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1 Purification of drug degradation products supported by analytical and 2 preparative supercritical fluid chromatography

3

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14

15 ABSTRACT

16

17 A stressed degradation (oxidation) was employed to produce metabolites from an active
18 pharmaceutical ingredient (API) with large molecular weight (about 900 g/mol). An analytical
19 chromatographic method was desired to compare the products generated by different degradation
20 methods while a multi-gram-scale preparative chromatographic method was necessary to purify the
21 produced metabolites. Supercritical fluid chromatography (SFC) was selected for both tasks as no
22 other chromatographic method had achieved the resolution of the API and metabolites (two isomeric
23 mono-oxide species and one di-oxide). First, an analytical-scale method was developed with ultra-high
24 performance supercritical fluid chromatography (UHPSFC). Achiral stationary phases containing sub-2
25 µm fully porous particles or sub-3 µm superficially porous particles, and chiral phases containing 3 and
26 5 µm fully porous particles were selected for a first screening with gradient elution (carbon dioxide –
27 methanol containing additives). The stationary phase providing the most promising results was
28 ACQUITY Torus 2-PIC (100 x 3 mm, 1.7 µm, Waters). A central composite design (CCD) was
29 conducted to optimize the gradient program and oven temperature. Final gradient conditions were as
30 follows: 50 to 70 % methanol in 3.8 min with oven temperature set at 36°C, back-pressure set at 11
31 MPa and flow-rate at 0.8 mL/min. The optimized method was employed to analyze samples obtained
32 with different degradation conditions. Then the method was adapted and transferred to preparative-
33 scale SFC on a 5 µm-particles Torus 2-PIC stationary phase (150 x 30 mm). The method was
34 modified to comprise an isocratic step followed by a gradient, favoring peak shape of the last eluting
35 compound and minimal volume of collected fractions. Batch injections in gradient mode were carried
36 out to purify six grams of crude product.

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38 **Keywords:** Degradation products; Supercritical fluid chromatography; Preparative SFC; Enhanced
39 Fluidity Liquid Chromatography

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Abbreviations:

ADMET	Absorption, distribution, metabolism, excretion, toxicity
API	Active pharmaceutical ingredient
CCD	Central composite design
DoE	Design of experiments
EFLC	Enhanced fluidity liquid chromatography
EtOH	Ethanol
HPLC	High-performance liquid chromatography
iPrOH	Isopropanol
MeOH	Methanol
SFC	Supercritical fluid chromatography
UHPSFC	Ultra-high performance supercritical fluid chromatography

1. Introduction

In the course of drug development, impurities, degradation products and possible metabolites [1] must be determined and purified in order to assess their toxicity for the human body. Degradation under stressed conditions are part of stability testing [2], governed by FDA and ICH guidelines [3]. The aim is to understand how a drug product will vary with time, using extreme conditions to mimic different climatic areas [4]. This degradation study may also have the purpose of producing metabolites observed in in-vivo degradations (or metabolism) when absorbing the drug, as part of the ADMET parameters (absorption, distribution, metabolism, excretion and toxicity) [5]. To identify degradation products or metabolites, high-performance liquid chromatography (HPLC) is most widely used [6]. When the degradation products and metabolites are identified, it is also necessary to obtain them in sufficient quantities for activity testing. For this purpose [7], but also to produce pure fractions for identification [8] or to generate individual enantiomers [9], preparative chromatographic techniques are most useful.

Supercritical fluid chromatography (SFC) is close to HPLC except that the main component of mobile phase is not a liquid solvent but a compressible fluid. The mobile phase composition most frequently employed in SFC is composed of a mixture of pressurized carbon dioxide [10] and a short-chain alcohol like methanol [11,12]. CO₂ and the organic co-solvent may be mixed in all proportions, although most SFC applications report mobile phase compositions with a larger proportion of CO₂ than co-solvent. When the proportion of solvent is larger than the proportion of carbon dioxide, the technique is often called “enhanced-fluidity liquid chromatography” (EFLC) [13]. This type of mobile phase composition was mostly employed by the group of S. Olesik, typically to analyze polar, water-soluble compounds such as amino acids [14], nucleosides [15] or protein [16]. When the compounds

81 have moderate polarity, the simple use of polar co-solvents and additives is often sufficient to achieve
82 satisfying elution, as was already discussed by T.A. Berger long ago [17].

83
84 The principal advantages of SFC mobile phases compared to HPLC ones, are the low fluid viscosity
85 allowing for high efficiencies at high flow rates, and the green features of CO₂. D. Speybrouck and E.
86 Lipka even said that “greenness is not the icing on the cake but the cake itself” [18]. CO₂ is recyclable,
87 produced as a side-product from other industries, therefore has a low cost, is non-flammable and less
88 toxic than most other solvents employed in HPLC [10]. Most of these useful characteristics are
89 desirable, not only at the analytical scale but also at preparative scale, particularly for
90 enantioseparations [9,19]. When employing small-particle fully porous (sub-2 μm) or superficially
91 porous (sub-3 μm) stationary phases, combined with modern instruments offering reduced dead
92 volumes, SFC offers comparable features to ultra-high performance liquid chromatography (UHPLC)
93 and may be termed “ultra-high performance supercritical fluid chromatography” (UHPSFC). The
94 separations obtained with UHPSFC have high efficiency and can be obtained very rapidly, especially
95 as the low fluid viscosity is not causing so high pressures as in UHPLC.

96
97 In this project, the product of interest is an active pharmaceutical ingredient (API), potential future drug
98 (stage I of development) with rather large molecular weight (about 900 g/mol). This product was
99 subjected to stressful degradation, and oxidation in particular. Partial structures of the API and
100 oxidation products can be seen in Fig. 1, to observe the position of oxidation sites relative to the whole
101 molecule. Therefore, the objective of this project was to analyze the products of stressful degradation
102 and to obtain enough purified oxidized compounds for future activity testing. Preliminary experiments
103 had shown that liquid chromatography (in the reversed-phase or normal-phase modes) was
104 unsuccessful to achieve the resolution of the API from its oxidation products. Indeed, because the
105 molecule is rather large, it was difficult to solubilize and the structural difference caused by the
106 introduction of one or two oxygen atoms in the large molecular structure was very small and difficult to
107 resolve. Because SFC is known to be effective in the separation of closely-related species [20,21], it
108 was investigated to achieve this task.

109 First, an analytical method was developed to analyze samples from different degradation experiments
110 conducted on the same active ingredient. The analytical conditions were optimized using a Design of
111 Experiments (DoE). This chemometric approach is very useful to determine the best experimental
112 parameters for an analysis. It allows evaluating combined interactions between parameters within a
113 short space of time (couple hours) [22]. The optimal analytical method then needed to be adapted and
114 scaled up to purify a few grams of the oxidized products. Robustness around the optimal conditions
115 (+/- 1%) was also examined to ensure a successful transfer [23].

116

117 **2. Material and methods**

118 **2.1. Chemicals and solvents**

119

120 The API and degradation products were produced at Servier Research laboratory in Orleans, France.
121 Their structure is confidential but their molecular weight is around 900 g/mol. Oxidative degradation
122 with hydrogen peroxide was carried out according to ICH guidance [3].

123

124 *At the University of Orleans, for the analytical-scale method development:*

125 Solvents employed were HPLC-grade methanol (MeOH), ethanol (EtOH) and isopropyl-alcohol
126 (iPrOH) provided by VWR (Fontenay-sous-Bois, France). Air Liquide (Paris, France) provided carbon
127 dioxide of industrial grade $\geq 99.7\%$. Ammonium acetate powder $\geq 98\%$ was supplied by Merck, formic
128 acid $\geq 99\%$ was obtained from VWR (Fontenay-sous-Bois, France) and an Elga Purelab Flex station
129 from Veolia (Antony, France) provided ultra-pure water.

130

131 *At Servier Research laboratories, for the preparative-scale method and analysis of fractions:*

132 Solvents used were MeOH and iPrOH for analysis-ACS-Reagent, provided by CARLO ERBA.
133 Honeywell supplied methylene chloride $> 99.9\%$ (used for product dissolution). Ammonium acetate
134 ($>98\%$) was obtained from ACROS and formic acid ($> 98\%$) from Merck. Messer provided carbon
135 dioxide ($\geq 99.995\%$) and ultra-pure water was obtained with a MiliQ® - Advantage A10 system.

136

137 2.2. Instrumentation

138 2.2.1. UHPSFC system

139

140 The analytical system used for analytical method development and analysis of fractions was an
141 ACQUITY Ultra Performance Convergence Chromatography™ (UPC²®) from Waters Corporation
142 (Milford, MA, USA). It was equipped with a binary solvent delivery pump, compatible with mobile
143 phase flow rates up to 4 mL/min and pressures up to 414 bar. The system had an autosampler that
144 included partial loop volume injection system, a backpressure regulator and 2-position column oven
145 compatible with 150 mm length columns. Two detectors were available: a photodiode-array (PDA)
146 detector and an ACQUITY QDa® single-quadrupole mass spectrometric (MS) detector with
147 electrospray ionization source (ESI). An isocratic solvent manager was used as a make-up pump and
148 was positioned before the mass detector. The main flow stream was then split by the on-board-flow-
149 splitter assembly. With this system, a portion of the column flow goes to the backpressure regulator
150 and another (unknown) portion goes to the mass detector. Empower®3 software was used for system
151 control and data acquisition.

152

153 2.2.2. Preparative-scale SFC system

154

155 An SFC-PICLab PREP 200 from PIC Solution™ (Avignon, France) was employed for the purification
156 of oxidation products. It was a 200 ml/min preparative SFC system designed for 20 and 30 mm i.d.
157 columns. It could stand up to 350 bar and host five columns (or four columns plus bypass). Thanks to
158 a recycling system, and depending on co-solvent percentage, CO₂ should be retrained in supercritical
159 conditions (for instance, more than 95% of CO₂ is recycled with 10% of co-solvent). The system had

160 five collection tracks, of which one served for waste. SFC PICLAB PREP ONLINE was the software
161 used for system control and data acquisition.

162

163 2.3. Stationary phases

164

165 In order to determine a stationary phase to achieve the best resolution of API and degradation
166 products, fifteen commercial columns were selected: six chiral and nine achiral stationary phases.
167 Their characteristics are gathered in Table 1. Waters (Guyancourt, France), Phenomenex (Le Pecq,
168 France) Supelco, Thermo Fisher Scientific (Villebon, France), Macherey-Nagel (Hoerd, France) and
169 Chiral Technologies (Illkirch-Graffenstaden, France) kindly provided the columns.

170

171 2.4. Chromatographic conditions

172

173 The screening of the different columns was performed with a large elution gradient from 10 to 100% of
174 co-solvent in CO₂ in 15 min. Indeed, because the API and oxidation products were strongly retained, it
175 was necessary to elevate the proportion of co-solvent to a high percentage to elute them from the
176 columns. The co-solvent was MeOH containing 2% pure water and 20 mM ammonium acetate (AA).
177 This mobile phase composition was previously defined as being most favorable to the elution of drug
178 candidates with good peak shapes, along with good ESI-MS response [24]. The flow rate was set at
179 0.5 mL/min with sub-2 μm columns and 1 mL/min with other column dimensions, to avoid reaching the
180 upper pressure limit before the end of the gradient program. Temperature was set at 25°C and the
181 outlet pressure was fixed at 15 MPa.

182 Samples were prepared at 1 mg/mL in methanol and 2 μL were injected. The wavelength of UV
183 detection was set at 210 nm, with frequency set at 20 pts/s and resolution at 1.2 nm.

184 The mass detector unit was pre-optimized by the manufacturer. Ion source temperature was fixed at
185 120°C, probe temperature at 600°C, scan rate at 8 pts/s, capillary voltage at 0.8 kV, cone voltage at
186 15 V and sampling frequency at 8 Hz. Nebulizing gas was nitrogen. The make-up solvent composition
187 was 98% MeOH, 2% pure water plus 1% formic acid with a 0.45 mL/min flow. The analytes were
188 detected in positive and negative electrospray ionization mode (m/z 400-1000).

189

190 2.5. Mobile phase optimization

191

192 After selecting the best stationary phase from the screening experiments (ACQUITY Torus 2-PIC), a
193 central composite design (CCD) was conducted to optimize the gradient program. Flow-rate was set at
194 0.8 mL/min and backpressure at 11 MPa in order to not generate system overpressure. Because three
195 analytical parameters had to vary (final co-solvent percentage, gradient time and oven temperature),
196 the CCD contains a factorial design with $2^3 = 8$ points (angles of the cube), six star points and a central
197 point with six replicates to assess the experimental error. The distance between the center of the
198 design space and a factorial point was ± 1 unit whereas the distance between the center of the design
199 space and a star point is $\alpha = 2^{3/4} = 1.682$ with α is the isovariance criterion by rotation [25]. Therefore,

200 each of the three analytical parameters was explored at five levels: -1.682; -1; 0; +1; +1.682 (see
201 Table 2). The software used to assist in this work was JMP® trial version, (S.A.S Institute Inc, Cary,
202 NC, USA).

203
204 Judging from the screening experiments, the initial co-solvent percentage was fixed at 50%. Again this
205 unusual initial percentage is due to the high molecular weight (~900 g/mol) of the API and degradation
206 product. Overall, 20 experiments were conducted, in a random order generated by the software.

207
208 *Optimum calculation:*

209 For each experiment, the separation criterion (S) was used (instead of resolution) to assess the
210 separation quality of two successive peaks [26]. It is defined as the difference between the retention
211 time at the beginning of the $n+1$ peak ($t_{B\ n+1}$) and the retention time at the end of the n peak ($t_{E\ n}$).
212 Peaks are baseline resolved if S is positive.

$$213$$
$$214 S = t_{B\ n+1} - t_{E\ n} \quad (1)$$
$$215$$

216 When the optimal point was found, because the result was still not considered sufficiently good, the
217 separation was further improved with the modification of co-solvent nature (MeOH and iPrOH). The
218 final mobile phase co-solvent was MeOH-iPrOH 70:30 (v/v), comprising 20 mM ammonium acetate
219 and 2% water.

220
221 Then, the robustness in optimized conditions was explored with variations of each of the three
222 optimized parameters (final solvent percentage, gradient time, temperature) of more or less 1%. Thus
223 six additional experiments were done to assess the changes caused by small variations around the
224 optimal point. The experiments and results are presented in Tables S1 and S2, in supplementary
225 material.

226

227

228 2.6. Purification conditions in preparative SFC

229

230 The SFC-PICLab PREP 200 was used to purify the active pharmaceutical ingredient (API) from its
231 oxidized degradation products. ACQUITY UPC² Torus 2-PIC column (150 x 30 mm, 5 μ m) was
232 employed and the mobile phase composition was the one optimized at the analytical scale. In
233 preparative SFC, stacked injection in isocratic elution mode is preferred to increase productivity [9]. In
234 addition, the lowest proportion of solvent is desirable to limit solvent consumption and evaporation
235 time. Therefore, methods in isocratic elution mode were also tested with 40%, 45%, 50%, 55% and
236 60% of co-solvent. It turned out that a rather good separation with poor peak shapes were obtained
237 with 45% of co-solvent in 15 minutes. However, in the present case, the last peak (di-oxidized
238 compound) showed a significantly tailing peak, probably due to the low solubility of this compound. In
239 order to improve the peak shape and minimize the volume of collected fractions for this peak, a

240 second gradient program was designed, which prevented the use of stacked injections. First, the
241 program included an isocratic step with 45% co-solvent during 10 min. Then, the co-solvent proportion
242 was raised to 65% in 1 min and maintained during 5 min to ensure the elution of the last compound.
243 Thanks to high diffusivities in the mobile phase, due to the presence of a large portion of pressurized
244 carbon dioxide, the column was reconditioned at 45% for only 4 min before the next injection. Cycle
245 time was then 18.1 min. The flow rate was set at 100 mL/min, oven temperature at 40 °C, outlet
246 pressure at 10 MPa and UV detection at 210 nm. Six grams of crude product were dissolved in 15 mL
247 methylene chloride. The injection volume was 300 µL; the injection lasted 10 s.

248

249 *Fractions quality control:*

250 The solvent from the collected fractions was evaporated with a rotary evaporator and the dry residue
251 was weighted. For purity analysis, ACQUITY UPC² system was used with Acquity Torus 2-PIC
252 (150x4.6 mm, 5 µm) column. The mobile phase was the same as used for compounds purification.
253 The elution gradient profile was: 55% co-solvent from 0 to 5 min, then an increase to 70% from 5 to 10
254 min and finally re-equilibration at 55% in 0.1 min for a 12 min total run. Flow rate was 2.5 mL/min,
255 oven temperature was fixed at 35 °C and a volume of 3 µL was injected. Detection wavelength was
256 set at 290 nm (instead of 210 nm) because no baseline drift appeared at this wavelength when
257 increasing co-solvent percentage from 55% to 70%. Mass detection was carried out by the QDa[®] with
258 the same previous settings except for the mass range (from 150-700 m/z). Make-up solvent for mass
259 analysis was MeOH/water (90/10) v/v with 0.1% formic acid. Flow rate was 0.5 mL/min.

260

261 **3. Results and discussion**

262 3.1. Stationary phase selection

263

264 Fifteen columns were selected for the initial screening (Table 1). Stationary phases with small particle
265 size (fully porous sub-2 µm or superficially porous 3-µm) were favored, apart from some chiral phases
266 that were not available with such dimensions. Although no chirality is involved in the desired
267 separation, both achiral and chiral stationary phases were selected, as chiral phases are also
268 interesting to separate analytes with small structural differences [27]. The columns selected cover a
269 wide range of selectivities, primarily for polar-type interactions as the separation process should target
270 the number and position of oxygen atoms from the oxidation process. Indeed, it was expected that
271 positional isomers of oxidation products would be produced by the forced degradation. The diversity of
272 selectivities in the chosen columns can be observed from the spider diagram [28] in Figure 2.

273

274 The aim of this first screening step was to find one stationary phase that would provide the best
275 separation of the API and all degradation products while maintaining a percentage of co-solvent as
276 low as possible. Indeed, while a large proportion of co-solvent is not causing any issue at the
277 analytical scale, it is not desirable for preparative-scale to have a large proportion of co-solvent
278 because the solvent consumption and waste would rise, and the volume of solvent to evaporate from
279 collected fractions would be unreasonable and time-consuming. At this stage, both UV and mass

280 spectrometric (MS) detection were employed: UV served at observing resolution between all species
281 in the samples while MS served at identifying the target analytes (API and oxidation products). Indeed,
282 no standard was available to compare to the samples, so the MS spectra were examined. In addition,
283 the UV chromatograms shows additional peaks that need not be purified.

284 To compare the fifteen different stationary phases, a gradient elution running from 10 to 100% co-
285 solvent was employed. This wide gradient thus comprises the usual domains of SFC and EFLC. The
286 API elution composition (C_e) was calculated according to Eq. 2 and corresponds to the co-solvent
287 percentage required to elute the compound of interest [29].

288

$$289 \quad C_e = C_i + \frac{(C_f - C_i)}{t_G} * (t_R - t_D) \quad (2)$$

290

291 C_i and C_f are respectively the initial and final compositions of the gradient, t_G is the gradient time, t_R is
292 the retention time of the compound and t_D is the system dwell time (0.46 min in our case).

293

294 As a result of the columns screening (chromatograms in electronic supplementary information, Figure
295 S1), with gradient conditions from 10 to 100% co-solvent, all columns yielding excessive retention
296 (elution composition of API above 50% co-solvent) were excluded: Acquity UPC² HSS C18 SB,
297 Kinetex HILIC, Accucore HILIC, Accucore Amide HILIC, Accucore Urea HILIC, Ascentis Express OH5,
298 Chiralpak IA, IB, IC, ID, IF and IG. The oxidation products were all more retained than the API, and
299 sometimes some of them did not elute at all.

300 The next step was to look at the number of detected peaks and the separation quality. At this stage,
301 Ascentis Express F5 was excluded because the peaks were broad. ACQUITY UPC² Torus 2-PIC from
302 Waters and Nucleoshell HILIC from Macherey-Nagel were the two stationary phases promising the
303 best results in terms of separation optimization (this is visible in the UV chromatograms in electronic
304 supplementary information, Figure S1). However, the gradient profile was not satisfactory with the
305 Nucleoshell HILIC column because the ammonium acetate breakthrough curve caused a bump in the
306 baseline that happened at the same time as one target peak. Indeed, the additives (ammonium
307 acetate, water) are present at a low but increasing concentration in the mobile phase. At the beginning
308 of the gradient, the additives adsorb on the stationary phase as any other mobile phase component.
309 When the stationary phase is saturated with additives, a small breakthrough curve is visible in the
310 baseline (gradient slope). On stationary phases where ionic interactions are strong (as is the case on
311 the sulfobetaine ligand in Nucleoshell HILIC column), the sudden rise of ammonium acetate
312 concentration in the mobile phase is usually causing the grouped elution of ionic analytes, causing a
313 loss of resolution. Also, because of the highest concentration of ammonium acetate at this moment, it
314 is undesirable to collect a fraction at that time, thus the ACQUITY UPC² Torus 2-PIC was retained as
315 the best column. Moreover, it was important that the selected column was available at preparative
316 scale.

317

318 **3.2. Design of experiments**

319

320 Twenty experiments were conducted according to the design of experiments (DoE) described in
321 experimental section to generate optimal gradient and temperature conditions. The whole set of DoE
322 experiments lasted around four hours, taking into account the column equilibration time. The best
323 resolution was desired, together with narrow and symmetric peaks. At the preparative scale, thin and
324 symmetric peaks essentially mean that the time window for fraction collection will be the lowest
325 possible, thereby ensuring more concentrate fractions and less solvent to evaporate to obtain the dry
326 product. Good resolution will also mean that the purity of the fractions collected shall be high.
327 The chromatogram in Fig. 3 represents the separation between the API and degradation products in
328 optimal analytical conditions determined by the DoE. The analytes were identified with the assistance
329 of ESI-MS detection. The first peak, with a 2 minutes retention time, was not interesting for this project
330 so it will not be further discussed. The second peak corresponded to the active pharmaceutical
331 ingredient with a molecular weight (M) near $M \sim 900$ g/mol. The third and fourth peaks were isomeric
332 mono-oxidized degradation products with $M+16$ molecular weight. The last peak had a $M+32$
333 molecular weight, corresponding to a di-oxidized degradation product.

334

335

336 To generate optimal conditions, five responses were selected and taken into account in the software.
337 Four separation criterions (S), calculated according to Eq. (1), in order to assess the separation quality
338 between the five main peaks, and the asymmetry of the last eluted peak, because it tended to tail
339 significantly.

340 For each of the five responses, desirability was defined. The highest S value was desired for the four
341 S criterions. For asymmetry criterion, we wanted values the closest to one. For example, iso-response
342 graphs are presented in Fig. 4 for the critical pair of peaks (3 and 4) corresponding to the two mono-
343 oxidized degradation products. These graphs allowed to model the DoE response: temperature vs.
344 final proportion of co-solvent (on the left) and temperature vs. gradient time (on the right). The darkest
345 area corresponds to a response zone where desirability is maximum. The crossing of the dashed
346 black lines represents optimal values (normalized values) determined by DoE. On this figure, it
347 appears that the overall optimum (defined by crossed interrupted lines) is not necessarily the same as
348 the local optimum for each pair of consecutive peaks. The overall optimum is therefore a compromise
349 to satisfy all five responses.

350

351 Optimal elution gradient conditions were found as follows: from 50 to 70.3% co-solvent (corresponding
352 to the normalized value 0.052) in 3.8 minutes (normalized value -1) with oven temperature set at 36°C
353 (normalized value +1).

354

355 *Modification of co-solvent nature:*

356 After the elution gradient optimization with MeOH containing 20 mM AA and 2% water as co-solvent,
357 the two mono-oxide peaks were not sufficiently resolved to hope for a productive purification (Fig. 3).
358 Therefore, it was decided to modify the mobile phase composition, introducing a portion of isopropyl-

359 alcohol (iPrOH). This solvent is less polar than MeOH and caused increased retention and separation
360 factors (Fig. 5). A MeOH / iPrOH 70/30 (v/v) mixture was finally selected as a middle ground between
361 separation quality, peak shape and run time. The final conditions for this analytical method may not be
362 SFC, but they may be EFLC.

363 The robustness of the method was assessed as described in experimental section and supplementary
364 material (Tables S1 and S2). It was observed that all parameters observed (retention time, peak area,
365 asymmetry and resolution) remained stable within 2%.

366 The method then served to compare different samples obtained after different conditions of stressed
367 degradation applied to the same API, prior to scale-up.

368

369 3.3. Method transfer to preparative-scale

370

371 The optimal SFC (or EFLC) method determined at the previous step was transferred to the preparative
372 scale as described in the experimental section. Four fractions were collected: the API from 5.47 to
373 6.67 min, two mono-oxidized degradation products from 6.83 to 8.3 min and from 9.2 to 10.7 min and
374 one di-oxidized degradation product from 11.92 to 15.43 min (Fig. 6). The whole purification
375 experiments lasted nearly 20 hours. Overall, 60 L of solvent and 57 L of carbon dioxide were eluted
376 through the system. Actually, the CO₂ consumption was much less than 57 L thanks to the recycling
377 system implemented. According to PIC Solution™, more than 90 % of CO₂ is recycled with 10% of co-
378 solvent and 85% is recycled with 30% of co-solvent. We may thus expect that a lower proportion of
379 CO₂ would be recycled in the present conditions. The collected volumes and masses of dry product
380 obtained for each fraction are indicated in Table 3. Obviously, the volume of solvent collected for the
381 fourth peak (di-oxidized product) is much larger than the others, due to (i) large proportion of this
382 compound in the sample injected and (ii) large proportion of co-solvent in this section of the
383 chromatogram, to elute this peak with reasonably short time frame. It is important to note that such
384 large proportions of solvent are quite unusual for SFC, but were necessary here to separate and elute
385 these high-molecular weight products. In terms of productivity, 306 mg of product were purified in an
386 hour for an average co-solvent consumption of 3 liters. For example, the purified mass is 14 times that
387 of the product purified in a paper from E. Landagaray et al. [22], but it is also 14 times more solvent
388 consuming. In terms of productivity, we have purified about 0.1 kg product per kg of stationary phase
389 per day (kkd), which may be compared to about 0.2 kkd in ref. [22]. While 0.1 kkd is by no means an
390 exceptional productivity, it may however be considered reasonably good [18], especially judging from
391 the difficulty in the separation and the fact that three compounds needed to be collected among other
392 undesirable species. If larger quantities had been desired, further optimization could have been done
393 (flow rate, injected mass and volume, dilution solvent, etc.).

394

395 3.4. Fractions analysis

396

397 Each fraction was analyzed in order to verify the quality of purified compounds. The results are
398 summarized in Table 3. We noticed that the API collection (fraction 1) was pure at 98.5% whereas for
399 the mono-oxidized degradation products (fractions 2 and 3), the presence of di-oxidized compound
400 (eluted later) was detected up to 2.6%. It was not a problem because the desired purity level of mono-
401 oxidized compounds was achieved (95.8% and 97.5%). The presence of di-oxidized compound in
402 these two fractions may result from further oxidation occurring after the preparative chromatography,
403 perhaps at the evaporation stage but this hypothesis was not verified.

404
405

406 **4. Conclusions**

407

408 Supercritical fluid chromatography at analytical-scale was used to solve the issue in degradation
409 products separation, emerged throughout first trials in HPLC. Thanks to this technique, three
410 degradation products (two isomeric mono-oxidized and one di-oxidized) were isolated from the API.
411 The fast and broad screening of stationary phases and design of experiments quickly provided an
412 optimal analytical method. Because of the large molecular weight of the analytes, inducing limited
413 solubility and strong interactions with the stationary phase, unusual SFC conditions were necessary to
414 obtain this separation, with co-solvent percentage in carbon dioxide up to 70%, which is probably more
415 relevant of enhanced-fluidity liquid chromatography (EFLC). Contrary to some previous reports from
416 Miller and Potter [30], scale-up from ultra-high performance chromatographic conditions to preparative
417 SFC was quite straightforward in this case and allowed efficient purification of all degradation products
418 for further activity testing.

419

420 **Conflicts of interest**

421

422 The authors declare no conflicts of interest.

423

424 **Acknowledgments**

425

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430

431

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- 520

521 **Figure captions**

522

523 **Fig. 1.** Partial structures of the API and oxidation products examined in this study.

524

525 **Fig. 2.** Linear Solvation Energy Relationships (LSER) classification showing the 15 tested columns
526 (achiral stationary phases in blue and enantioselective stationary phases in purple).

527

528 **Fig. 3.** Chromatogram of the sample from forced degradation obtained on Acquity Torus 2-PIC column
529 (100 x 3.0 mm, 1.7 μ m) with the optimal elution gradient conditions as defined by the DoE: 100%
530 MeOH co-solvent + 20mM AA + 2% water in mobile phase; gradient elution as indicated in the figure.
531 UV detection 210 nm. Red star is the API (M); blue diamonds are the two isomeric mono-oxide
532 species (M+16); green circle is the di-oxidized degradation product (M+32).

533

534 **Fig. 4.** Iso-response graphs of separation criterion S for the critical pair of peaks (peaks 3 and 4). The
535 crossing of the dashed black lines represents optimal values of the DoE.

536

537 **Fig. 5.** Chromatogram of the sample from forced degradation obtained on Acquity Torus 2-PIC column
538 (100 x 3.0 mm, 1.7 μ m) with optimal elution gradient and modified mobile phase composition: co-
539 solvent 70%MeOH / 30% iPrOH comprising 20 mM AA + 2% water in mobile phase; gradient elution
540 as indicated in the figure. UV detection 210 nm. Red star is the API (M); blue diamonds are the two
541 isomeric mono-oxide species (M+16); green circle is the di-oxidized degradation product (M+32).

542

543 **Fig. 6.** Fraction collection (purple areas) in preparative supercritical fluid chromatography with elution
544 gradient profile (green line). Separation done with Torus 2-PIC column (150 x 30 mm, 5 μ m) and
545 mobile phase co-solvent comprising 70% MeOH and 30% iPrOH comprising 2% water and 20 mM
546 ammonium acetate. UV detection 210 nm.

547

548

549 **Table 1**

550 The 15 columns used to screen the samples in this study

Column Name	Manufacturer	Particle type	Bonded ligand	Dimensions (mm)	Particle size (µm)
ACQUITY UPC ² Torus 2- PIC	Waters	Fully porous hybrid silica	2 Picolyl-amine	100 x 3.0	1.7
ACQUITY UPC ² HSS C18 SB	Waters	Fully porous high strength silica	Octadecyl, non endcapped	100 x 3.0	1.8
Kinetex HILIC	Phenomenex	Superficially porous silica	-	150 x 4.6	2.6
Accucore HILIC	Thermo	Superficially porous silica	-	150 x 4.6	2.6
Accucore Amide-HILIC	Thermo	Superficially porous silica	-	150 x 4.6	2.6
Accucore Urea- HILIC	Thermo	Superficially porous silica	-	150 x 4.6	2.6
Ascentis Express OH5	Supelco	Superficially porous silica	Penta-hydroxyl	150 x 4.6	2.7
Ascentis Express F5	Supelco	Superficially porous silica	Penta-fluorophenyl	150 x 4.6	2.7
Nucleoshell HILIC	Macherey-Nagel	Superficially porous silica	Sulfobetaine	150 x 3.0	2.7
Chiralpak IA	Chiral Technologies	Fully porous silica	Amylose <i>tris</i> -(3,5- dimethylphenylcarbamate)	150 x 4.6	3.0
Chiralpak IB	Chiral Technologies	Fully porous silica	Cellulose <i>tris</i> -(3,5- dimethylphenylcarbamate)	150 x 4.6	3.0
Chiralpak IC	Chiral Technologies	Fully porous silica	Cellulose <i>tris</i> -(3,5-dichloro- phenylcarbamate)	150 x 4.6	5.0
Chiralpak ID	Chiral Technologies	Fully porous silica	Amylose <i>tris</i> -(3-chloro- phenylcarbamate)	150 x 4.6	5.0
Chiralpak IF	Chiral Technologies	Fully porous silica	Amylose <i>tris</i> -(3-chloro-4- methylphenylcarbamate)	150 x 4.6	3.0
Chiralpak IG	Chiral Technologies	Fully porous silica	Amylose <i>tris</i> -(3-chloro-5- methylphenylcarbamate)	150 x 4.6	3.0

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557 **Table 2**

558 Design of experiments

	Final solvent percentage	Gradient time	Oven temperature
-α	60 %	3.0 min	20°C
-1	64 %	3.8 min	24°C
0	70 %	5.0 min	30°C
+1	76 %	6.2 min	36°C
+α	80 %	7.0 min	40°C

559

560

561

562

563 **Table 3**

564 Fractions analysis: collected masses and volumes, quality control for each fraction

	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Collected volume (L)	3.5	4.3	5.0	14.8
Collected mass (g)	ND	0.3	0.3	3
Fractions purity				
API	98.5 %	0.8%	ND	ND
Mono oxide 1	<1	95.8 %	0.1 %	ND
Mono oxide 2	<1	ND	97.5 %	ND
Di oxide	ND	2.6 %	2.1 %	99.3 %

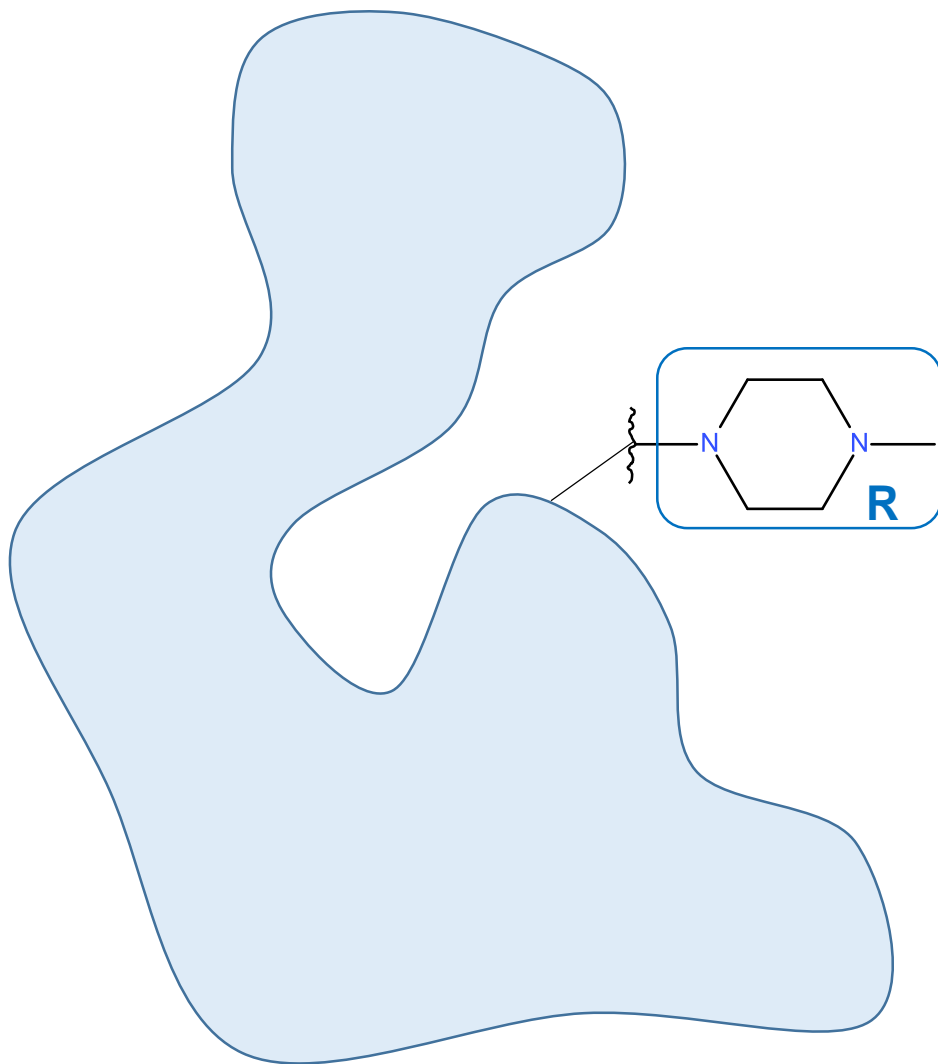
565

566 *ND = Not determined

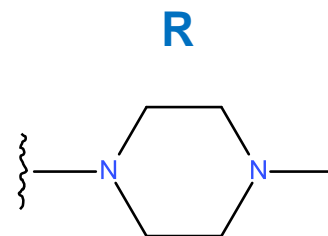
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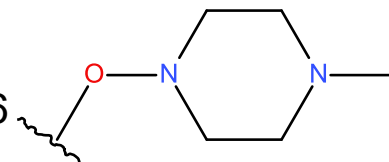
Fig. 1



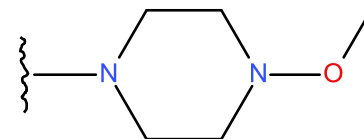
★ API:
M ~ 900 Da



◆ Mono oxidized
product 1: M+16



◆ Mono oxidized
product 2: M+16



● Di oxidized
product : M+32

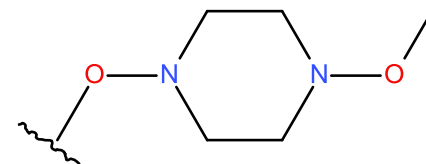


Fig. 2

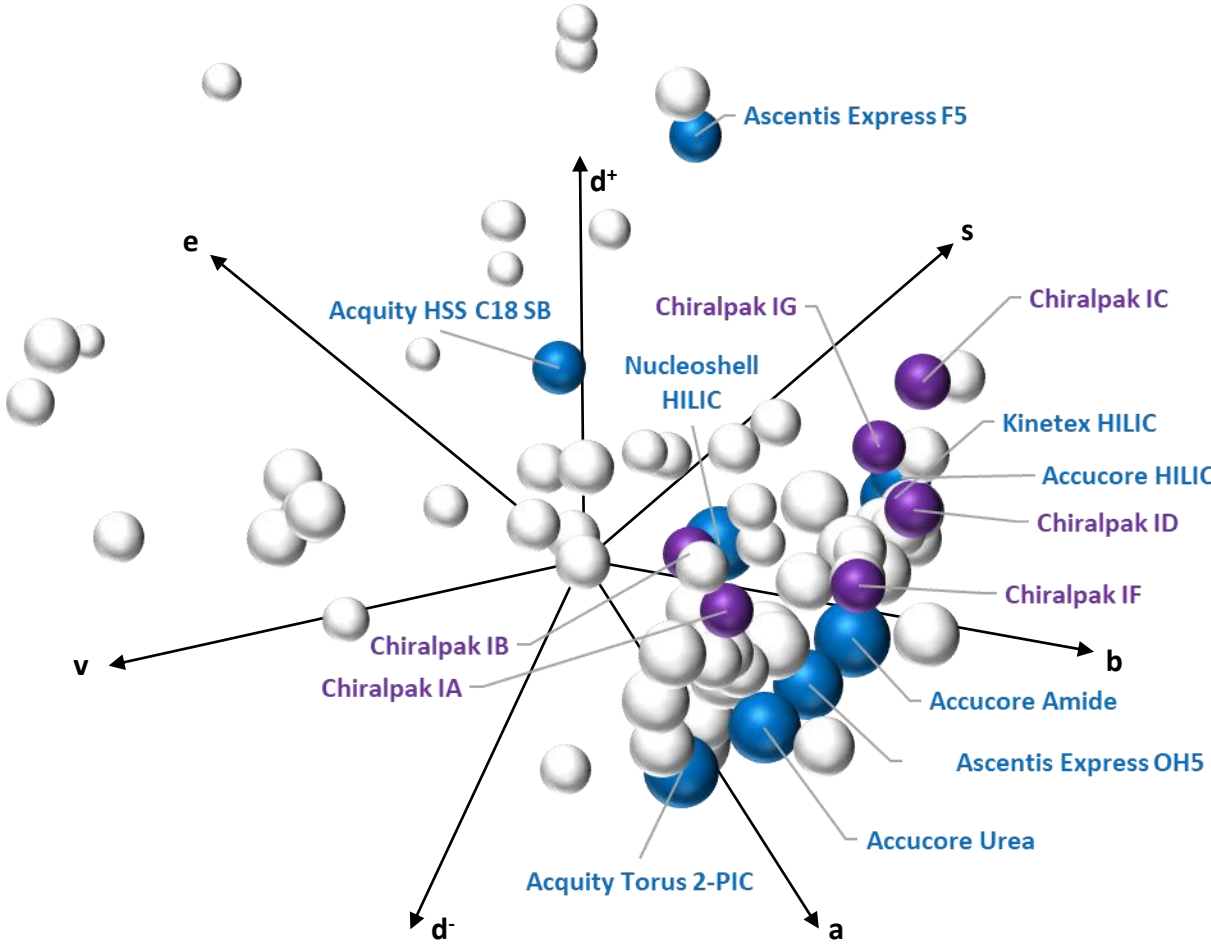


Fig. 3

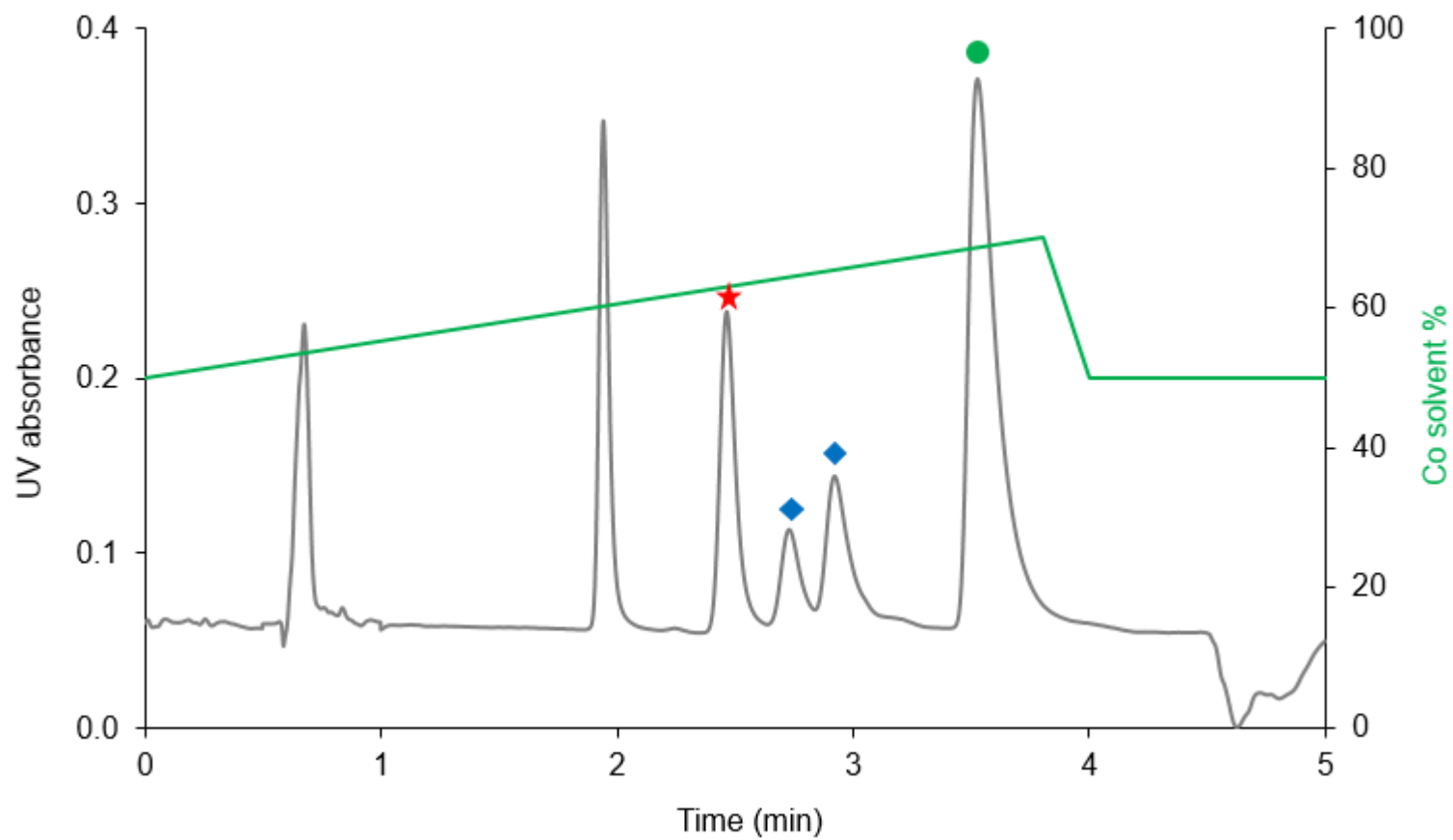


Fig. 4

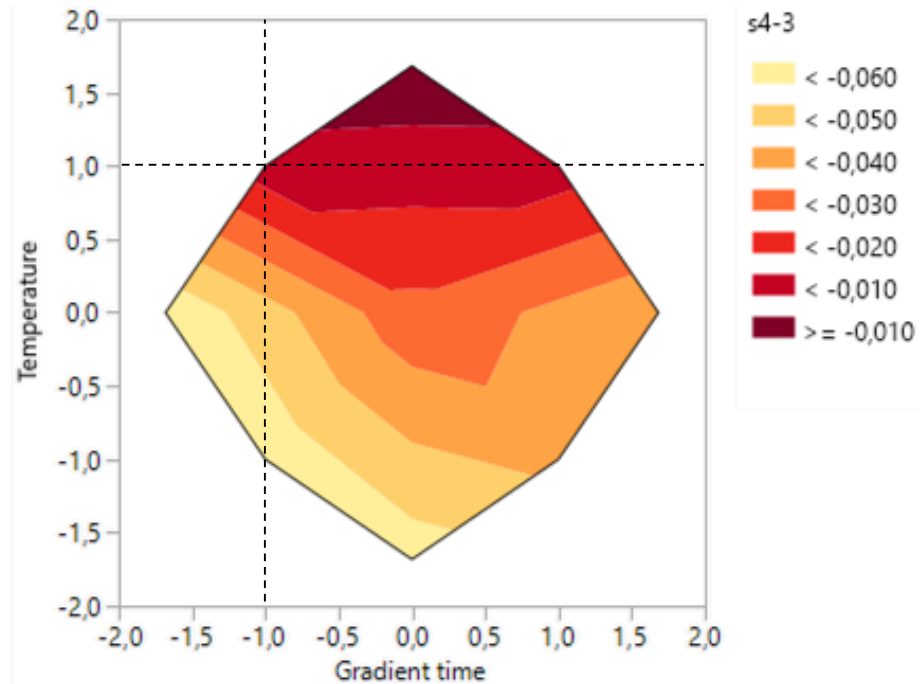
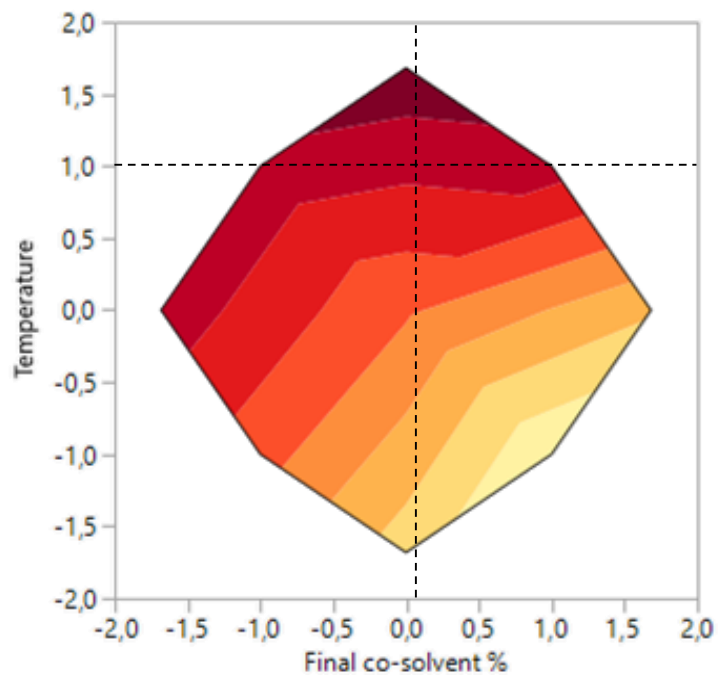


Fig. 5

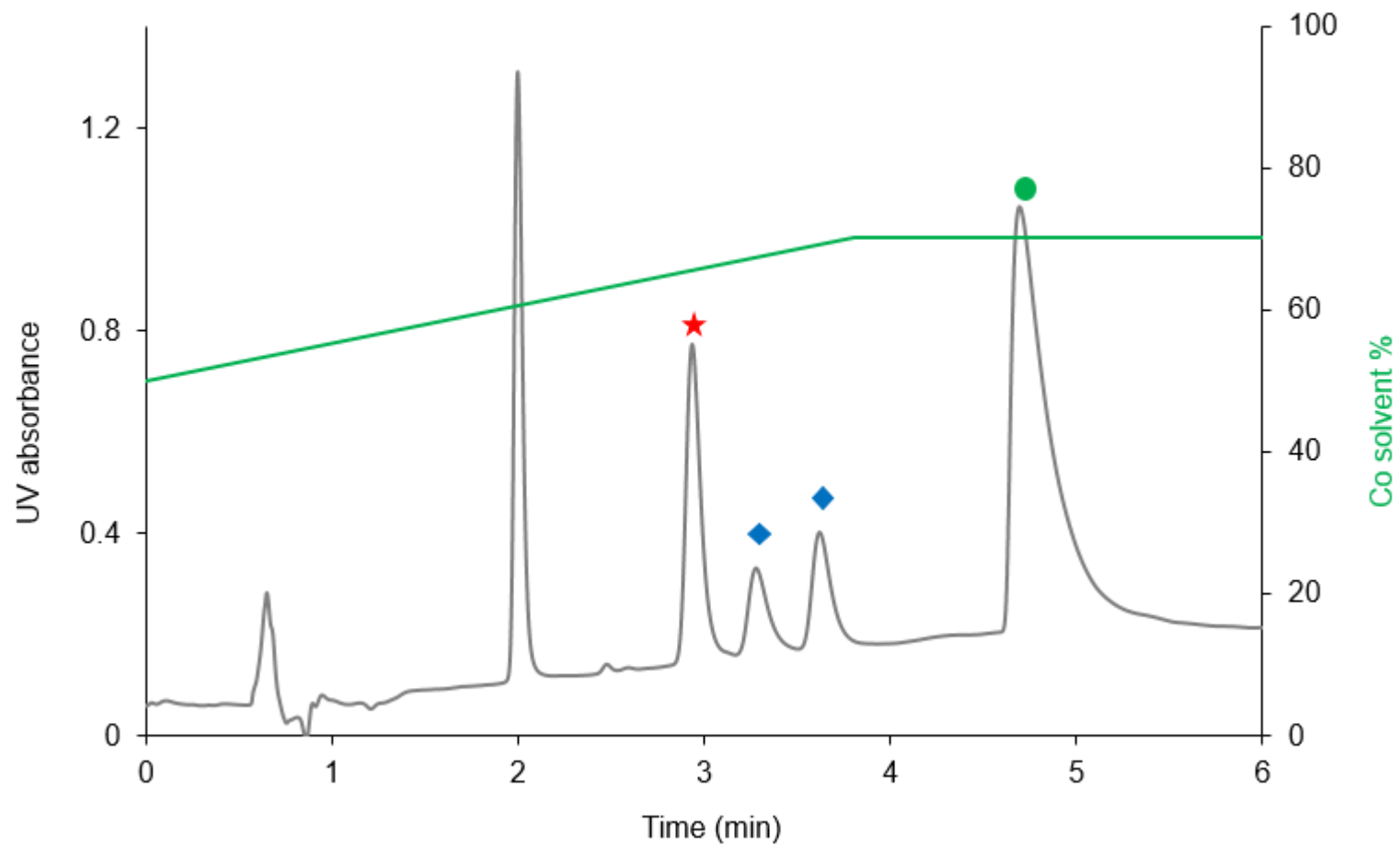
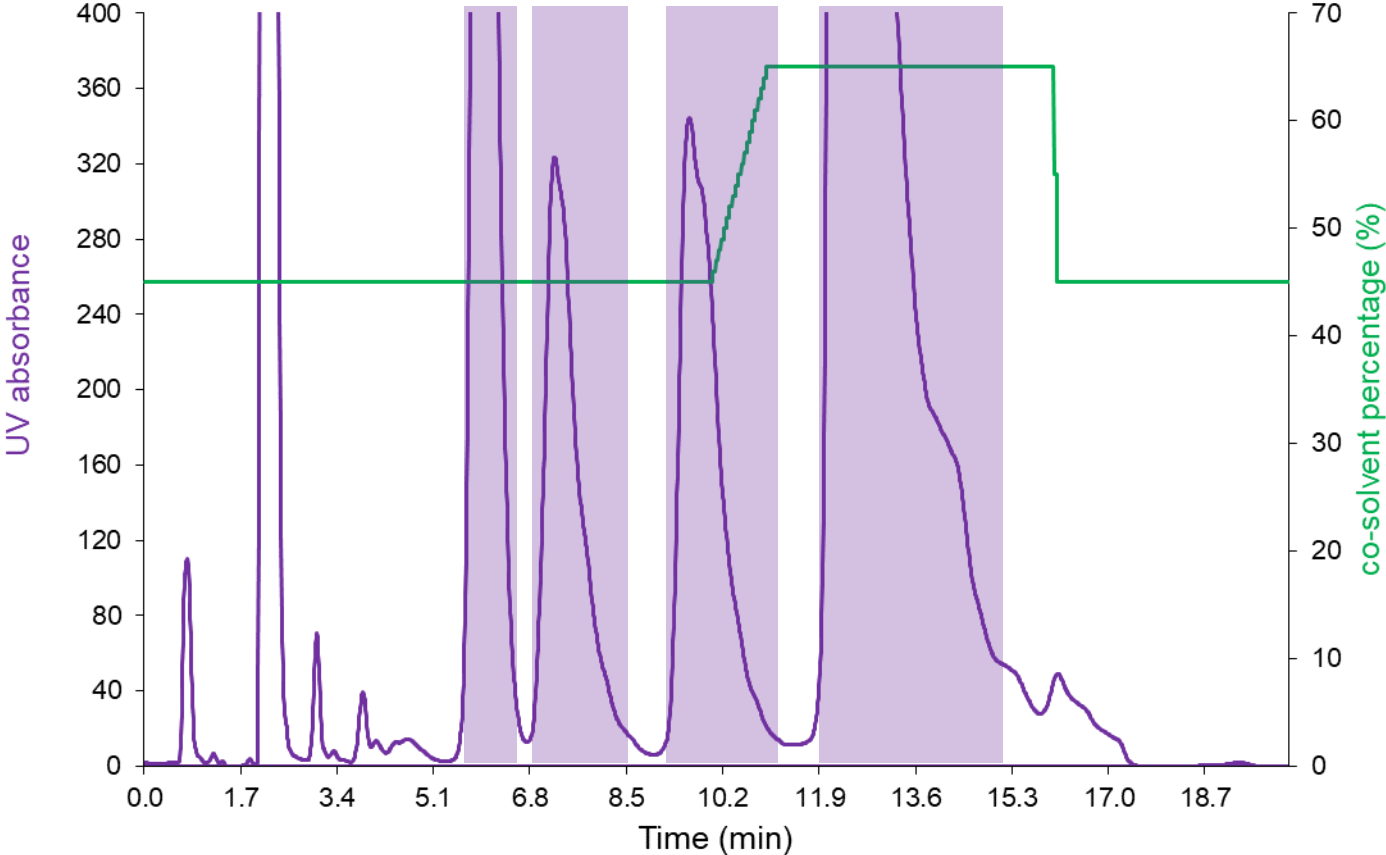
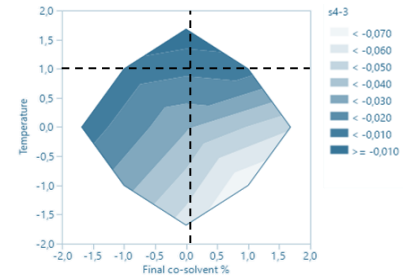
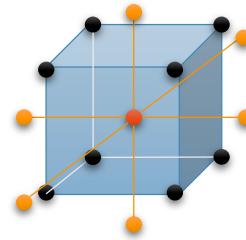
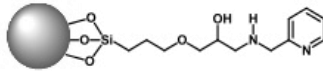


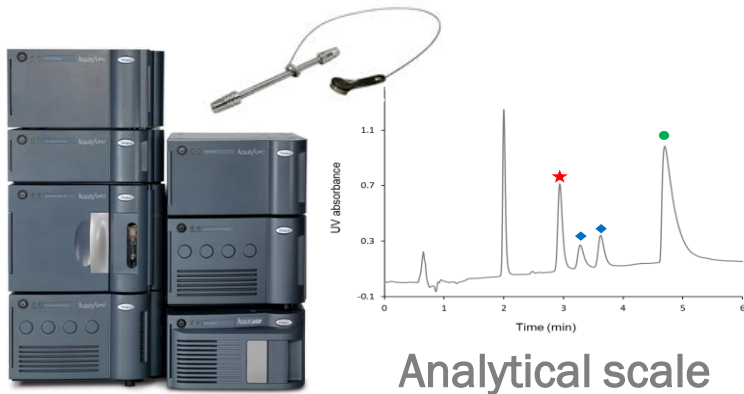
Fig. 6



Supercritical fluid chromatography

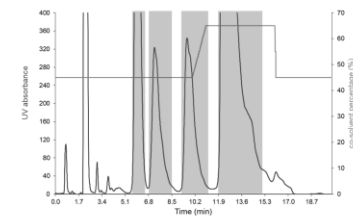


Design of experiments



Method optimization

Scale - up



Preparative scale