Two-dimensional multi-heart cutting centrifugal partition chromatography–liquid chromatography for the preparative isolation of antioxidants from Edelweiss plant

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1 **Two-dimensional multi-heart cutting CPC-LC for the preparative isolation of antioxidants**
2 **from Edelweiss plant**

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Abstract

The Edelweiss plant has been recognized as a very valuable source of anti-aging principles due to its composition of antioxidants compounds: leontopodic acid A and 3,5-dicaffeoylquinic acid. In this work, off-line multi-heart cutting CPC-LC separation was set up at industrial scale in order to isolate and produce new high quality reference material of these two antioxidants from Edelweiss. For this purpose, CPC and HPLC methods were developed and optimized at laboratory scale and a comprehensive CPCxHPLC analysis of the crude extract was established. Thereby, the CPC method led to a first separation of the target compounds according to their partition coefficient in the solvent system and the HPLC method was performed on the recovered fractions to lead to a second separation. A 2D CPCxHPLC plot was established in order to know the fractions to select at the industrial scale. Then, the CPC and HPLC methods were transferred at industrial scale and the multi-heart cutting CPC-LC was performed in off-line mode. Using CPC with methyl ter-butyl ether-water 1:1 (v/v) solvent system and LC with Denali C18 column, 2 g of crude extract sample were injected and leontopodic acid A and 3,5-dicaffeoylquinic acid were recovered with purity over 97%. The compounds were identified by MS and NMR.

25

1. Introduction

Leontopodium alpinium, commonly known as Edelweiss, is one of the most famous plants of the European Alps. In folk medicine, extracts of Edelweiss are used for the therapy of abdominal aches, angina, bronchitis, cancer, diarrhea, dysentery and fever for humans as well as for livestock [1, 2]. Indeed, this plant shows a wide diversity of secondary plant metabolites such as phenolic acids, lignans, flavonoids, sesquiterpenes, coumarins, benzofuran and others [3-5]. Wild Edelweiss is protected by the law but the plant is now cultivated in Switzerland and extracts of the aerial parts are used for their anti-oxidative properties [1, 3, 6]. From the early 2010's, the Edelweiss plant extracts have been recognized as a very valuable source of skin anti-aging principles by the cosmetic industry and the main actives substances are phenylpropanoids such as leontopodic acids [7, 8].

In order to improve clinical research and provide new high quality standards, the production of two antioxidants from Edelweiss plant is required, namely: leontopodic acid A and 3,5-dicaffeoylquinic acid. In recent years, Schwaiger et al. [9] and Ganzera et al. [10] developed different chromatographic methods for the identification and the quantification of major phenolic Edelweiss constituents such as high performance liquid chromatography and micellar electrokinetic capillary chromatography. The analysis exhibit the complexity of these plant extracts. However, currently no preparative assay of

42 these phenylpropanoids is available to isolate and produce the compounds of interest. As the plant
43 extract is complex, the isolation of compounds using preparative HPLC cannot be considered.

44 For the isolation and production of phenolic compounds and phenylpropanoids from natural products,
45 Countercurrent Chromatography (CCC) is widely used [11-14]. The countercurrent chromatography is
46 a liquid chromatography technique that uses two immiscible liquid phases without any solid support.
47 This technique eliminates irreversible adsorptive loss of samples onto the solid support matrix used in
48 conventional chromatography and has the advantage to be versatile due to the huge choice of solvent
49 combinations. With a loadable stationary phase, CCC is a very interesting preparative separation
50 technique [15].

51 The purpose of the work is the purification and isolation of multiple targets from complex natural
52 product. For this purpose, a complementarity of techniques was developed in order to combine two
53 different selectivities through two different mechanisms of separation. A two-dimensional (2D)
54 chromatography methodology coupling independent separation techniques provides higher peak
55 capacity, resolution and selectivity. The countercurrent chromatography technique is set to be the first
56 dimensional separation due to the loadable stationary phase. The HPLC technique, more efficient, is
57 used as second dimensional separation. Several preparative applications using this strategy was
58 reported in these recent years, especially in natural products field. The hyphenation can be on-line and
59 comprehensive [16] with flow programming CCC [17], on-line and heart-cutting [18, 19] with stop-and-
60 go CCC [20] or off-line and heart-cutting [21].

61 In the present study, an off-line multi-heart-cutting hyphenation of countercurrent chromatography
62 using Centrifugal Partition Chromatography device (CPC) and preparative liquid chromatography was
63 established in order to produce the two antioxidants from the Edelweiss extract. The CPC and HPLC
64 methods were first developed separately at laboratory scale. Then the 2D hyphenation CPCxLC was
65 performed off-line in a comprehensive mode at laboratory scale. Finally, the methods were transferred
66 at industrial scale for the production of the two antioxidants by multi-heart-cutting CPC-LC meaning
67 that only the fractions of interest were sent from the CPC to the second LC dimension.

68

69 **2. Experimental**

70 *2.1. Chemicals and materials*

71 All solvents used for the preparation of the sample, the HPLC analysis and the CPC separation were of
72 analytical grade. Methyl ter-butyl ether was purchased from Acros Organics (Fisher Scientific, Illkirch,
73 France). HPLC grade solvents for HPLC were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier,
74 France).

75 The extract of the aerial part of Edelweiss was provided by the company Extrasynthese (Genay,
76 France). It had to be stored away from light at -20°C in a freezer to avoid any degradation. Leontopodic
77 acid A and 3,5-dicaffeoylquinic acid authentic samples were provided by Prof. Schwaiger (Innsbruck
78 University, Austria).

79

80 *2.2. Instrumentation*

81 All instruments used in this study are commercially available.

The CPC instrument employed at the laboratory scale was the FCPC-C from Kromaton Rousselet-Robatel (Annonay, France) with interchangeable columns (or rotors). The column had an exact volume of 34.5 ml. The apparatus was equipped with a Shimadzu preparative pump LC-20AP (Noisiel, France), a Shimadzu UV/VIS detector SPD-20A set up at 220 nm, a manual sample injection valve with a 350 µL sample loop and a fraction collector. The rotor was thermostated at 21°C. The data were collected with Azur software provided by Datalys (Le Touvet, France).

The CPC instrument employed at industrial scale was the SCPC-1000 from Armen Instrument (Saint-Avé, France). The column had an exact volume of 1.088 liters. The apparatus was equipped with a Spot Prep II integrated system from Armen Instrument. This equipment is the assembly of a quaternary pump, an automatic sample injection valve fitted on a 20 ml sample loop, a UV/VIS spectrophotometer dual wavelength set up at 220 nm and 330 nm and a fraction collector. The Armen Glider Prep software installed in the integrated computer allowed the control of the apparatus and the data acquisition.

The HPLC system used at laboratory scale as second dimension was an Alliance 2690 system from Waters (Saint-Quentin-en-Yvelines, France) using a binary solvent delivery system, an autosampler and a Photodiode Array detector Waters 996 set up at 330 nm. A reversed-phase Grace Vydac Denali C18 column (150 mm x 4.6 mm i.d., 5 µm) was used as the second dimensional stationary phase. The data acquisition was performed by EmPower software from Waters.

The HPLC system used at preparative scale was composed of a Reveleris integrated system for injection, detection and collection, set up with 20 ml sample loop and a wavelength of 330 nm. A reversed-phase Grace Vydac Denali C18 column (300 mm x 50 mm i.d., 10 µm) was used as the stationary phase.

All the analysis for the methods development and the fractions control were performed using an Acquity Ultra Performance Liquid Chromatography system from Waters. The system was equipped with a binary solvent delivery system, an autosampler and a Photodiode Array detector set up at 330 nm. The data acquisition was performed by EmPower software from Waters. A reversed-phase Acquity UPLC CSH Phenyl-Hexyl column (100 mm x 2.1 mm i.d., 1.7 µm) was used as stationary phase.

2.3. Selection of CPC solvent system

The solvent system was selected according to the partition coefficient (K_D) of each target component to separate: leontopodic acid A et 3,5-dicaffeoylquinic acid. The K_D value was measured by HPLC analysis using the Shake-Flask methodology. A suitable amount of crude extract was added to a test tube and 2 ml of each of the equilibrated two-phase solvents was added. The tube was shaken vigorously to equilibrate the compounds between the two phases. After partition equilibrium the upper and lower phases were separately taken into vials and 20 µl of each phase was analyzed by HPLC-UV. The peak area of the target compound in the upper phase was recorded as A_{upper} and the one in the lower phase as A_{lower}. The K_D value was calculated according to the following equation: $K_D = A_{upper}/A_{lower}$.

2.4. Preparation of two-phase solvent system and sample solutions

A two-phase solvent system composed of methyl ter-butyl ether-water pH 3 at a ratio of 1:1 (v/v) was developed. At the laboratory, according to the selected ratio, 1000 ml solvent system was thoroughly

123 equilibrated in 1 l bottle at room temperature for 10 min. At industrial scale, 8 l of solvent system was
124 prepared in a 10 l bottle and directly used in CPC.

125 The sample solution for UHPLC analysis of crude extract was prepared by dissolving 10 mg of crude
126 extract in 1 ml of water-acetonitrile 1:1 (v/v).

127 For the CPCxLC method development at laboratory scale, 1, 2, 20 and 50 mg of crude extract was
128 dissolved in 1 ml of methyl ter-butyl ether. The solutions were vortexed for 4 min then sonicated for
129 20 min and centrifuged for 20 min at 4000 rpm. The supernatant was taken and 350 µl was injected.
130 Finally, for the developed CPCxLC hyphenation at laboratory scale, the sample solution was prepared
131 by dissolving 50 mg of crude extract in 1 ml of methyl ter-butyl ether. For the HPLC method
132 development at laboratory scale, 10 mg of crude extract was dissolved in 2 ml of water-acetonitrile
133 80:20 (v/v) and then filtrated.

134 For the CPC-LC hyphenation at industrial scale, the sample solution was prepared by dissolving 2 g of
135 crude extract into 40 ml of total solvent system (1:1 v/v). The whole upper phase was injected in CPC
136 which correspond to a 20 ml sample solution.

137

138 *2.5. Optimization of CPC separation at laboratory scale*

139 The CPC column was first filled with the upper phase as stationary phase. The rotation of the column
140 was started at the minimum speed 600 rpm and gradually increased to 1720 rpm. The lower phase as
141 mobile phase was pumped into the column at a flow-rate of 5 ml/min in descending mode, leading to
142 a stationary phase volume ratio (Sf) of 60%. When the hydrodynamic equilibrium was established in
143 the column and the mobile phase started emerging in the effluent (P = 60 bars), the sample solution
144 was injected through the injection valve. The effluent was continuously monitored with a UV detector
145 at 220 nm. The total run time was 40 min. The fractions were collected every minute with the fraction
146 collector which correspond to a recovery of 40 fractions of 5 ml. All fractions were put in 1 ml vial for
147 UHPLC analysis to identify peak compounds.

148

149 *2.6. Optimization of HPLC separation as second dimension*

150 A reversed-phase Grace Vydac Denali C18 column (150 mm x 4.6 mm i.d., 5 µm) was used as the second
151 dimensional stationary phase. This column was a strategic choice for the transfer at industrial scale.
152 The mobile phase was composed of water-acetonitrile with 0.1% of formic acid under isocratic elution
153 mode. The flow-rate of the mobile phase was 1 ml/min. All effluents trough the HPLC column were
154 monitored by a PDA detector at 330 nm. Data for two-dimensional plots were processed with Matlab
155 software.

156

157 *2.7. Transfer of off-line 2D CPC-LC separation to industrial scale*

158 The CPC column was first filled with the upper phase as stationary phase. The rotation of the column
159 was performed at 900 rpm. The column was equilibrated by pumping a ratio of 60:40 (upper
160 phase:lower phase v/v) of the system solvent at a flow-rate of 55 ml/min in descending mode. This
161 solvent system introduction sets the stationary phase volume ratio at 60% while saving time and
162 solvent consumption. When the hydrodynamic equilibrium was established in the column and the
163 mobile phase started emerging in the effluent (P = 70 bars), 20 ml of the sample solution was injected

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through the injection valve. The effluent was continuously monitored with a UV detector at 220 nm and 330 nm. The total run time was 120 min. The fractions were collected every 0.41 minute with the fraction collector which correspond to a recovery of 287 fractions of 23 ml each.

The fractions corresponding to leontopodic acid A (compound B) were gathered from the middle of the rise to the middle of the descent of the peak. The same gathering was performed for 3,5-dicaffeoylquinic acid (compound A). Thereby two main fractions were recovered: one for leontopodic acid A called F1 (F1 = 450 mg) and one for 3,5-dicaffeoylquinic acid called F2 (F2 = 180 mg). The analysis of the fractions were carried out using UHPLC. The two recovered fractions F1 and F2, treated with acidic methyl ter-butyl ether and evaporated to dryness, were dissolved into 8 ml of the LC mobile phase to be sent to the second dimensional separation. A reversed-phase Grace Vydac Denali C18 column (300 mm x 50 mm i.d., 10 μm) was used as the stationary phase. The mobile phase was composed of water-acetonitrile with 0.1% of formic acid at a ratio of 75:25 (v/v) and 78:22 (v/v) under isocratic elution mode for leontopodic acid A and 3,5-dicaffeoylquinic acid fractions, respectively. The flow-rate of the mobile phase was 110 ml/min. All effluents through the HPLC column were monitored by a PDA detector at 220 nm and 330 nm. The total runs were 20 minutes.

The HPLC fractionation was determined in order to eliminate impurities in the head and the tail of the peaks. Two final fractions were recovered: the fraction F3 gathering the fractions of interest of leontopodic acid A peak (F3 = 298.5 mg) and the fraction F4 gathering the fractions of interest of 3,5-dicaffeoylquinic acid peak (F4 = 44.6 mg). The two final fractions, F3 and F4, were concentrated then extracted with acidic methyl ter-butyl ether and finally evaporated to dryness for UHPLC assessments.

2.8. UHPLC analysis of crude extract and purified fragments

The crude Edelweiss extract sample, the fractions collected from the CPC separations during development, the two intermediate fractions F1 and F2 collected from industrial CPC and the final products from fractions F3 and F4 were analyzed using the Acquity Ultra Performance Liquid Chromatography system from Waters. The analysis were carried out on an Acquity UPLC CSH Phenyl-Hexyl column (100 mm x 2.1 mm i.d., 1.7 μm) from Waters. The mobile phase used was water + 0.1% of formic acid (A) and acetonitrile + 0.1% of formic acid (B) in a gradient mode as following: B at 5% for 0.29 min, B from 5% to 38% in 17.15 min, B at 38% for 0.52 min, B from 38 to 80% in 0.52 min, B at 80% for 0.52 min, B from 80 to 5% in 0.52 min and B at 5% for 1.56 min. The total elution time was 21.08 minutes. The flow-rate of the mobile phase was 0.4 ml/min and the effluents were monitored at 330 nm by a DAD detector.

2.9. Structure identification of components

Identification of the compounds was carried out by ESI-MS in negative mode and ^{1}H, ^{13}C NMR. For the ESI-MS analysis, the compounds were directly introduced in a SQ Detector 2 from Waters. For 3,5-dicaffeoylquinic acid, the MS measurements were made using a 0.85 kV capillary voltage, 47 V cone voltage and a source temperature of 500°C. For leontopodic acid A analysis, the MS measurements were made using a 2.02 kV capillary voltage, a 32 V cone voltage and a source temperature of 500°C.

A Bruker apparatus was used for 1D and 2D NMR identifications of the 3,5-dicaffeoylquinic acid (^{1}H set up at 400.13 MHz and ^{13}C set up at 100.6 MHz) and the leontopodic acid A (^{1}H set up at 500.13 MHz and ^{13}C set up at 125.77 MHz) recovered in CD$_3$OD for ^{1}H identification and D$_2$O for ^{13}C identification.

3. Results and discussion

The Edelweiss plant contains a lot of compounds which represents a complex matrix. In order to extract the compounds of interest from the plant according to the desired application, the crude sample undergo an extraction. Thus, the obtain extract is a simplified sample of the crude sample but still complex regarding the number of compounds and the close chemical structures. Thereby, whatever the extraction solvent, the crude extract remains complex and this complexity depends on the way the extraction is performed.

3.1. UHPLC analysis of the crude extract

As shown in Fig. 1, the UHPLC analysis of the crude extract indicates that there are several minor compounds with some major compounds including the two compounds of interest. This chromatogram shows the complexity of the sample extract. The peaks are in the same time retention window which means that they have relatively close chemical structures. Furthermore, the resolution of the separation is sufficient for analytical purpose while method and column are optimized. Unfortunately the isolation of the compounds of interest by HPLC at preparative scale cannot be considered due to insufficient resolution on available preparative stationary phase.

[Here Fig. 1]

In order to increase the selectivity and the peak capacity, a first dimensional CPC separation was performed on the crude extract. This technique brings a different selectivity than HPLC and can separate compounds in first dimension that could not be separated in the second dimension.

3.2. Solvent system selection for first dimensional CPC separation

Solute partition coefficient (K_D) and retention of the stationary phase are important factors for the selection of the best solvent system to be implemented in CPC method. In literature, three main criteria have been developed in order to select the suitable solvent system for effective separations [22]. First, the partition coefficient of the compounds should be within the range of $0.5 \leq K_D \leq 5$. With values under 0.5, the separation resolution can be poor and with values over 5, there is a risk of band broadening. Then, the separation factor α ($\alpha = K_2/K_1$) should be greater than 1.5 to obtain an effective separation. And finally, the retention of the stationary phase should be high to improve the resolution of the separation. Due to the high polarity of the target compounds, a series of polar solvent systems were tested. Table 1 shows the partition coefficients of the two target compounds in the different solvent systems.

[Here Table 1]

244 The K_D values of the other compounds are really close to 0 for each test tube (data not shown) meaning
245 that they are more soluble and have more partition in the mobile phase. Thus, the suitable solvent
246 system should be the one in which target compounds have K_D values different from 0. The solvent
247 system that meets the required specifications is methyl ter-butyl ether-water 1:1 (n°13) because the
248 target compounds have K_D values in the suitable range and different from 0 and the separation factor
249 is higher than 1.5.

250

251 *3.3. Optimization of CPC separation at laboratory scale*

252 Although the selection of the two-phase system is important, the flow-rate of the mobile phase and
253 the rotation speed also play critical roles in the separation process, especially with regard to retention
254 of the stationary phase. The lower phase was chosen as the mobile phase and was introduced through
255 the column. The flow-rate of the mobile phase was set up at 5 ml/min and the rotation speed was
256 performed at 1720 rpm. These optimized parameters were established to obtain the maximum
257 stationary phase retention without reaching the maximum threshold pressure which was 75 bars. The
258 solvent system methyl ter-butyl ether-water 1:1 generates a high pressure value due to the viscosity
259 of methyl ter-butyl ether. With this solvent system, a stable retention of 60% stationary phase was
260 obtained at 1720 rpm with a flow-rate of 5 ml/min providing a maximum pressure of 60 bars.

261

262

263 In order to inject the maximum amount of sample in CPC, the sample concentration was investigated.
264 Different sample concentrations were tested for the CPC separation, from 1 mg/ml to 50 mg/ml. The
265 injection volume was set up at 350 µl which represents 1% of the column volume. This volume was
266 found to be the compromise injection volume allowing the maximum volume injected while
267 maintaining a satisfactory resolution. If the injection volume is higher, the resolution can be lower due
268 to the peaks widening.

269 Chromatograms of CPC separations are shown in Fig. 2. For all separations, the quality of the
270 separation is still conserved. The concentration value was finally raised to 50 mg/ml which was the
271 sample solubility limit in the solvent. The chromatogram of the 50 mg/ml sample solution shows the
272 conservation of the quality separation as well as the conservation of the selectivity between the peaks
273 of interest while the return to baseline is not observed. This is not compulsory in two-dimensional
274 separation as we will discuss later on. For optimized CPC parameters, the sample concentration was
275 adjusted to 50 mg/ml.

276

277 [Here Fig. 2]

278

279 Through this investigation, CPC optimized parameters were settled as following: injection of 350 µl of
280 a 50 mg/ml centrifuged sample solution, on a methyl ter-butyl ether-water 1:1 (v/v) solvent system in
281 descending mode, rotation speed at 1750 rpm, flow-rate at 5 ml/min. As presented in Fig. 2,
282 leontopodic acid A elutes at 14.2 minutes which corresponds to K_D value around 2.8 and 3,5-
283 dicaffeoylquinic acid elutes at 26.7 minutes which corresponds to K_D value around 5.8. These values
284 are close to the expected values. The polar compounds, which had expected K_D values close to 0,
285 correspond to the two first peaks in Fig. 2 and have shorter retention times than leontopodic acid A as

286 expected. It can be noticed that selectivity in CPC is different than in HPLC for the two compounds of
287 interest since the elution order is switched as seen on Fig. 1 and Fig. 2.

288

289 *3.4. Development of HPLC separation as second dimension*

290 The second dimensional separation was performed on HPLC using Denali C18 column. This column was
291 chosen for industrial reasons. Therefore, the Denali C18 column was employed at laboratory and
292 industrial scales.

293 To develop this HPLC separation, two parameters were studied. The elution conditions were first
294 investigated in order to optimize the separation and obtain a satisfied selectivity between compounds
295 of interest without dispersed peaks. A volume of 5 µl of extract sample was injected to test the mobile
296 phase ratio of 75:25, 78:22 and 80:20 (v/v). Then, the injection volume of the sample was optimized
297 by testing injection volumes of 20, 50 and 100 µl.

298

299   *3.4.1. Elution conditions*

300 The settled mobile phase was water + 0.1% of formic acid and acetonitrile + 0.1% of formic acid under
301 isocratic elution. Three ratios of mobile phase were examined: 75:25, 78:22 and 80:20. The
302 chromatograms are shown in Fig. 3. A satisfying separation as second dimensional separation is a
303 separation which gives the desire selectivity while maintaining a satisfying efficiency. The ideal criteria
304 are a separation factor between peaks α higher than 1.5 and a peak width as small as possible to obtain
305 resolute peaks. In order to transfer the separation at industrial scale, peaks had to be separated with
306 high selectivity and require efficiency to be able to inject as much as possible and keep the return to
307 baseline between them.

308 On the chromatogram corresponding to the 75:25 mobile phase ratio in Fig. 3, peaks show a very
309 satisfying efficiency because the width of the peaks is really small. However, the selectivity between
310 peaks around 3,5-dicaffeoylquinic acid peak is too low for satisfying separation because there is no
311 return to baseline. The same observation is done for the leontopodic acid A peak. On the
312 chromatogram corresponding to the 78:22 mobile phase ratio in Fig. 3, the same observations can be
313 established even if the selectivity between peaks is a little higher but does not allow the baseline return
314 of the signal between peaks. The 80:20 ratio of the mobile phase was also tested to observe the
315 evolution of the resolution and the selectivity between peaks. As presented on the chromatogram in
316 Fig. 3, peaks retention shows the best selectivity between the peaks. However, the efficiency of the
317 separation is not satisfactory because the width of the target peaks is too high thus, when recovered,
318 the compounds can be mixed with another compounds due to the large width of the peak and the
319 compounds are dilute in a large volume of solvent. These conditions are not satisfactory conditions for
320 preparative purpose. Thereby, the best compromise to carry out second dimensional HPLC is the use
321 of 78:22 (v/v) mobile phase ratio. Indeed, the first dimensional CPC separation brings a first separation
322 of the compounds with a larger selectivity than HPLC separation. Thus, the efficiency of the two
323 methods are combined to offer a higher peak capacity. The return to baseline in separated methods is
324 not compulsory for two-dimensional separation due to the efficiency combination. Both methods have
325 to be a compromise for optimized parameters to satisfy the two-dimensional separation. Thereby, the
326 optimized second dimensional HPLC was performed at a mobile phase ratio of 78:22 at laboratory
327 scale.

328

329 [Here Fig. 3]

330

331 *3.4.2. Injection volume for second dimension usage*

332 During method development it is necessary to be able to observe the behavior of compounds of
333 interest as well as the neighboring impurities that may hinder their purification. Since CPC is a highly
334 diluting process, it is hence required to transfer to the subsequent LC separation a sufficient amount
335 of sample to monitor such impurities. Of course, at production scale, this concern does not apply as
336 the whole selected CPC fraction will be treated on the LC column.

337 In order to observe the maximum signal on HPLC chromatogram, and thus, try to see the minor
338 neighboring impurities of the compounds of interest, the CPC fraction containing the maximum
339 amount of compounds was injected in HPLC. This fraction corresponds to the fraction eluting at 14.2
340 minutes in Fig. 2, concentration at 50 mg/ml, which is the fraction of the peak top of leontopodic acid
341 A. Then, three different injection volumes were tested: 20 µl, 50 µl and 100 µl. The mobile phase was
342 set up at a 75:25 ratio for a gain of time. Using an injection volume of 20 µl gives a low peak intensity
343 which is not interesting for the laboratory scale analysis because minor compounds are not observed.
344 The injection volume of 100 µl gives a high peak intensity which is high for the HPLC detector and a
345 risk of signal saturation can occur. The injection volume of 50 µl gives peak intensity high enough to
346 hope to observe both peaks of interest and impurities. This HPLC injection volume was maintained for
347 CPCxLC separation at laboratory scale.

348

349 *3.5. Application of off-line 2D CPCxLC analysis at laboratory scale*

350 The optimized CPC and HPLC methods were carried out successively in a comprehensive way meaning
351 that fractions from CPC separation were collected every 1 minute and later injected in HPLC for second
352 dimensional separation (corresponding to a total of 40 HPLC analyses).

353 The first CPC dimension was set up with optimized conditions: methyl ter-butyl ether solvent system
354 1:1 (v/v), flow-rate of 5 ml/min, rotation speed of 1720 rpm, sample extract of 50 mg/ml prepared by
355 centrifugation and injection volume of 350 µl. The second LC dimension on Denali C18 column was set
356 up with optimized conditions: mobile phase of water + 0.1% of formic acid and acetonitrile + 0.1% of
357 formic acid at a ratio of 78:22, injection volume of 50 µl and flow-rate of 1 ml/min. The CPC
358 chromatogram and the LC chromatograms of the fractions were recorded. The 2D CPCxLC analysis was
359 plotted and the plot is shown in Fig. 4.

360 As seen on the figure, major compounds and minor compounds are present. This 2D plot allows the
361 well-understanding of the crude extract sample composition. Moreover, as said previously and as
362 expected, the efficiency of both methods are combined and the compounds can be separated through
363 the two-dimensional separation.

364 The goal of this 2D CPCxLC plot is to allow to select the first-dimension fractions containing the target
365 compounds that will be cut at industrial scale (blue squares on vertical chromatogram). The target
366 compounds, 3,5-dicaffeoylquinic acid (compound A) and leontopodic acid A (compound B), are
367 spotted on the map with the black squares. Then the corresponding fractions of interest to be
368 recovered in CPC and HPLC dimensions could be determined. Therefore, the cut of the target fractions
369 in the first dimensional CPC separation for industrial scale was decided through this map in order to
370 eliminate the impurities which could hinder the recuperation of the target compounds in second

371 dimensional HPLC separation. The collected fractionation in both dimensions was shown on Fig. 4
372 through the dotted lines.

373

374 [Here Fig. 4]

375

376 *3.6. Transfer of off-line 2D CPC-LC separation to industrial scale*

377 The CPC and HPLC methods were transferred at industrial scale. For first dimensional CPC separation,
378 the method transfer was made on a 1 l rotor. The solvent system was the same methyl ter-butyl ether-
379 water 1:1 (v/v) solvent system. The flow-rate of the mobile phase and the rotation speed were
380 optimized to maintain the same separation quality. Due to pressure limitation, the maximum rotation
381 speed reached was 900 rpm with a maximum flow-rate of 55 ml/min using a forced stationary phase
382 volume ratio of 60%. This compromise was chosen to obtain the higher efficiency with the shorter
383 elution time.

384 The maximum charge was examined and was limited by the solubility of the sample in the upper phase
385 of the solvent system. During the CPC run, the eluent was collected and fractionated every 0.41
386 minute. These fractions from the first dimensional CPC separation were gathered in two main fractions
387 as shown in Fig. 5 (vertical chromatogram). Two fractions were recovered from first dimension: one
388 fraction of leontopodic acid A called F1 and one fraction of 3,5-dicaffeoylquinic acid called F2. These
389 two fractions were sent to second dimensional LC separation after concentration, extraction and
390 evaporation.

391 In order to save solvent and time consumption, LC separation of F1 was accelerated by increasing the
392 eluent strength of the mobile phase. Thus the second dimensional LC separation for leontopodic acid
393 A fraction was carried out with a mobile phase ratio of 75:25 (v/v), as opposed to 78:22 (v/v) during
394 method development. Consequently, the impurities present on Fig. 4 before leontopodic acid A
395 (retention times of 5.5 min and 6.5 min on HPLC axis) are coeluted under the peak eluting at 8.5 min
396 on Fig. 5. The impurities present on Fig.4 after leontopodic acid A (retention time of 10 min on HPLC
397 axis) are in the tail of leontopodic acid A peak on Fig. 5.

398 For the 3,5-dicaffeoylquinic acid fraction, as the neighboring impurities are very close to the peak of
399 interest, the second dimensional LC separation was carried out with the initially selected mobile phase
400 ratio of 78:22 (v/v). Final fractions, F3 and F4, were recovered as shown in Fig. 5 (horizontal
401 chromatogram) by eliminating the tail and the end of the peaks of interest to recover compound with
402 a high purity value.[Here Fig. 5]

403 Indeed, the purity of the two target compounds is higher than 97% (estimated purity with peak area).
404 The chemical structures of those isolated compounds were identified as 3,5-dicaffeoylquinic acid and
405 leontopodic acid A by ESI-MS in negative mode and NMR [6, 23, 24] (MS and NMR spectra provided in
406 Supplementary Information). 3,5-dicaffeoylquinic acid was clearly identified through its ion $[M-H]^-$
407 m/z=514.99 and its ion fragment $[M-H]^-$-$C_9H_6O_3$ m/z=353.01. Moreover, the NMR spectra are coherent
408 with the known structure. Similarly, leontopodic acid A identity was confirmed through its ion $[M-H]^-$ m/z=781.28 and its ion fragments $[M-H]^-$-$C_9H_6O_3$ m/z=619.13 and $[M-H]^-$-$2xC_9H_6O_3$ m/z=457.07.
410 Moreover, the NMR spectra are coherent with the known structure.

411 From a 2g crude extract containing 172.5 mg of 3,5-dicaffeoylquinic acid and 345.9 mg of leontopodic
412 acid A, the CPC-LC process leads to a 3,5-dicaffeoylquinic acid fraction (44.6 mg, purity of 97%) and a

leontopodic acid A fraction (298.5 mg, purity of 97%). Because of the purity specifications thus a strict selection of fractionation, the recovery is low (25.1% for 3,5-dicaffeoylquinic acid and 83.7% for leontopodic acid A). The overall 2D CPC-LC process is able to produce new reference materials at a yield of 22mg/g of crude extract for 3,5-dicaffeoylquinic acid and 145mg/g of crude extract for leontopodic acid A.

4. Conclusion

In this work, a 2D comprehensive CPCxHPLC protocol was designed at laboratory scale to enhance capability and resolving power for the separation of Edelweiss plant extract. This protocol allowed to implement a multi-heart-cutting CPC-HPLC at industrial scale for the purification and the isolation of two antioxidants from Edelweiss plant extract as new reference materials. The CPC and HPLC methods were developed in parallel at laboratory scale and the off-line hyphenation was successfully performed to identify the antioxidants. Both methods were transferred at industrial scale and the separation quality was maintained. The CPC-LC protocol was successfully performed at industrial scale. A recovery of 83.7% and 25.1% were obtained for leontopodic acid A and 3,5-dicaffeoylquinic acid compounds, respectively, with a purity higher than 97%. The structures of the isolated compounds were confirmed by MS and 1D and 2D NMR.

To the best of our knowledge, this is the first report using the combination of CPC and HPLC for the production of leontopodic acid A and 3,5-dicaffeoylquinic acid. These compounds were simultaneously isolated at preparative scale for the first time.

The above results show that this off-line CPC-LC protocol is an effective and high-purity technique which has potential applications in preparative extraction and purification of multiple target components from complex natural products.

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Figure captions

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520 Fig. 1. UHPLC analysis of the crude extract (10 g/l) on Phenyl-Hexyl column (100 mm x 2.1 mm i.d., 1.7
521 μm), mobile phase of water + 0.1% of formic acid and acetonitrile + 0.1% of formic acid in gradient
522 mode, flow-rate at 0.4 ml/min, detection at 330 nm. Peaks A: 3,5-dicaffeoylquinic acid; B: leontopodic
523 acid A.

524 Fig. 2. CPC chromatograms of the crude extract solution (2, 20, 50 mg/ml in upper phase). Methyl ter-
525 butyl ether-water 1:1 (v/v) solvent system, flow-rate at 5 ml/min, 1720 rpm, detection at 220 nm.
526 Peaks A: 3,5-dicaffeoylquinic acid; B: leontopodic acid A.

527 Fig. 3. HPLC chromatograms of the crude extract (5 g/l) on Grace Vydac Denali C18 column (150 mm x
528 4.6 mm i.d., 5 μm), mobile phase of water + 0.1% of formic acid and acetonitrile + 0.1% of formic acid
529 under isocratic elution at a ratio of 75:25, 78:22 or 80:20 (v/v), injection volume of 5 μl, flow-rate at 1
530 ml/min and detection at 330 nm. Peaks A: 3,5-dicaffeoylquinic acid; B: leontopodic acid A.

531 Fig. 4. 2D plot of the CPCxLC analysis of the crude extract. CPC parameters: 50 mg/ml centrifuged
532 extract sample, injection volume of 350 μl, methyl ter-butyl ether 1:1 (v/v) solvent system, flow-rate
533 of 5 ml/min, 1720 rpm and detection at 220 nm. LC parameters: Grace Vydac Denali C18 column (150
534 mm x 4.6 mm i.d., 5 μm), mobile phase of water + 0.1% of formic acid and acetonitrile + 0.1% of formic
535 acid (78:22 v/v), injection volume of 50 μl, flow-rate of 1 ml/min and detection at 330 nm. Peaks A:
536 3,5-dicaffeoylquinic acid; B: leontopodic acid A.

537 Fig. 5. Industrial CPC-LC separation of Edelweiss crude extract. CPC protocol: methyl ter-butyl ether
538 solvent system 1:1 (v/v), 2 g of crude extract in 40 ml of solvent system (1:1), injection of upper
539 phase, flow-rate of 55 ml/min, 900 rpm and detection at 220 nm. LC protocol: Grace Vydac Denali
540 C18 column (300 mm x 50 mm i.d., 10 μm), mobile phase of water-acetonitrile with 0.1% of formic
541 acid at 75:25 (v/v) for F1 fraction and 78:22 (v/v) for F2 fraction, flow-rate of 110 ml/min, detection
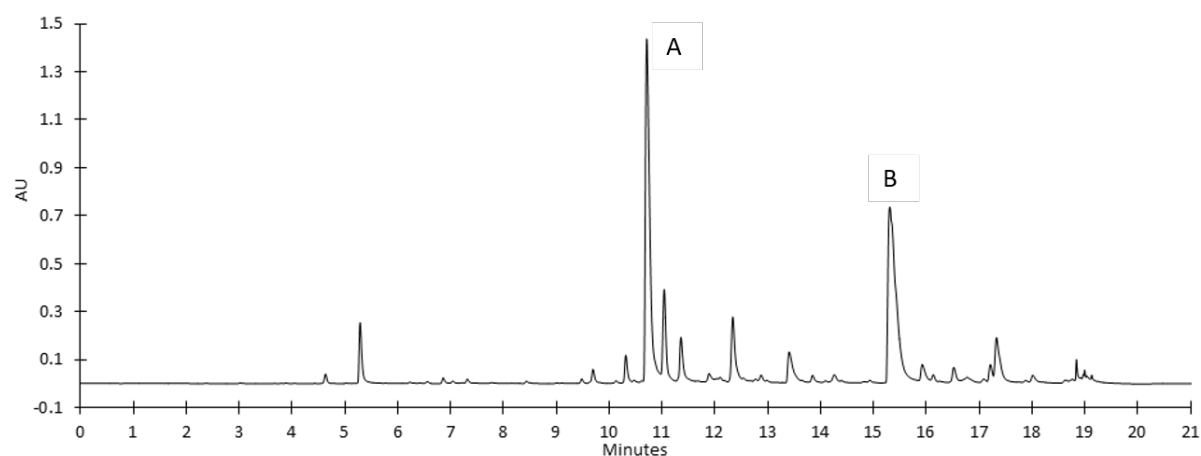542 at 330 nm.

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Fig. 1. UHPLC analysis of the crude extract (10 g/l) on Phenyl-Hexyl column (100 mm x 2.1 mm i.d., 1.7 µm), mobile phase of water + 0.1% of formic acid and acetonitrile + 0.1% of formic acid in gradient mode, flow-rate at 0.4 ml/min, detection at 330 nm. Peaks A: 3,5-dicaffeoylquinic acid; B: leontopodic acid A.
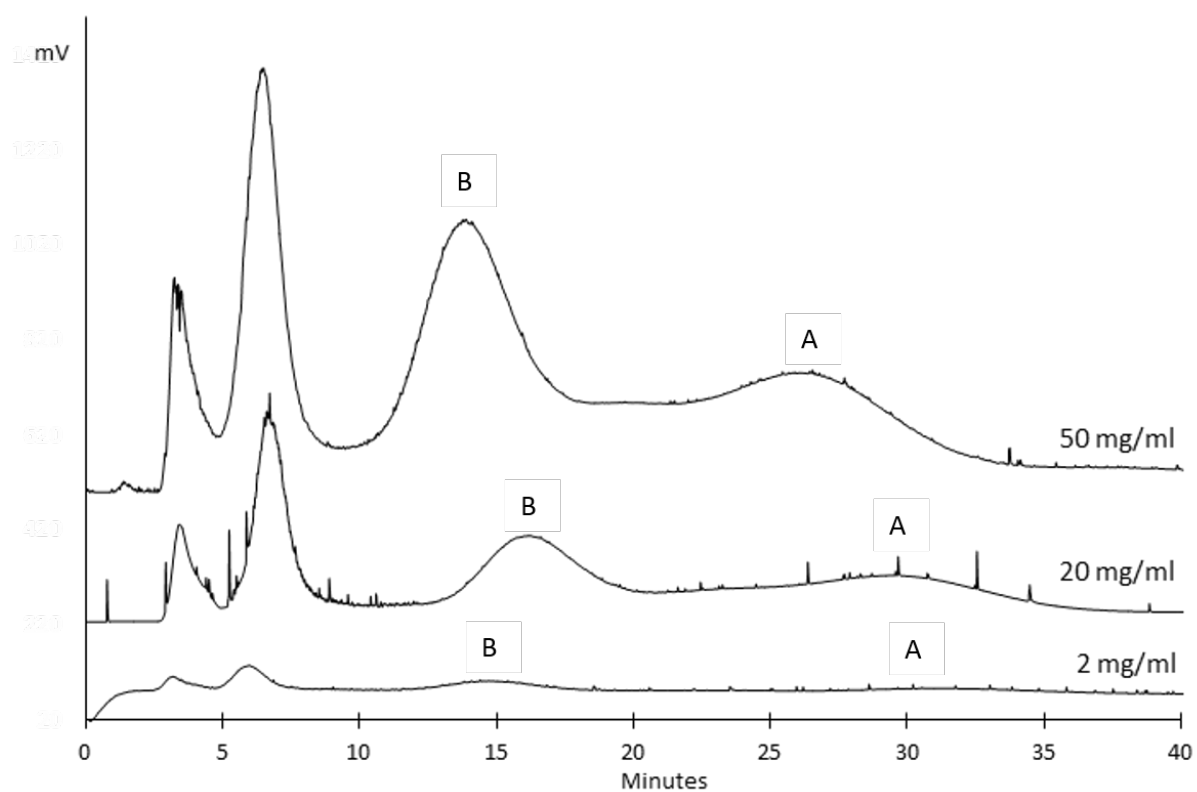
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559 Fig. 2. CPC chromatograms of the crude extract solution (2, 20, 50 mg/ml in upper phase). Methyl ter-
560 butyl ether-water 1:1 (v/v) solvent system, flow-rate at 5 ml/min, 1720 rpm, detection at 220 nm.
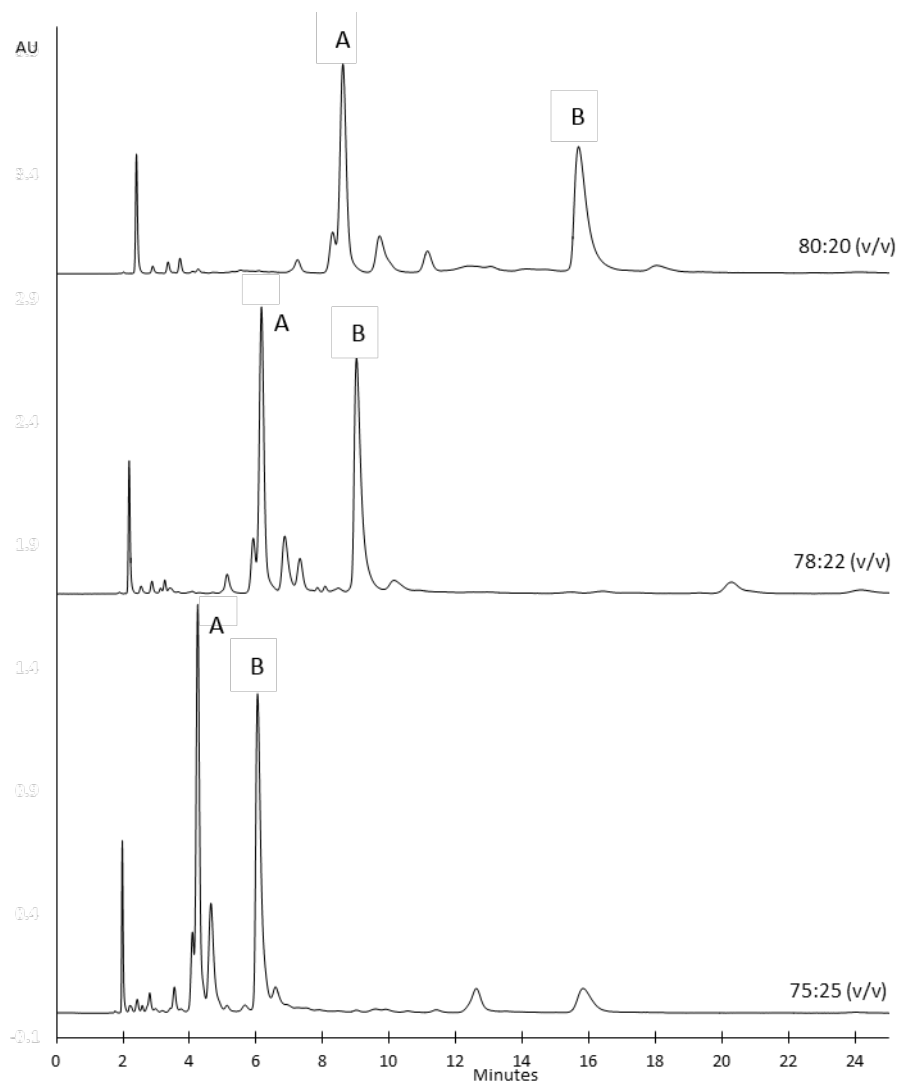561 Peaks A: 3,5-dicaffeoylquinic acid; B: leontopodic acid A.

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Fig. 3. HPLC chromatograms of the crude extract (5 g/l) on Grace Vydac Denali C18 column (150 mm x 4.6 mm i.d., 5 μm), mobile phase of water + 0.1% of formic acid and acetonitrile + 0.1% of formic acid under isocratic elution at a ratio of 75:25, 78:22 or 80:20 (v/v), injection volume of 5 μl, flow-rate at 1 ml/min and detection at 330 nm. Peaks A: 3,5-dicaffeoylquinic acid; B: leontopodic acid A.
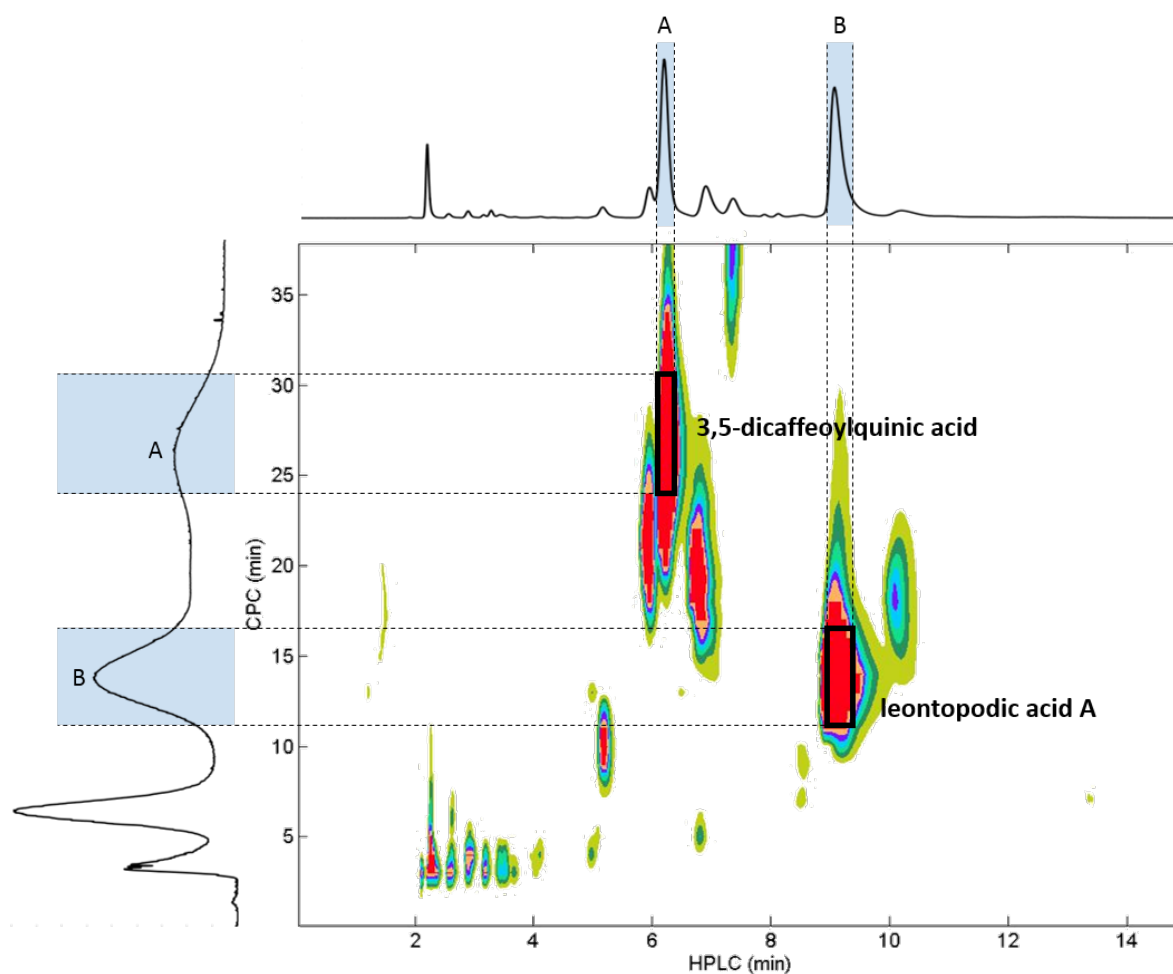
Fig. 4. 2D plot of the CPCxLC analysis of the crude extract. CPC parameters: 50 mg/ml centrifuged extract sample, injection volume of 350 µl, methyl ter-butyl ether 1:1 (v/v) solvent system, flow-rate of 5 ml/min, 1720 rpm and detection at 220 nm. LC parameters: Grace Vydac Denali C18 column (150 mm x 4.6 mm i.d., 5 µm), mobile phase of water + 0.1% of formic acid and acetonitrile + 0.1% of formic acid (78:22 v/v), injection volume of 50 µl, flow-rate of 1 ml/min and detection at 330 nm. Peaks A: 3,5-dicaffeoylquinic acid; B: leontopodic acid A.

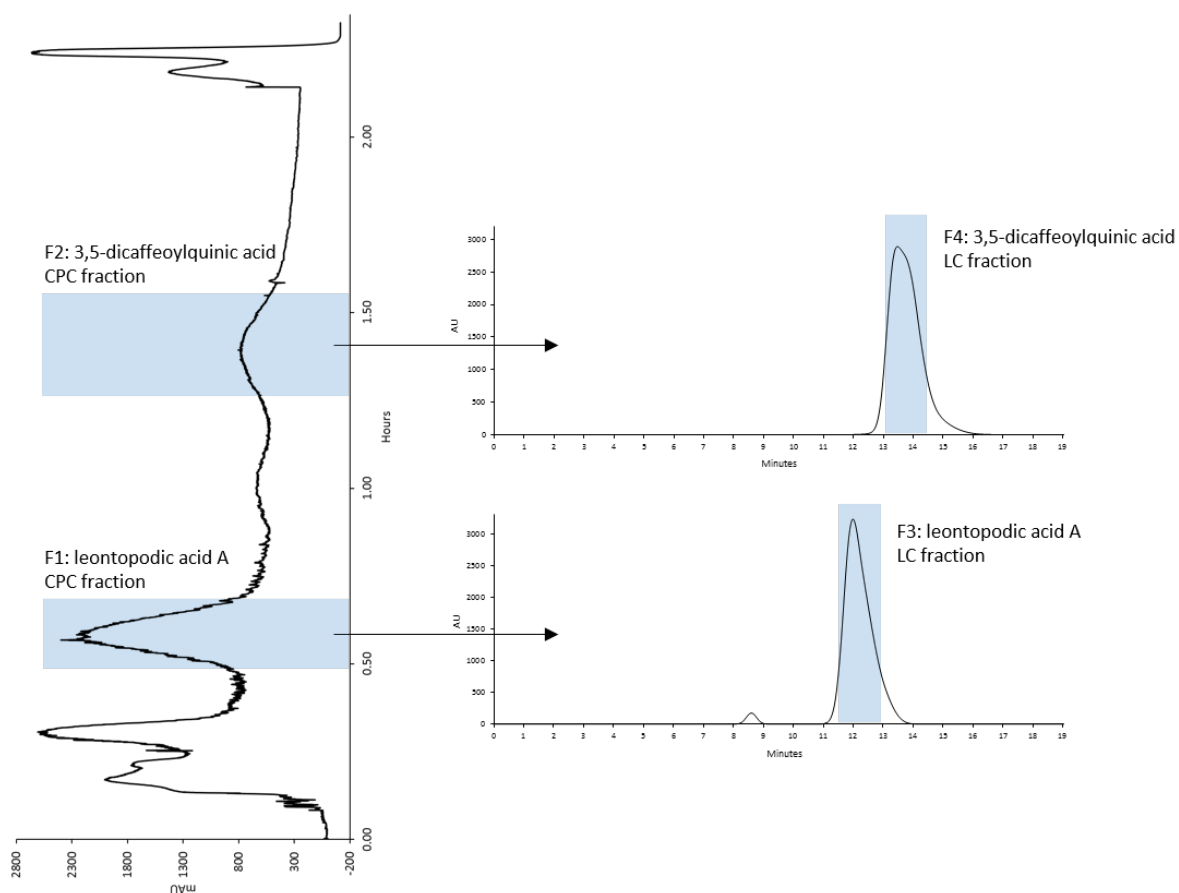*1st dimensional CPC*   *2nd dimensional LC*

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582   Fig. 5. Industrial CPC-LC separation of Edelweiss crude extract. CPC protocol: methyl ter-butyl ether
583   solvent system 1:1 (v/v), 2 g of crude extract in 40 ml of solvent system (1:1), injection of upper phase,
584   flow-rate of 55 ml/min, 900 rpm and detection at 220 nm. LC protocol: Grace Vydac Denali C18 column
585   (300 mm x 50 mm i.d., 10 μm), mobile phase of water-acetonitrile with 0.1% of formic acid at 75:25
586   (v/v) for F1 fraction and 78:22 (v/v) for F2 fraction, flow-rate of 110 ml/min, detection at 330 nm.

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