

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

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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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# 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

scale instruments. The quality of separation is compared using 2D-contour plots.

The prepLCxLC exhibits a large separation space that leads to an overall large peak capacity, which is of great interest for complex samples. But its limited loading capacity involves a large number of <sup>2</sup>D

runs increasing the running costs for preparative purposes. On the other hand, CPCxLC provides a

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 second dimension strictly identical throughout the study. 48 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

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

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

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

The crude extract was prepared from the dried aerial part of Edelweiss plant by macerating 4 g of the

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

concentrated 10 times with the rotary evaporator to get 20 ml of the crude extract in water. The final

rude extract was filtrated through Nylon membrane 0.45 μm.

79 2.4. First-dimension separations

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

For the prepLC method, a reversed-phase XSelect CSH Phenyl-Hexyl column (150 mm x 10 mm i.d.
5 μm, column dead volume 7.7 ml) was used as the stationary phase, thermostated at 30°C. The
mobile phase was composed of water at 10 mM of ammonium acetate (pH 7) (A) and ACN (B) in
linear gradient: 1% to 25% B for 19.2 minutes, 25% to 95% B for 0.8 minute, 95% to 1% B for
0.8 minute and 1% B for 4.2 minutes. The flow-rate was 9.5 ml/min. After equilibration of the
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 μm, column dead volume 1.6 ml) was used as the second dimension HPLC column. For <sup>1</sup>D fraction analysis, an injection 95 volume of 20  $\mu l$  (1% of the column volume) was selected. For the loading step, 65% of the  $^2D$  column 96 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

The HPLC chromatogram of the Edelweiss extract is shown in Figure 1. In this crude sample, the two compounds of interest from our previous study [18], namely 3,5-dicaffeoylquinic acid (compound A) and leontopodic acid A (compound D), are present in high quantity. Three other compounds, B, C and E, are unknown compounds that we suspect to be isomers and products of degradation of compound D. As shown on the chromatogram and in our previous study, the isolation of the five compounds of interest with high purity level cannot be considered by one-dimensional preparative LC or CPC due to 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 provides better resolution and hence purification quality. Hence in this comparison, two preparative 114 115 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 <sup>1</sup>D and the second is noted <sup>2</sup>D. To get an estimation of the pros and cons of each first-dimension technology, the second dimension 121 122 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  $\mu$ m, column 126 dead volume 1.6 ml) for the development of our 2D strategy (Figure 2). The off-line 2D separations are carried out, during production, in a selective comprehensive mode 127 where only the fractions of interest from first dimension are sent to the second dimension (Figure 2, 128 129 production step). This imposes that the <sup>1</sup>D and <sup>2</sup>D fractions are finely selected and that the fraction

130 transfer between <sup>1</sup>D and <sup>2</sup>D 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 comprehensive mode with the second dimension implemented at smaller scale. In the first step, the 132 orthogonality of the two dimensions is validated and/or optimized by monitoring the position of each 133 134 compound of interest (Figure 2, analytical step). It requires only aliquots of the <sup>1</sup>D fractions to be analyzed, while in the second stage, the <sup>2</sup>D lab-scale column is loaded in such a way to simulate the 135 136 full transfer of <sup>1</sup>D 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 <sup>1</sup>D fractions and this specification will 139 remain during the study.

In order to conduct a fair comparison of the first-dimension technology, it is decided that the 2D 140 141 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 <sup>2</sup>D runs for comprehensive separations. Moreover, the 146 same sample load of 1 ml is injected in <sup>1</sup>D 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

With the objective to isolate as much targeted compounds as possible at the lowest cost, it is important to visualize the distribution of the targeted peaks regarding the others in the given separation space, because the distances between peaks usually relate to the loading capacity of a preparative method [20], providing that practical transfer conditions are favorable. Hence, the <sup>1</sup>D separations, either CPC or prepLC, were performed and only 20-µl aliquots of the 50 recovered fractions were transferred in the <sup>2</sup>D HPLC separation. The resulting 2D-contour plots are shown in 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*

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

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

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

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

173 The 2D theoretical sample peak capacity being the product of sample peak capacities in each

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

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

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 line. Indeed, to reduce the costs of investment, we experimented here an orthogonality 185 186 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.

The prepLCxLC separation with a high theoretical sample peak capacity allows the spread of the
whole matrix in the separation space, and an homogeneous distribution of compounds, at the
detriment of selectivity.

201

## 202 3.2.2. Peak distribution of CPCxLC separation

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

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

207 With N the number of theoretical plates during the separation,  $Vr_1$  and  $Vr_n$  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 <sup>1</sup>D 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 <sup>2</sup>D 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 <sup>2</sup>D 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
- as second dimension brings more efficiency to finally isolate targeted compounds.

235 3.2.3. Practical condition assessment

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 <sup>1</sup>D and <sup>2</sup>D resolutions. To maintain the <sup>1</sup>D and <sup>2</sup>D resolutions during the 2D
- 240 separation, the fractionation of the <sup>1</sup>D eluent and the transfer of the fractions to the second
- dimension are of great concern [1, 24, 27].

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

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

246 With t<sub>s</sub> the sampling time of the first-dimension eluent and  ${}^{1}\sigma_{t,obs}$  the  ${}^{1}D$  peak width.

247 To preserve the <sup>1</sup>D separation to the second dimension, the factor  $\alpha$  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 <sup>1</sup>D 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 implemented in isocratic elution, the width of the peaks becomes broader with the retention. For the 253 254 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  $\alpha$  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 <sup>1</sup>D eluent contained in transferred fractions and the <sup>2</sup>D mobile phase is another major issue. In order to 260 261 maintain the <sup>2</sup>D resolution, it is of importance to avoid peaks widening during the <sup>2</sup>D 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 treatment is carried out, these peak band broadening due to injection effects can be evaluated 268 269 through the correction factor noted  $\beta$  [29]:

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

271 With  $\delta i^2$  a parameter related to the shape of the injection band (equal to 4 in a practical case), <sup>2</sup>Vi the 272 <sup>2</sup>D injection volume, <sup>2</sup> $\sigma_{v,col}^2$  the standard deviation resulting from the column dispersion and C<sub>F</sub> the 273 compression factor.

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

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

279 With  ${}^{2}k_{e,1}$  the solute retention factor on the  ${}^{2}D$  column, in  ${}^{1}D$  mobile phase at elution (fraction 280 solvent) and  ${}^{2}k_{e,2}$  the solute retention factor on the  ${}^{2}D$  column in  ${}^{2}D$  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 dimension shows a reverse-phase mechanism and runs with a mobile phase composed of 88% water 283 284 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 <sup>2</sup>D injection volume but these effects are 293 visible during the loading step with higher <sup>2</sup>D injection volume (Figure 3c). When CPC was implemented as first dimension, and run in isocratic mode, all <sup>1</sup>D fractions are composed of 93.5% 294 295 water (with MtBE as organic solvent), which is obviously favorable going into a 88%-65% <sup>2</sup>D 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.

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

307 Where  $\delta^2 i$  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  ${}^1Vi$  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 load on a 4.6 mm internal diameter column can be achieved by injecting 65% of its volume, i.e. 1 ml. 318 319 The 2D-contour plots resulting from the loading step are shown in Figures 3c and 3d. 320 3.3.1. Resulting peaks resolution on second dimension 321 322 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 <sup>2</sup>D injection effect factor  $\beta$ . As the <sup>2</sup>D injection volume increases from 1% to 65% of the <sup>2</sup>D column 324 volume, the ratio  ${}^{2}Vi^{2}/{}^{2}\sigma_{v,col}{}^{2}$  is 2500 times higher than during the analytical step. These effects do not 325 326 appear with the use of <sup>1</sup>D 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<sub>D</sub> contained in

330 fraction #30 (from 14.5 to 15 minutes) and for CPCxLC, the critical pair is compounds A and B

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

It can be seen that the resolution is totally lost between peaks D and i<sub>D</sub> from <sup>1</sup>D prepLC #30 when the 333 334 <sup>2</sup>D 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 <sup>2</sup>D 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 <sup>2</sup>D 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 <sup>2</sup>D 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.

Because <sup>1</sup>D prepLC and CPC have here very different elution phases, it is interesting to optimize the
 transferred volume for each configuration.

349

355

350 3.3.2. Charge capacity on second dimension for prepLCxLC separation

The injection volume of the critical fraction #30 from <sup>1</sup>D prepLC was incrementally increased to achieve baseline return between peak D and its impurity. For the quantitative evaluation of the return to baseline [41], the free-space between peaks, noted  $\Delta V$  (expressed in ml), was calculated with the following equation:

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

With the retention volumes of peaks 1 and 2 and their corresponding standard deviation in volume unit. As the peaks are not symmetric with the load, the standard deviations were calculating from the right peak width for peak 1 and left peak width for peak 2. Statistically, 99.7% of the solute molecules can be found within the 6- $\sigma$  baseline width which was a satisfactory criterion for the evaluation of the baseline return. Thus, using the <sup>1</sup>D prepLC #30 fraction as sample, the  $\Delta$ V was calculated for the injection volume of 1%, 6%, 13%, 19%, 25%, 31% and 65% <sup>2</sup>D LC column. In our study, the maximum 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 second one, the use of a 150 x 10 mm i.d. prepLC as <sup>2</sup>D imposes that the <sup>1</sup>D fractions need to be 364 reduced down to 1.5 ml each. It then represents a sampling time of 0.16 min on the <sup>1</sup>D prepLC and 365 366 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 <sup>1</sup>D undersampling 368 factor  $\alpha$  closer to 1 and thus favorable for the separation. Concerning the <sup>2</sup>D injection effect factor  $\beta$ , 369 as the injection volume on the second dimension decreases from 65% to 19% of the column dead volume, the ratio  ${}^{2}Vi^{2}/{}^{2}\sigma_{v,col}{}^{2}$  will be 11 times minimized leading to factor closer to 1 and thus the 370 negative effects will be more negligible. Injecting 19% of the column dead volume on a 150 x 4.6 mm 371 i.d. column or on a 150 x 10 mm i.d. prepLC column, the ratio  ${}^{2}Vi^{2}/{}^{2}\sigma_{v,col}{}^{2}$  remains the same and so 372 373 the <sup>2</sup>D injection effect factor  $\beta$ .

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

380

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

382 It was previously found that injecting <sup>1</sup>D CPC fraction to the extent of 65% <sup>2</sup>D 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 <sup>1</sup>D 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 <sup>1</sup>D undersampling factor  $\alpha$  of 0.8 lower than previously but still very 390 favorable for the separation. Concerning the <sup>2</sup>D injection effect factor  $\beta$ , the injection volume 391 increases from 65% to 313% of the column dead volume leading to a 25 times increase of the ratio  $^{2}Vi^{2}/^{2}\sigma_{v,col}^{2}$  and so a lower  $\beta$  factor than previously but as the compression factor is very important, 392 393 the resolution is kept.

394

### 395 *3.4. Final 2D separations comparison*

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

The <sup>1</sup>D prepLC (150 x 10 mm i.d.) requires 475 ml of solvent for a total duration of 50 minutes (including blank run). For the implementation of CPC in first dimension, a total volume of 325 ml of solvent was used (mobile and stationary phases included) for a total time of 65 minutes (including equilibration and separation time). With the implementation of a <sup>2</sup>D prepLC column with the very same dimensions (no supplementary investment), 157 fractions of 1.5 ml can be collected from <sup>1</sup>D prepLC. Using a selective comprehensive mode to isolate only the five targets, the injection of 29 fractions on second dimension is necessary with total time of 775 minutes. In the case of CPCxLC separation, 11 fractions of 24 ml can be collected from <sup>1</sup>D CPC to be sent on the
prepLC column (150 x 10 mm i.d.). In selective comprehensive mode, this combination requires the
injection of only 7 fractions on second dimension for a total time of 240 minutes.

In order to minimize the second-dimension investment cost, alternatives can be implemented. For

410 the prepLCxLC separation, a higher internal diameter <sup>2</sup>D column (column 150 x 19 mm i.d.) can be 411 implemented allowing 3.3 times less fractions to be injected on the <sup>2</sup>D column. Additional investment 412 costs have to be considered. For the CPCxLC separation, the <sup>2</sup>D 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 ( $\alpha$  and  $\beta$  factors). If less 416 fractions are to be collected and injected on <sup>2</sup>D column in order to further minimize the second-417 dimension running costs, a heart-cut mode can be implemented. In this case, the <sup>1</sup>D undersampling 418 factor  $\alpha$  and the <sup>2</sup>D injection effect factor  $\beta$  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

409

### 424 **4. Conclusion**

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

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



Figure 3. 2D-contour plots of off-line comprehensive two-dimensional separations (a) prepLCxLC at
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.



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



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 μm),
- 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.
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- 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.
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- 533 Figure 3. 2D-contour plots of off-line comprehensive two-dimensional separations (a) prepLCxLC at
- 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	
	Spread the solutes over a large	Separate target compounds from	
Strategy	separation space to get better	the others by finely tuning the	
	chance to separate targets	solvent system	
Development method	Fast	Slow	
<sup>1</sup> 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	
Nc,2D,theo	900	< 300	
Selectivity	Low	High	
<sup>2</sup> D column load	19% of column dead volume	313% of column dead volume	
	using a 150 mm x 10 mm <sup>2</sup> D column		
Fractions volume	1.5 ml	24 ml	
<sup>1</sup> 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 <sup>2</sup> D 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	