to factor closer to 1 and thus the
371 negative effects will be more negligible. Injecting 19% of the column dead volume on a 150 x 4.6 mm
372 i.d. column or on a 150 x 10 mm i.d. prepLC column, the ratio $^2V_i^2/\sigma_{v,col}^2$ remains the same and so
373 the ²D injection effect factor β .

374 Another solution, to avoid the huge running cost of 157 ²D runs is to increase the size of the ²D
375 column to decrease the number of ¹D fractions. The maximal fraction volume that can be collected
376 while keep reasonable ¹D undersampling factor α is 4.75 ml. To handle such an injection volume
377 without overloading effects, a prepLC column with an internal diameter of 19 mm is necessary.
378 Hence, 50 fractions of 4.75 ml can be transferred leading to a lower α factor and an unmodified β
379 factor.

380

381 *3.3.3. Charge capacity on second dimension for CPCxLC separation*

382 It was previously found that injecting ¹D CPC fraction to the extent of 65% ²D column was easily
383 achieved without any resolution loss. Increasing the transferred volume, we noticed that it was
384 possible to inject the entire CPC fractions of 5 ml on the analytical LC column (150 x 4.6 mm i.d.)
385 meaning 313 % of the LC column dead volume without any resolution loss.
386 Using the prepLC column (150 x 10 mm i.d.) as second dimension, 24-ml fractions could then be
387 injected on the second dimension. This is obtained by increasing the ¹D sampling time to 4.8 min,
388 which results in the collection of only 11 fractions from first dimension. In this case, the sampling
389 time is maximal leading to a ¹D undersampling factor α of 0.8 lower than previously but still very
390 favorable for the separation. Concerning the ²D injection effect factor β , the injection volume
391 increases from 65% to 313% of the column dead volume leading to a 25 times increase of the ratio
392 $^2V_i^2/\sigma_{v,col}^2$ and so a lower β factor than previously but as the compression factor is very important,
393 the resolution is kept.

394

395 *3.4. Final 2D separations comparison*

396 In order to fairly compare off-line CPCxLC and prepLCxLC selective comprehensive separations based
397 on the purification of 1 ml rare Edelweiss extract, the various optimized options are here compiled
398 together with solvent consumption and duration in Table 1.

399 The ¹D prepLC (150 x 10 mm i.d.) requires 475 ml of solvent for a total duration of 50 minutes
400 (including blank run). For the implementation of CPC in first dimension, a total volume of 325 ml of
401 solvent was used (mobile and stationary phases included) for a total time of 65 minutes (including
402 equilibration and separation time). With the implementation of a ²D prepLC column with the very
403 same dimensions (no supplementary investment), 157 fractions of 1.5 ml can be collected from ¹D
404 prepLC. Using a selective comprehensive mode to isolate only the five targets, the injection of 29
405 fractions on second dimension is necessary with total time of 775 minutes.

406 In the case of CPCxLC separation, 11 fractions of 24 ml can be collected from ¹D CPC to be sent on the
407 prepLC column (150 x 10 mm i.d.). In selective comprehensive mode, this combination requires the
408 injection of only 7 fractions on second dimension for a total time of 240 minutes.

409 In order to minimize the second-dimension investment cost, alternatives can be implemented. For
410 the prepLCxLC separation, a higher internal diameter ²D column (column 150 x 19 mm i.d.) can be
411 implemented allowing 3.3 times less fractions to be injected on the ²D column. Additional investment
412 costs have to be considered. For the CPCxLC separation, the ²D column used during method
413 development (column 150 x 4.6 mm i.d.) can be implemented for preparative purposes leading to 4.8
414 times more fractions to be injected on second dimension but with no need to purchase a prepLC
415 All these options can be implemented with favorable practical conditions (α and β factors). If less
416 fractions are to be collected and injected on ²D column in order to further minimize the second-
417 dimension running costs, a heart-cut mode can be implemented. In this case, the ¹D undersampling
418 factor α and the ²D injection effect factor β would not be favorable anymore leading eventually to a
419 re-mix of the compounds from first-dimension separation and peaks deformation during the transfer
420 and thus an important loss of the resolution between peaks. Other alternatives to either reduce the
421 fraction volume or increase the compression factor by make-up addition would be of interest but
422 may greatly increase the overall separation process duration.

423

424 **4. Conclusion**

425 This study represents the first comparison between CPC and prepLC used as first dimension in an off-
426 line two-dimensional separation. The two-dimensional CPCxLC and prepLCxLC separations were
427 performed in comprehensive mode at lab-scale in order to develop the selective comprehensive 2D
428 separations for the isolation of five targeted compounds from Edelweiss plant. As the investment
429 cost on second dimension was initially stated for both separations, the first dimensions CPC and
430 prepLC performances could be compared.

431 While the conventional LCxLC setup demonstrates once again its powerful peak capacity and
432 resolution, it requires a large investment and exhibits solvent compatibility issues. The CPC technique
433 may be an appropriate alternative when dealing with these solvent compatibility issues since the
434 choice of the chemical nature of the CPC columns is much wider and can, if finely tuned, be of great
435 interest for preparative purposes. In both cases, CPC and prepLC are attractive techniques with
436 advantages and drawbacks, offering a great deal of possibilities for the development of 2D
437 preparative separations for rare samples.

438

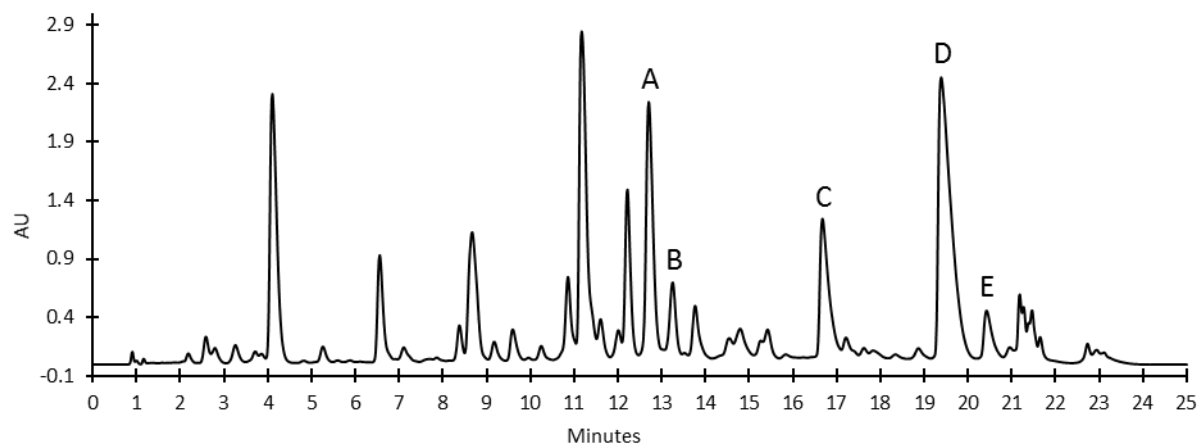
439 **Acknowledgment**

440 The authors wish to deeply thank Extrasynthese company for providing the aerial part of Edelweiss
441 plant.

442 **References**

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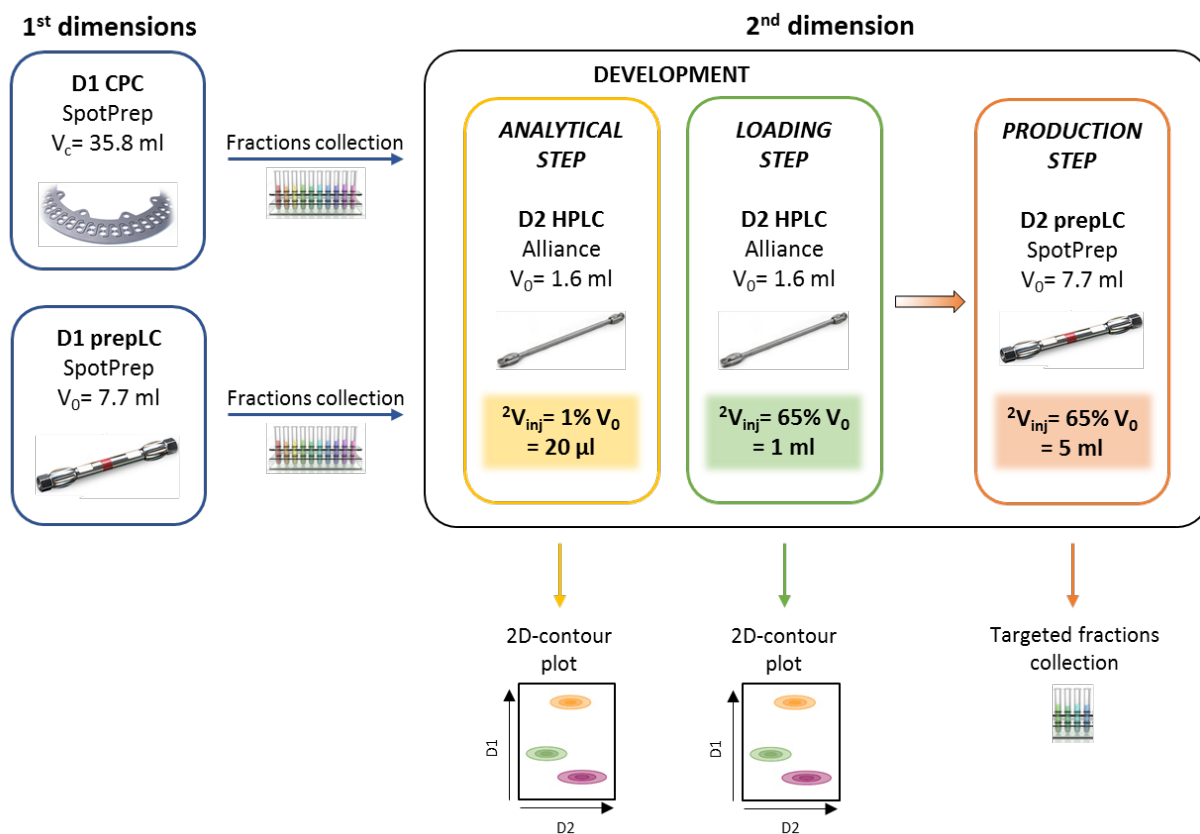
509

510 Figure 1. HPLC analysis of the crude sample on Phenyl-Hexyl column (150 mm x 4.6 mm i.d., 5 μ m),

511 mobile phase of water+0.1% of formic acid (A) and acetonitrile+0.1% of formic acid (B) in gradient

512 mode: 12% B for 1.1 min, 12 to 35% B in 18.4 min, 35 to 95% B for 0.8 min, flow-rate 2 ml/min,

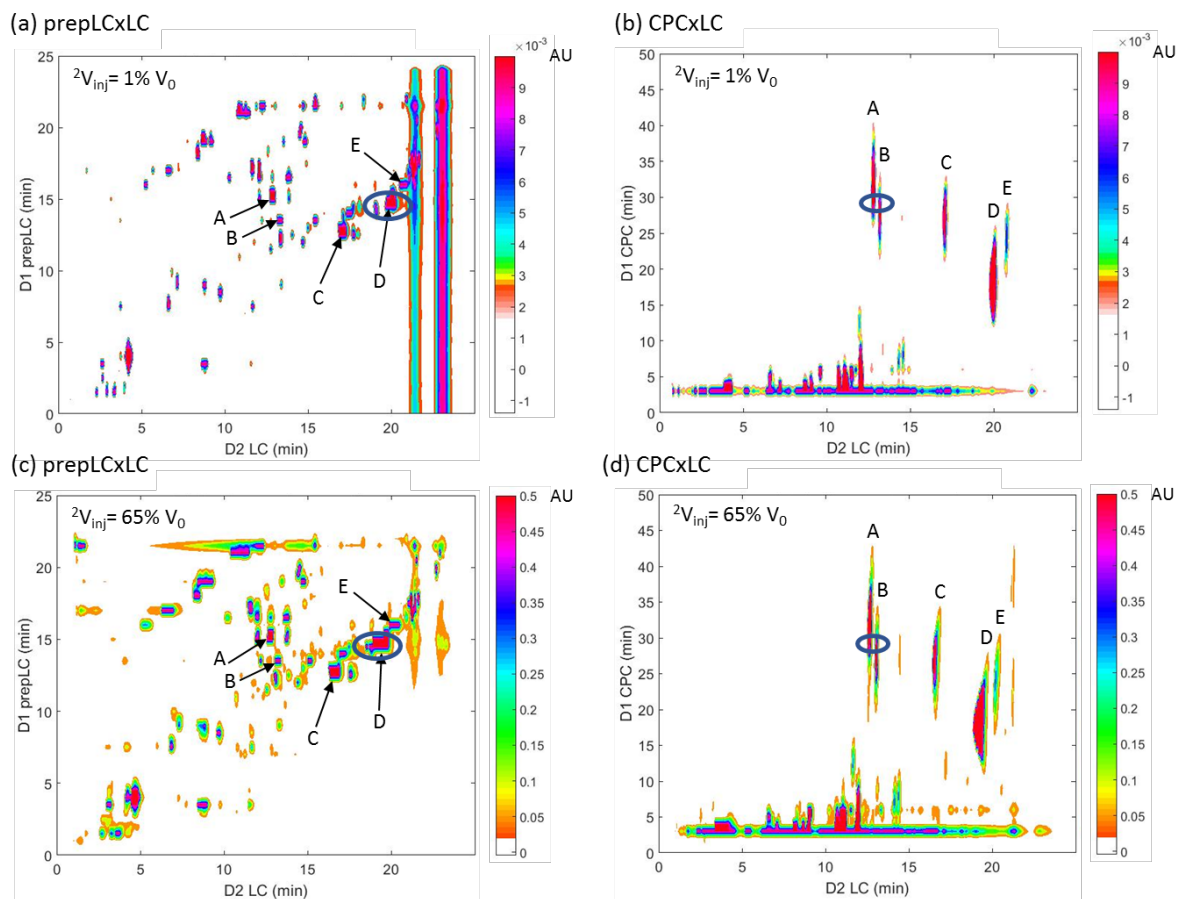
513 detection 330 nm.



514

515 Figure 2. Two-dimensional process with CPC and prepLC as first dimensions and LC as second

516 dimension with the various sizes corresponding to the successive development steps.

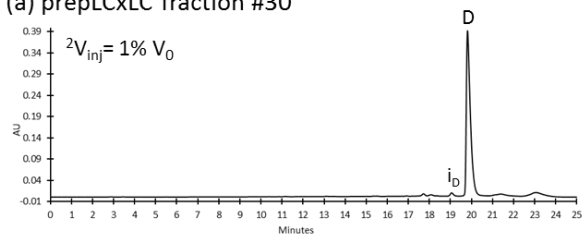


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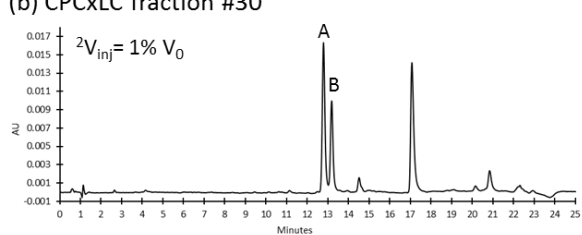
518 Figure 3. 2D-contour plots of off-line comprehensive two-dimensional separations (a) prepLCxLC at
 519 analytical step (b) CPCxLC at analytical step (c) prepLCxLC at loading step (d) CPCxLC at loading step.

520 In circles are the critical pairs of each configuration.

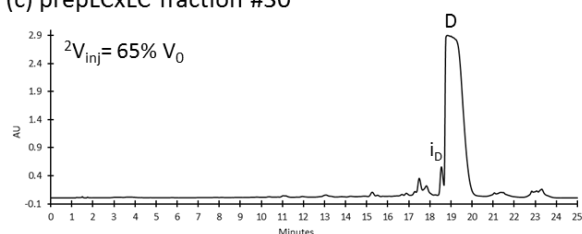
(a) prepLCxLC fraction #30



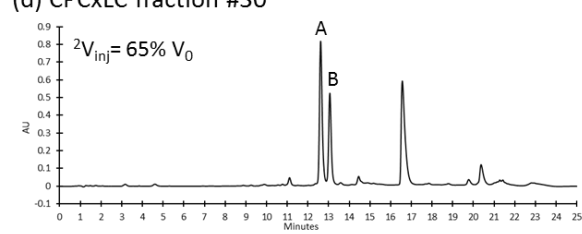
(b) CPCxLC fraction #30



(c) prepLCxLC fraction #30



(d) CPCxLC fraction #30



521

522 Figure 4. 2nd-dimension LC chromatograms at analytical step of (a) fraction #30 of prepLC (b) fraction

523 #30 of CPC and 2nd-dimension LC chromatograms at loading step of (c) fraction #30 of prepLC (d)

524 fraction #30 of CPC.

525 Figure 1. HPLC analysis of the crude sample on Phenyl-Hexyl column (150 mm x 4.6 mm i.d., 5 μ m),
526 mobile phase of water+0.1% of formic acid (A) and acetonitrile+0.1% of formic acid (B) in gradient
527 mode: 12% B for 1.1 min, 12 to 35% B in 18.4 min, 35 to 95% B for 0.8 min, flow-rate 2 ml/min,
528 detection 330 nm.

529

530 Figure 2. Two-dimensional process with CPC and prepLC as first dimensions and LC as second
531 dimension with the various sizes corresponding to the successive development steps.

532

533 Figure 3. 2D-contour plots of off-line comprehensive two-dimensional separations (a) prepLCxLC at
534 analytical step (b) CPCxLC at analytical step (c) prepLCxLC at loading step (d) CPCxLC at loading step.
535 In circles are the critical pairs of each configuration.

536

537 Figure 4. 2nd-dimension LC chromatograms at analytical step of (a) fraction #30 of prepLC (b) fraction
538 #30 of CPC and 2nd-dimension LC chromatograms at loading step of (c) fraction #30 of prepLC (d)
539 fraction #30 of CPC.

540 Table 1. Comparison of prepLCxLC and CPCxLC separations for the isolation of five targets from 1 ml
 541 Edelweiss extract.

	prepLCxLC	CPCxLC
Strategy	Spread the solutes over a large separation space to get better chance to separate targets	Separate target compounds from the others by finely tuning the solvent system
Development method	Fast	Slow
¹ D column	150 mm x 10 mm	35.8 ml rotor
Cost and duration of first dimension (including equilibration, blanks)	475 ml of solvent 50 min	325 ml of solvent 65 min
$n_{C,2D,theo}$	900	< 300
Selectivity	Low	High
² D column load	19% of column dead volume	313% of column dead volume
	using a 150 mm x 10 mm ²D column	
Fractions volume	1.5 ml	24 ml
¹ D sampling time	0.16 min	4.8 min
Comprehensive strategy	157 fractions	11 fractions
Selective comprehensive strategy (5 targets)	29 fractions	7 fractions
	using alternative ²D column size	
Second dimension alternative	Column 150 mm x 19 mm	Column 150 mm x 4.6 mm
Fractions volume	4.75 ml	5 ml
Comprehensive strategy	50 fractions	50 fractions
Selective comprehensive strategy (5 targets)	9 fractions	34 fractions

542