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# Analysis of short-chain bioactive peptides by unified chromatography-electrospray ionization mass spectrometry. Part II. Comparison to reversed-phase ultra-high performance liquid chromatography

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1 **Analysis of short-chain bioactive peptides by unified**  
2 **chromatography-electrospray ionization mass spectrometry. Part II.**  
3 **Comparison to reversed-phase ultra-high performance liquid**  
4 **chromatography**

5  
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17

18

19 **Abstract**

20

21 In the first part of this study, a unified chromatography (UC) analysis method, which is  
22 similar to supercritical fluid chromatography (SFC) but with wide mobile phase  
23 gradients of pressurized CO<sub>2</sub> and solvent, was developed to analyse short-chain  
24 peptides, with UV and mass spectrometry (MS) detection. In this second part, the  
25 method is compared to a reference reversed-phase ultra-high-performance liquid  
26 chromatography (RP-UHPLC) method, based on the analysis of 43 peptides, including  
27 10 linear peptides and 33 cyclic ones.

28 First, the orthogonality between the two methods was examined, based on the  
29 retention patterns. As the UC method was developed on a polar stationary phase  
30 (Ascentis Express OH5), the elution orders and selectivities were expected to be  
31 significantly different from RPLC on a non-polar stationary phase (ACQUITY CSH  
32 C18). Secondly, the success rate of the methods was examined, based on successful  
33 retention / elution of the peptides and the absence of observed co-elutions between  
34 the main peak and impurities. A successful analysis was obtained for 81% of the  
35 peptides in UC and 67% in RPLC. Thirdly, the performance of the methods for the  
36 intended application of impurity profiling of peptide drug candidates was assessed,  
37 based on the comparison of peak purities, the number of impurities detected and the  
38 thorough examination of impurity profiles. Excellent complementarity of the two  
39 methods for the specific task of impurity profiling, and for the separation of isomeric  
40 species was observed, with only one isomeric pair in this set remaining unresolved.  
41 The method sensitivity was however better with RPLC than UC. Finally, the  
42 operational costs in terms of solvent cost per analysis were the same between the two  
43 methods.

44

45

46 **Keywords**

47 Peptides; Reversed-phase liquid chromatography; Supercritical fluid chromatography;  
48 Unified Chromatography

49

## 1. Introduction

The use of biomolecules as active pharmaceutical ingredients (API) has strongly intensified since the beginning of this century. Among them, peptides are amino-acid oligomers and polymers, with a wide diversity of molecular weights and polarities. Peptides have been used as APIs since the introduction of insulin for diabetes treatment in the 1920s' [1]. Compared to the small synthetic chemical molecules mostly used in occidental medicine, the low toxicity usually associated with peptide drugs is attractive to favor patient's safety [2].

Historical perspectives on therapeutic peptides were clearly exposed in previous reviews [3,4]. In addition, pharmaceutical peptides of biological or synthetic origin under 5 000 Da and commercialized in northern countries (Europe, Japan and North America) for therapeutic and diagnostic applications are all freely listed in the PepTherDia database (<http://peptherdia.herokuapp.com/>) [5]. This database currently comprises around 100 compounds information such as physicochemical and pharmacokinetic properties. Examining this database, it appears that short-chain peptides (between 300 Da and 2000 Da) stand for 75% of commercialized pharmaceutical peptides. Among them, about a third (a quarter of the total) has a molecular weight below 1000 Da. Until the 1990s, most of the therapeutic peptides entering in clinical study were short-chain peptides [4]. Then, as the synthesis and manufacturing technologies improved [6,7], peptides with higher molecular weights were more often described. Short-chain peptides however continued to be developed in a steady fashion.

Reversed-phase liquid chromatography (RPLC) is the most employed method for peptide analysis [8–11] because of its robustness, high-resolution power, short analysis times and compatibility to mass spectrometry (MS). Different authors described RPLC as a flexible and versatile technique, which could also explain its attractiveness for peptides investigations [11,12]. However, RPLC-UV-MS is not always enough to assess peptide structure and purity as co-elutions may occur, especially with isobaric compounds are isomeric species are frequent in such mixtures. Complementary techniques are always desirable to obtain a more complete picture of a sample [13]. Besides, solubilization of the peptides in adequate dilution solvent and carryover effects have been reported as significant issues in RPLC [14,15]. In addition, retention in RPLC is mostly governed by hydrophobic interactions

84 between the analyte and aqueous mobile phase, and by dispersive interactions  
85 between hydrophobic residues and hydrophobic stationary phase ligands. As a result,  
86 the more polar peptides (typically encountered in short-chain peptides) are not always  
87 adequately retained in RPLC chromatographic systems. Polar peptides should be best  
88 analyzed with hydrophilic interaction liquid chromatography (HILIC), which is  
89 frequently used as an orthogonal method for peptide purification and identification [16–  
90 18]. The solvent-rich mobile phases employed in HILIC are particularly advantageous  
91 for sensitive electrospray ionization (ESI) MS detection [16,19]. However, in our  
92 experience, HILIC methods often lack the sufficient robustness to be suitable for a  
93 generic method in the R&D laboratory. Capillary electrophoresis is also a favorite  
94 method to analyze therapeutic biomolecules [20,21]. However, when purification is  
95 desirable, transfer to preparative scale is impossible.

96 Looking for complementary methods that could cover a wide polarity range for  
97 short-chain and long-chain peptides, and could possibly be transferred to preparative  
98 scale when needed, Unified Chromatography (UC) appears as an interesting option.  
99 Indeed, UC mobile phase gradients may start with a non-polar composition comprising  
100 large proportions of pressurized carbon dioxide (usual working range of supercritical  
101 fluid chromatography – SFC), and end with a rather polar composition containing large  
102 proportions of liquid solvents (usual working range of enhanced fluidity liquid  
103 chromatography – EFLC), possibly ending in pure-liquid solvent conditions (LC). This  
104 promises the possible analysis of non-polar and polar analytes in a single run [22], or  
105 makes it possible to propose a single and generic method to analyze both non-polar  
106 and polar peptides. Although carbon-dioxide mobile phases are usually perceived as  
107 being incapable to elute polar analytes, SFC, EFLC and UC have all been  
108 demonstrated to be useful for this purpose [23–27]. Some examples of peptides and  
109 proteins separations by SFC, EFLC or UC appeared in literature recently [28–30].  
110 However, most of the peptides appearing in previous works had molecular weights  
111 above 1000 Da, which set different issues in terms of solubility and multiple  
112 conformations than short-chain peptides [31]. Further information on the topic of  
113 biomolecules analysis with SFC, EFLC and UC can be found in a recent review [32].

114 In the first part of this series of papers [33], a UC method with UV and ESI-MS  
115 detection was developed for the purpose of determining the purity of peptide drug  
116 candidates, with molecular weights below 1000 Da. The method was developed on a  
117 modern instrument included reduced dead volumes, and with a stationary phase

118 based on superficially porous particles offering improved efficiencies, thus the method  
119 could be called “ultra-high-performance UC”. In this second part, a thorough  
120 comparison between this UC-MS method and a reference, previously optimized,  
121 reversed-phase ultra-high-performance liquid chromatography - mass spectrometry  
122 (RP-UHPLC-MS) method is proposed. Previously, we had conducted a similar  
123 comparison between ultra-high-performance SFC-MS and RP-UHPLC-MS for small  
124 chemical drugs [34] that demonstrated a high level of orthogonality between the two  
125 methods. It was therefore of interest to examine the complementarity and compare  
126 method performance in more details on these short-chain peptides. For this purpose,  
127 43 peptides comprising 10 linear tripeptides and 33 cyclic pentapeptides were  
128 analyzed with both methods. The comparison was focused on performance for the  
129 intended application to purity analysis, thereby examined the impurities detection and  
130 profile, overall purity comparison, and quality attributes like the symmetry of the main  
131 peak and observed peak capacity. Some attention was paid to the separation of  
132 isomeric species, which are often encountered in synthetic mixtures of peptides.

133

## 134 **2. Material and methods**

135

### 136 **2.1. Chemicals and solvents**

137 From the 76 peptides used to develop the UC-MS method in the first part of this  
138 series [33], 43 peptides were available in sufficient quantity to ensure reliable method  
139 comparison. They comprised 10 linear tripeptides and 33 cyclic pentapeptides. These  
140 compounds have confidential structures not exposed in this paper. They are however  
141 described by their significant features in Table S1. It can be observed in Figure S1  
142 (supplementary information) that adequate diversity was retained with this shorter  
143 selection, as the molecular weights ranged from 392 to 736 g/mol, calculated log P  
144 values ranged from -6.9 to 2.9 and topological polar surface area (TPSA) ranged from  
145 130 to 292. These features were determined with Vortex v2019.04.82972.15-s  
146 (Dotmatics Limited). The scattering of points according to these significant features  
147 can be compared to that of the larger set of peptides used to develop the UC-MS  
148 method.

149 The peptides were provided in dimethylsulfoxide (DMSO) solutions at 10 mM.  
150 Some of them having basic residues were present as trifluoroacetic acid salts, as  
151 indicated in Table S1. The DMSO solutions were further diluted at 1 mM with (i)

152 methanol (MeOH) for UC analysis and (ii) water/acetonitrile/trifluoroacetic acid  
153 98/2/0.1 (v/v/v) for LC analysis.

154 For UC analysis, gradient-grade MeOH was purchased from VWR (Fontenay-  
155 sous-bois, France) and CO<sub>2</sub> with a purity of 99,995% was provided by Air Liquide  
156 (Bagneux, France). Water was provided by Purelab flex from Veolia (Wissous,  
157 France). Ammonium hydroxide solution was obtained from Sigma-Aldrich (Merck,  
158 France). For RPLC analysis, HPLC-grade acetonitrile (ACN) was purchased from  
159 Merck (VWR international SAS, France), water was obtained from a Milli-Q Purification  
160 System from Millipore (Millipore SAS, France) and trifluoroacetic acid (TFA) for  
161 spectroscopy was obtained from Merck (VWR International SAS, France).

162

## 163 **2.2. UC-MS Instrument**

164 The ultra-high performance supercritical fluid chromatography system was a  
165 Waters Corporation (Guyancourt, France) ACQUITY Ultra Performance Convergence  
166 Chromatography™ (UPC2®) instrument, equipped with a photodiode-array (ACQUITY  
167 PDA) detector and an ACQUITY QDa® single-quadrupole mass detector with electro-  
168 spray ionization source. Instrument details were provided in the first part of this work  
169 [33]. Empower® 3 was used for system control, data acquisition and integration of  
170 peaks for symmetry and peak capacity measurements.

171

## 172 **2.3. UC-MS method**

173 The UC-MS method development was previously described [33]. The stationary  
174 phase was Ascentis Express OH5 (150 x 4.6 mm; 2.7 μm) from Sigma Aldrich. The  
175 mobile phase was a CO<sub>2</sub>-cosolvent gradient, with a cosolvent composed of MeOH  
176 containing 2% H<sub>2</sub>O (v/v) and 20 mM NH<sub>4</sub>OH. Two different methods were used. The  
177 first one, referred to as “generic gradient”, is the gradient optimized in the first part of  
178 this series. The co-solvent gradient starts at 5% and reaches 80% in 10 min, together  
179 with a reverse flow-rate gradient from 3.0 to 1.5 ml/min (see details in Table 1a). Total  
180 run duration is 15 min. A second gradient, referred to as “focus gradient”, was  
181 developed in the course of this study, to enhance the resolution of impurities eluting  
182 close to the target peptide. The co-solvent gradient is adjusted individually for each  
183 target peptide based on its elution composition  $C_e$ , corresponding to the proportion of  
184 co-solvent necessary to elute the analyte with the generic gradient. A slow gradient is  
185 then conducted with a co-solvent proportion varying from  $C_e - 5\%$  to  $C_e + 5\%$  in 10

186 min. Before and after this slow gradient, a fast gradient is applied, so the total analysis  
187 duration is 15 min. Because the  $C_e$  values were all rather large, a constant flow-rate of  
188 1.5 ml/min was used in this case to avoid reaching the pressure limit of the pumping  
189 system (414 bar). Details are provided in Table 1b.

190 For both gradient methods, the back pressure was set at 120 bar, the oven  
191 temperature was set at 60°C and the sample compartment temperature at 10°C. UV  
192 detection wavelength was set at 210 nm with resolution 1.2 nm. ESI-MS parameters  
193 were set as follows: both positive and negative ionization single-ion-recordings (SIR)  
194 were observed, together with positive ionization for total ion chromatograms (TIC). The  
195 needle temperature was set at 600°C and the cone voltage at 10 V. For positive TIC  
196 and SIR acquisition, the capillary voltage was set at 0.3 kV, while it was set at -0.8 kV  
197 for negative SIR. Injection volume was 5  $\mu$ l for every blank and peptide analysis.

198 When the proportion of co-solvent is low (at the beginning of UC gradient), it is  
199 useful to introduce an additional flow of liquid prior to entering the MS. It is however  
200 unnecessary when the proportion of co-solvent reaches high values (at the end of UC  
201 gradient). Consequently, the MS make-up flow-rate for “large gradient” also applied a  
202 reverse gradient from 0.4 to 0 ml/min in 3 min, as previously optimized. However, no  
203 make-up fluid was necessary in the case of focus gradients, because all peptides  
204 analyzed with a focus gradient were eluted with a large proportion of co-solvent. The  
205 composition of make-up fluid was identical to that of the chromatographic co-solvent  
206 (MeOH comprising 2% H<sub>2</sub>O and 20 mM NH<sub>4</sub>OH).

207

## 208 **2.4. UHPLC-MS Instrument**

209 The ultra-high-performance liquid chromatography system was an ACQUITY  
210 UPLC® I-Class from Waters Corporation. It was equipped with a binary solvent  
211 delivery pump compatible with the conditions of the present method (mobile phase  
212 flow rate of 0.7 mL/min and pressures up to 528 bar), an autosampler with flow-  
213 through-needle injection system, two 2-position column ovens compatible with 150 mm  
214 length columns and a photodiode-array (ACQUITY PDA) detector. The main flow  
215 stream was splitted by a CORONA VEO RS splitter (Thermo Scientific) placed before  
216 the MS entrance. With this system, the split ratio is about 1/10: only 1/10 of the column  
217 flow goes to the MS. ACQUITY TQMQ XEVO® triple-quadrupole mass detector  
218 (Waters Corporation) with electrospray ionization source was used for mass  
219 spectrometry identification. MassLynx® software (V4.1) was used for system control



220 and data acquisition and Empower®3 for integration of peaks for column efficiency  
221 measurements. Waters Data Converter (V2.1) was used to convert data from  
222 MassLynx to Empower.

223

## 224 **2.5. RP-UHPLC-MS method**

225 The RP-UHPLC-MS method serving here as reference has been routinely used at  
226 Servier Research Laboratories since 2016 as a generic method to analyze peptides of  
227 varied sizes and polarities. The stationary phase was ACQUITY CSH C18 (100 × 2.1  
228 mm, 1.7 µm) from Waters. Mobile phase A was H<sub>2</sub>O / ACN / TFA 100/1/0.1 (v/v/v) and  
229 mobile phase B was ACN / H<sub>2</sub>O / TFA 100/1/0.1 (v/v/v). Similarly to the UC methods,  
230 two different gradient programs were used: a large gradient referred to as “RPLC  
231 generic gradient” and a slower gradient focused on the target peptide, individually  
232 adapted to each peptide, referred to as “RPLC focus gradient”. In the generic gradient,  
233 the B solvent proportion was increased from 0 to 60% in 11 min (see details in Table  
234 1c). The total analysis time was 14.3 min. In focus gradient, the B solvent proportion  
235 was slowly increased from C<sub>e</sub> -3% to C<sub>e</sub> +3. Details are provided in Table 1d.  
236 Flow-rate was set at 0.7 ml/min in both methods with an oven temperature settled at  
237 70°C and sample temperature at 20°C. Injection volume was held at 1 µl for every  
238 blank and peptide analysis. The lower injected volume was necessary to maintain  
239 comparable ratios of injected volume to column volume between the UC and RPLC  
240 methods (0.3 vs. 0.4%).

241 UV detection wavelength was set at 210 nm with resolution 1.2 nm. For MS  
242 detection, mass to charge ratio between 100 and 1000 Da were recorded with a scan  
243 time of 0.2 s. For positive TIC and SIR, acquisition, the MS parameters were set as  
244 follows: capillary voltage at 3.8 kV, cone voltage at 30 V, extractor 3 V, source  
245 temperature 150°C, desolvation temperature 600°C, cone gas flow 0 L/h, desolvation  
246 gas flow 1000 L/h, collision gas flow 0.14 mL/min.

247

## 248 **2.6. Design of experiment for UC robustness assessment**

249

250 To assess the method robustness, a Design of Experiments (DoE) with  
251 Doehlert matrix was carried out. The DoE was prepared and evaluated with Ellistat  
252 software version 6.5 2021/01 (Ellistat, Chavanod, France). Three parameters were  
253 considered: back-pressure (ranging from 111.3 to 128.7 bar), column oven

254 temperature (ranging from 51.8 to 68.2°C) and the final co-solvent concentration in  
255 gradient elution (ranging from 70 to 90%). The full matrix is presented in  
256 supplementary information (Figure S3).

257

258

### 259 **3. Results & Discussion**

260

#### 261 3.1. Orthogonality of the two methods

262 Before comparing the performance of the methods, it is useful to examine their  
263 orthogonality. Because the UC method was developed on a polar stationary phase  
264 (polyhydroxylated ligand), it was expected that elution order would be most different  
265 from the elution order obtained in RPLC on an octadecylsiloxane stationary phase. For  
266 this purpose, we followed the method proposed by D'Attoma *et al.* [35] and plotted the  
267 peptides eluted with both methods in terms of the mobile phase composition  
268 (percentage of organic solvent) at the moment of elution. Considering the regression  
269 line and the 95% confidence limits, a polygon is drawn to provide a view of the  
270 distribution of target peaks in the retention space. The elution composition was  
271 computed according to the method proposed by Fekete *et al.* [36] using Eq. (1):

272

$$273 \quad C_e = C_i + \frac{(C_f - C_i)}{t_g} * (t_R - t_D) \quad (1)$$

274

275 Where  $C_e$  corresponds to the percentage of ACN (for RPLC analysis) and MeOH  
276 (for UC analysis) required to elute the peptide;  $C_i$  and  $C_f$  are the initial and final  
277 compositions of the gradient, respectively;  $t_g$  is the gradient time;  $t_R$  is the retention  
278 time of the peptide and  $t_D$  is the system dwell time. The UC dwell time was 0.46 min  
279 and the RPLC dwell time was 0.51 min.

280 The plot obtained is presented in Figure 1. Note that less than 43 points are visible  
281 on this figure because some analytes eluted with the same elution composition with  
282 both techniques (typically, the un-retained analytes in RPLC that were strongly  
283 retained in UC).

284 Firstly, it appears that these small peptides elute very early in the RPLC method,  
285 with proportions of organic solvent never exceeding 40%. It must be noted that this  
286 method was not specifically purposed for such small peptides but is a generic method

287 used for a variety of peptides with all sizes and polarities. However, small polar  
288 molecules are unlikely to be well retained and resolved in RPLC mode, even with an  
289 optimized mobile phase. On the contrary, the UC method that was specifically  
290 designed for these small peptides shows adequate scattering of the target peaks, with  
291 elution compositions varying between 40 and 100% organic solvent. It is also worth  
292 noting that the scales of the figure were adjusted to have a better view of the  
293 scattering of points. The figure with identical scales is visible in supplementary  
294 information Figure S2, where it clearly appears that the points are mostly grouped at  
295 the bottom of the figure (low proportion of solvent in RPLC mode). The scattering of  
296 linear and cyclic peptides in this figure was also interesting to illustrate that this smaller  
297 analyte set (compared to the larger set used in the first part of this study) was however  
298 providing sufficient diversity.

299 Secondly, there appears to be little correlation between the two methods. As could  
300 be expected, a negative trend is observed, as those peptides that are less retained in  
301 the RPLC method were most retained in UC and vice-versa. The polygon drawn with  
302 interrupted lines in Figure 1 is showing the retention space covered by the two  
303 techniques, based on 95% confidence limit. Truly orthogonal methods would have a  
304 square figure for this polygon, while the present polygon leaves significant areas of  
305 white. However, reasonable orthogonality was observed, with a determination  
306 coefficient equal to 0.58. This suggests that the two methods will provide adequate  
307 complementarity also for the purpose of separating impurities from the main peak.

308

### 309 3.2. General chromatographic achievement

310 The main objective of this study was to compare the performance of the UC and  
311 RPLC methods for impurity profiling of short-chain peptide drug candidates. To better  
312 understand the purpose of this method, the general context must be explained. In the  
313 laboratory where this analysis must be conducted, drug candidates are received from  
314 the chemists at an early stage of drug development. At this stage, the possible  
315 impurities are unknown and a quick answer is desirable as to the overall purity of the  
316 main peak, to decide on the necessity to further purify it. Any information about the  
317 number, proportion and identity of impurities is useful and retained for further  
318 development. For this purpose, we first considered the success or failure of each  
319 analysis method for each of the 43 peptides examined. This achievement was  
320 measured based on 3 decision criteria:

- 321 (i) First, the peptide needed to be retained in the chromatographic system and  
322 eluted within the gradient time frame. In other words, a measurable peak  
323 should be observed for the target peptide. In addition, because impurities  
324 are expected to be eluted both before and after the main peak, the fact that  
325 the main peak elutes with sufficient but not excessive retention should be  
326 favorable to the observation of impurities. An elution of the main peptide  
327 occurring 1 min after the dead time and initial baseline disturbances was  
328 considered as “sufficient” retention. Indeed, the major disturbance caused  
329 by DMSO elution occurred at about 2 min in UC, and some baseline  
330 disturbances were observed before 2 min in RPLC, thus “sufficient retention”  
331 was considered at 2 min in RPLC and 3 min in UC. As no peptide was totally  
332 retained on the column, no excessive retention was defined with this set of  
333 analytes.
- 334 (ii) Secondly, the target peptide should show no co-elution with impurities,  
335 based on the examination of UV and MS spectra.
- 336 (iii) Finally, the target peak should not be eluted in a chromatographic area  
337 where some baseline disturbances occur. Indeed, the presence of additives  
338 in the mobile phase, adsorbing on the stationary phase and causing  
339 breakthrough waves when the stationary phase is saturated, is responsible  
340 for baseline disturbances. In the first part of this study [33], the UC generic  
341 gradient had been particularly optimized to minimize the impact of such  
342 baseline disturbances on impurity profiling.

343 The overall achievement considering these three criteria was controlled a first time  
344 based on the generic gradient analyses. In cases of failure, a “focus gradient”,  
345 individually tailored to each peptide, was applied and the achievement was assessed a  
346 second time based on this second, supposedly more resolutive analysis.

347 The results are presented in Figure 2.

348 In UC with the generic gradient, all target peptides were eluted and were  
349 sufficiently retained to ensure adequate measurement of peak purity. A “successful  
350 analysis” was obtained for 81% of the peptides (35 out of 43). The 8 failed cases all  
351 resulted from co-elution of impurities. Consequently, they were re-analyzed with  
352 tailored focus gradients. After this second analysis, only 3 peptides (7% of the cases)  
353 remained as “unsuccessful”, as co-eluted impurities could still be detected. In real-life  
354 operation, the gradient program would be further optimized to achieve complete

355 resolution of impurities [37], but naturally this would require time and efforts, while the  
356 objective here was to compare the methods based on minimal effort.

357 In RPLC with generic gradient, only 42% of the analyses were successful (18 out of  
358 43). Two types of failure were observed. The first case of failure was due to the  
359 absence of retention for 8 target peptides, which eluted in the dead volume, or too  
360 early to ensure accurate purity measurement (19% of cases). Considering the large  
361 proportion of water already present at the beginning of the generic gradient, changes  
362 in the mobile phase gradient are unlikely to solve this issue and a change of stationary  
363 phase would be required to improve their retention. The second case of failure was  
364 due to co-elution of impurities with the target peptide (39% cases). Again, in this case  
365 tailored focus gradients were carried out for each peptide with observed co-elution.  
366 After this second set of experiments, the overall success rate increased to 67% (29  
367 peptides), as 14% of cases (6 peptides) still showed co-elution of impurities.

368 Clearly, the overall success rate of the UC method on this set of peptides was  
369 superior to the success rate of RPLC method. However, an interesting point is that the  
370 two methods appeared to be complementary. Indeed, after the whole process of  
371 generic and focus gradients, only 1 peptide remained as a “failed case”, as it was  
372 unretained in RPLC but showed co-elution of impurities in UC. However, this peptide  
373 was strongly retained in UC method, thus optimization would still be possible in  
374 changing the mobile phase composition or other operating parameters. In conclusion,  
375 all peptides in this set except one could be successfully analyzed with either one or the  
376 other method. This process is summarized in Figure 2.

377 A final comment is that the UC method also seems more advantageous as it is less  
378 labor-intensive to achieve satisfactory results. Indeed, the total number of experiments  
379 carried out in UC was equal to 51 (43 generic gradients plus 8 focus gradients), while  
380 the total number of experiments carried out in RPLC was 61 (43 generic gradients plus  
381 18 focus gradients). An overall performance criterium, defined as the successful cases  
382 divided by the total number of experiments: would yield the following numbers:  $40/51$   
383  $= 0.78$  in UC, and  $29/61 = 0.48$  in RPLC, which is largely in favor of the UC method.

384

### 385 3.3. Performance for impurity profiling

386 After this first general view on the data, it was necessary to go into more details and  
387 assess the methods specifically for the purpose of impurity profiling. For this purpose,  
388 12 peptides (3 linear and 9 cyclic ones) were retained, which were all eluted in a

389 reasonable time (not too early nor too late) with the generic gradients in both methods.  
390 More precisely, the peptides retained for this detailed examination were eluted  
391 between 5.7 and 12.4 min in UC, and between 2.1 and 8.1 min in RPLC. Thus the  
392 performance of the two methods would be evaluated solely on successful cases.

393

### 394 *3.3.1. Purity assessment*

395 A first information that is expected from the method is the overall purity measured  
396 for the target peptide, expressed as a percentage of all peak areas integrated,  
397 measured on the UV chromatograms. Each chromatogram was compared to blank  
398 injection acquired in identical conditions (UC or RPLC, generic or focus gradient) to  
399 assign the minor peaks as impurities. All peaks with peak area superior to 0.1% of the  
400 major peptide were considered. Supposing that all impurities elute within the  
401 chromatographic time frame but are not in dead volume nor are totally retained in the  
402 column, the lowest value obtained for the overall purity should indicate the best  
403 method, because a lower value means that more impurities were resolved from the  
404 main peak. In Figure 3, the values of peak purity are compared for the 12 selected  
405 peptides. In most cases (with the one exception being far from the first bisector), they  
406 appear to be very similar. As they are distributed both sides of the first bisector, none  
407 of the method is consistently better than the other to provide the lowest purity value.  
408 The nature of the peptide (linear or cyclic) seems to have no impact on this  
409 measurement.

410

### 411 *3.3.2. Detection of impurities*

412 The second information expected is the number and concentration of impurities  
413 present in the synthesized peptide. First, we focused on the number of impurities  
414 detected with each method, again considering only those peaks with UV peak area  
415 superior to 0.1% of the main peptide. The best method should be the one showing the  
416 largest number of impurities. In Figure 4a, it appears that, when the number of  
417 impurities was low, the two methods were comparable, with a slight advantage to UC.  
418 On the contrary, when the number of impurities was large, the best method was  
419 always RPLC. No relation to the nature of peptide (linear or cyclic) was observed in  
420 this small set. In the larger set of 43 peptides, more impurities were generally  
421 observed in linear peptides than in cyclic ones, possibly because the cyclic peptides  
422 are more stable. The largest difference observed was of 10 impurities detected in

423 RPLC versus 1 impurity detected with UC. This most significant difference was  
424 observed for the peptide that was the most retained in UC (12.4 min), so it can be  
425 supposed that the missing impurities were too strongly retained to be eluted within the  
426 gradient time. Perhaps, this is an indication that above 12 min retention in UC, the  
427 peptide could be considered as being too much retained to provide meaningful  
428 information on purity assessment, but a single case is not enough to be conclusive.

429 Moreover, to better assess the differences between the two methods, all impurities  
430 observed were further categorized depending on their relative peak area with UV  
431 detection: abundant impurities (area < 1%) and minor impurities ( $0.1\% < \text{area} \leq 1\%$ ).  
432 Impurities below 0.1% were not considered. The results are shown in Figure 4b. It  
433 appears that the advantage of the RPLC method seems mostly related to minor  
434 impurities. This is consistent with the fact that overall purity measurements did not  
435 show much difference between the two methods (Figure 3).

436 There may be two reasons for the better performance of RPLC method. A first  
437 reason is that the resolving power in RPLC would be superior, thus showing more  
438 peaks resolved. In UC, these impurities would be either co-eluted with the major peak  
439 of the target peptide, or co-eluted with other impurities (thereby reducing the overall  
440 number of peaks observed). The impurities might also be eluted in areas where they  
441 cannot be measured, very early or very late in the chromatograms, or where a  
442 baseline disturbance is complicating accurate measurement. Such cases would be  
443 related to the cases in Figure 3 where the measured purity of the target peak was  
444 larger in UC than in RPLC (points below the first bisector).

445 To examine the hypothesis of a higher resolving power in RPLC, peak capacity  
446 was measured. The evaluation of peak capacity was carried out on the 12 peptides.  
447 For each of them, the peak width at 50% height was measured. It is interesting to note  
448 that peak width in UC showed no relation to retention time and was rather constant  
449 (similar to LC), confirming that the strategy of progressively reducing the mobile phase  
450 flow rate to better approach the optimum flow rate [24,33] that is decreasing along the  
451 gradient [38,39] was not detrimental to peak width. In Table 2, the average maximal  
452 peak capacity was calculated for both generic gradient methods. A large difference  
453 appears, as UC peak capacity ( $P_c = 189$ ) is significantly lower than RPLC ( $P_c = 314$ ).  
454 Gradient times were similar (10 vs. 10.9 min) so this significant difference principally  
455 issues from the larger peaks observed in UC (about 60% larger, on average). To a  
456 certain extent, this may be related to the different column and particle formats

457 employed in this study as the RPLC method was done with sub-2  $\mu\text{m}$  fully porous  
458 particles, while the UC method was done with sub-3  $\mu\text{m}$  superficially porous particles.  
459 This observation is also in line with previous studies comparing RPLC to SFC analysis  
460 of small chemical drugs on sub-2  $\mu\text{m}$  fully porous particles [34], where the measured  
461 efficiency was higher in RPLC than SFC. It is also possible that the slow interaction  
462 kinetics due to polar interactions in UC would cause larger band broadening than the  
463 hydrophobic interactions in RPLC. It is also worth mentioning that peak asymmetry  
464 was not responsible for the larger peaks observed in UC, as the average peak  
465 symmetry in this set was  $A_{\text{SEP}} = 1.10$ , slightly better than the average 1.23 measured  
466 in LC. In general, it was observed that the peak symmetry on the main peak was better  
467 in UC chromatograms than in LC chromatograms, where peak asymmetry frequently  
468 exceeded 1.2. In UC chromatograms, asymmetry was slightly worse for late-eluting  
469 peaks than for early-eluting peaks.

470 A second reason that could explain the superior performance of the RPLC method  
471 is that the sensitivity would be higher, which should principally show in the number of  
472 minor impurities detected. Indeed, as appears in Figure 4b, the increased number of  
473 impurities observed in RPLC was essentially due to minor impurities ( $0.1\% < \text{area} \leq$   
474  $1\%$ ). For the set of 12 well-eluted peptides, the UV signal-to-noise ratio was higher in  
475 RPLC than in UC. Independently of the UV detector performance, this is consistent  
476 with larger peaks observed in UC. This sensitivity issue is also well known by SFC-UV  
477 chromatographers. Indeed, although modern instruments have largely improved  
478 sensitivity compared to older instruments, it is still not as good as comparable  
479 instruments in liquid conditions. This is partly inherent to the way the system is  
480 working: pressure regulation causes a constant variation of fluid density, which is  
481 causing a constant variation of refractive index, thus causing baseline noise in UV [40].  
482 Baseline compensation is somewhat improving this problem but not enough to reach  
483 the sensitivity of LC systems.

484

### 485 3.3.3. Impurity profiling

486 The 12 peptides were investigated in great details on their impurity profiles, trying  
487 to match the information from both methods based on peak area and MS spectra. Two  
488 representative examples are shown in Figure 5 with a linear peptide (Figure 5a) and a  
489 cyclic peptide (Figure 5b). In these figures, the main peptide is identified with a red  
490 square, and was of course detected with both methods. The impurities that were



491 observed with both methods and could be matched are identified with yellow circles.  
492 The impurities that were observed with only one method are identified with green  
493 triangles. On this figure, they are placed on the right and top of the figure to indicate  
494 that they were seen with UC (abscissa) or with RPLC (ordinates) only.

495 For the linear peptide, 4 impurities were observed with both methods (yellow  
496 circles). In addition, 1 impurity was observed with UC only (green triangle at the  
497 bottom), while 4 impurities were observed with RPLC only (green triangles on the left).  
498 It also appears that the impurities were eluted over a wider time frame in UC than in  
499 RPLC. This is consistent with the above observation that retention times of the target  
500 peptides in UC were scattered in a wider time frame than in RPLC (Figure 1).

501 For the cyclic peptide, 6 impurities were observed with both methods (yellow  
502 circles). In addition, 3 impurities were observed with UC only (green triangles at the  
503 bottom), while 5 impurities were observed with RPLC only (green triangles on the left).  
504 For this sample, some impurities appeared at the end of the UC gradient that were  
505 difficult to identify. As the proportion of co-solvent is high at this point, MS sensitivity is  
506 somewhat less than at the beginning of the gradient. Further improvement of MS  
507 parameters might improve identification of late-eluting impurities.

508 Among the 12 cases examined in detail, the complementarity of the two techniques  
509 was evident. While the RPLC method often showed more minor impurities than the UC  
510 method, the two methods mostly provided different information.

511

#### 512 3.4. Resolution of isomers

513 The separation of isomeric species is of interest as isomeric impurities are often  
514 present in synthesized peptides. In the set of 43 peptides that could be analyzed with  
515 both UC and RPLC methods, no isomeric pairs were present for the linear peptides,  
516 but 10 isomeric pairs or trios were present for cyclic peptides. When enough retention  
517 could be obtained in RPLC, the resolution of isomeric pairs was generally good with  
518 RPLC method. In UC, where all peptides were eluted in an adequate retention time,  
519 some isomeric pairs were well resolved while others remained co-eluted. Because the  
520 set of compounds was very small for this comparison, it is impossible to draw any  
521 conclusion as to the reason for success or failure of isomers separation in one or the  
522 other method. Sample chromatograms are presented in Figure 6, to illustrate the  
523 different capabilities for isomer separation. In Figure 6a, a case where UC offered  
524 superior resolution, is observed with positional isomers: CP 18791 was -R<sub>1</sub>-R<sub>2</sub>-R<sub>3</sub>-R<sub>4</sub>-

525 R<sub>5</sub>- while CP 18787 was -R<sub>1</sub>-R<sub>4</sub>-R<sub>3</sub>-R<sub>2</sub>-R<sub>5</sub>-, *i.e.* the positions of R<sub>2</sub> and R<sub>4</sub> were  
526 exchanged. In Figure 6b a case where RPLC offered superior resolution is observed  
527 with a diastereomeric pair, where CP 19846 was -R<sub>1</sub>-(D-R<sub>2</sub>)-R<sub>3</sub>-R<sub>4</sub>-R<sub>5</sub>- and CP 19850  
528 was -R<sub>1</sub>-(L-R<sub>2</sub>)-R<sub>3</sub>-R<sub>4</sub>-R<sub>5</sub>-, *i.e.* one amino acid had opposite absolute configuration in  
529 the two peptides (D/L). Figure 6 also shows that the two techniques provided  
530 comparable chromatographic quality, as previously mentioned when comparing  
531 significant features. Finally, only one isomeric pair could not be resolved with any of  
532 the two methods, again supporting the high complementarity of the UC and RPLC  
533 methods.

534

### 535 3.5. Robustness

536

537 The robustness of the well-established LC method was not assessed. The UC  
538 method, however, required robustness assessment as it was little explored so far. For  
539 this purpose, a Doehlert design of experiment [41] was achieved with three variable  
540 parameters: back-pressure, column temperature and final gradient composition. The  
541 limit values of the three parameters were voluntarily chosen to be large (20 bars, 20°C  
542 and 20 % of co-solvent). Indeed, they are much larger than what would occur in  
543 normal operation of the instrument, where the accepted variations of instrument  
544 control are about 2 bar, 0.5°C and 2% respectively for back-pressure, temperature and  
545 co-solvent proportion. The DoE matrix is detailed in in Figure S3 (supplementary  
546 information). Retention factor for one peptide eluting about mid-gradient (with retention  
547 time of about 7 min) was examined to assess its variability with these three  
548 parameters. The results are presented in Figure 7 and Table S2. The model was well-  
549 fitted, as appears in Figure S3 with  $R^2 = 0.982$  and a confidence interval of 0.2.  
550 Unsurprisingly, the most influent parameter was the solvent proportion, while back-  
551 pressure and temperature were found to be insignificant. Indeed, the large proportion  
552 of co-solvent along the gradient and particularly at the end of the gradient make it a  
553 low-compressibility fluid, on which pressure and temperature can have only little  
554 impact. Whatever the fluid density and compressibility, the effect of changing mobile  
555 phase composition is still significant as it will impact the elution strength through  
556 changes in polarity, similar to what would be observed in liquid chromatography.  
557 However, this impact is largely acceptable as it appears that the normal range of  
558 variation that is considered acceptable in instrument control (typically less than 2%

559 variation in the proportion of co-solvent) would yield a variation of retention factor of  
560 about 0.1. This corresponds to a variation in retention time of about 0.06 min. As a  
561 result and taking account of the instrument reliability, the method looks sufficiently  
562 robust to be applied as a generic method in the research and development context.

563

### 564 3.6. Operational cost comparison

565

566 Finally, comparing the operational cost of the two techniques may be of use. For  
567 this purpose, the solvent consumption per analysis was computed for the generic  
568 gradient methods. The volume of solvent used (and thus requiring waste treatment  
569 after analysis) with the RPLC method was about 10 mL acetonitrile-water, while the  
570 volume of solvent used (and requiring waste treatment) with the UC method was 11.9  
571 mL methanol-water. Because acetonitrile is much more expensive than methanol and  
572 because carbon dioxide is relatively cheap, the final cost per sample was nearly the  
573 same: about 2.3 € per sample. Of course, the UC equipment is somewhat more  
574 expensive than the RPLC equipment. However, we must point out that the high  
575 success rate of the UC generic method is also providing operational cost savings as it  
576 does not require additional expenses to re-analyse the sample with focus gradient or  
577 another analytical method.

578

579

## 580 4. Conclusions and perspectives

581

582 Orthogonality between the UC and RPLC methods on this set of peptides is  
583 somewhat less significant than was previously observed when small chemical drugs  
584 were compared between SFC and RPLC. However, the UC method is particularly  
585 interesting for the cases when RPLC retention is too low, which occurred frequently for  
586 these small, rather polar molecules. In the present test set, 19% of the samples could  
587 not be analyzed with the RPLC method while all of them had adequate retention in  
588 UC. When adequate retention could be obtained, less effort was required with UC than  
589 with RPLC to obtain satisfying results, as the generic gradient was most successful  
590 while the RPLC method often required a second experiment with focus gradient. In  
591 terms of capability for impurity profiling, the RPLC method seems superior as it was  
592 capable to detect more minor impurities, either due to higher peak capacity or possibly

593 due to higher sensitivity with UV detection. However, the UC and RPLC methods are  
594 most complementary as different impurities could be observed between the two  
595 methods. As a conclusion, the joint use of the two methods is advisable to improve the  
596 chances of successful analysis with a minimum effort and to obtain a maximum  
597 amount of information on a single sample.

598

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600 The authors declare they have no conflict of interest.

601

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614

### 615 **CRedit author statement**

616 **Jérémy Molineau:** Methodology, Formal analysis, Investigation, Writing – Original  
617 Draft, Visualization, **Maria Hideux:** Resources, Writing – Review & Editing,  
618 Supervision, **Philippe Hennig:** Conceptualization, Resources, Funding acquisition,  
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623

624

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776



777 **Figure captions**

778

779 **Figure 1.** Comparison of the values of elution composition obtained with the LC  
780 method and the UC method, based on the set of 43 peptides and according to Eq. (1).  
781 Blue dashes are linear peptides; orange circles are cyclic peptides. The central grey  
782 line is the correlation line; the grey interrupted lines are the 95% confidence limits  
783 (indicating that 95% of the points in a random set of analytes should be comprised  
784 within these lines) and delimit the retention space covered by the two techniques.

785

786 **Figure 2.** Cases of failures observed among the 43 peptides analyzed with both  
787 gradient methods (generic and focus) in UC and RPLC

788

789 **Figure 3.** Comparison of peptide purity measured with both generic gradient methods,  
790 for 12 peptides that were successfully analyzed with both methods. Blue dashes are  
791 linear peptides; orange circles are cyclic peptides. The interrupted grey line is the first  
792 bisector, indicating identical values measured with both methods.

793

794 **Figure 4.** Comparison of the number of impurities observed with both generic gradient  
795 methods, for 12 peptides that were successfully analyzed with both methods. (a) Total  
796 number of impurities observed for linear peptides (blue dashes) and cyclic peptides  
797 (orange circles). (b) Impurities with large concentration (>1%, black diamonds) and low  
798 concentration (<1%, open squares). The interrupted grey line is the first bisector,  
799 indicating identical values measured with both methods.

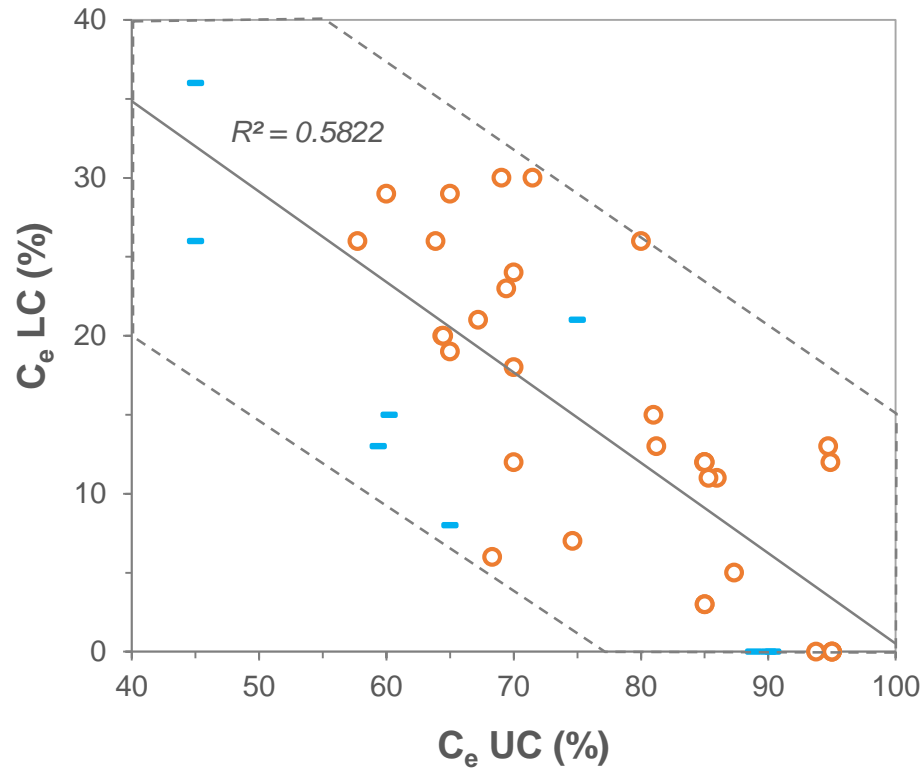
800

801 **Figure 5.** Comparison of retention times obtained for the API (red square) and  
802 impurities. Yellow circles are impurities observed with both RPLC and UC methods,  
803 green triangles are impurities observed with only one method: green triangles at the  
804 top of the figure were seen with UC only, green triangles at the right of the figure were  
805 seen with RPLC only.

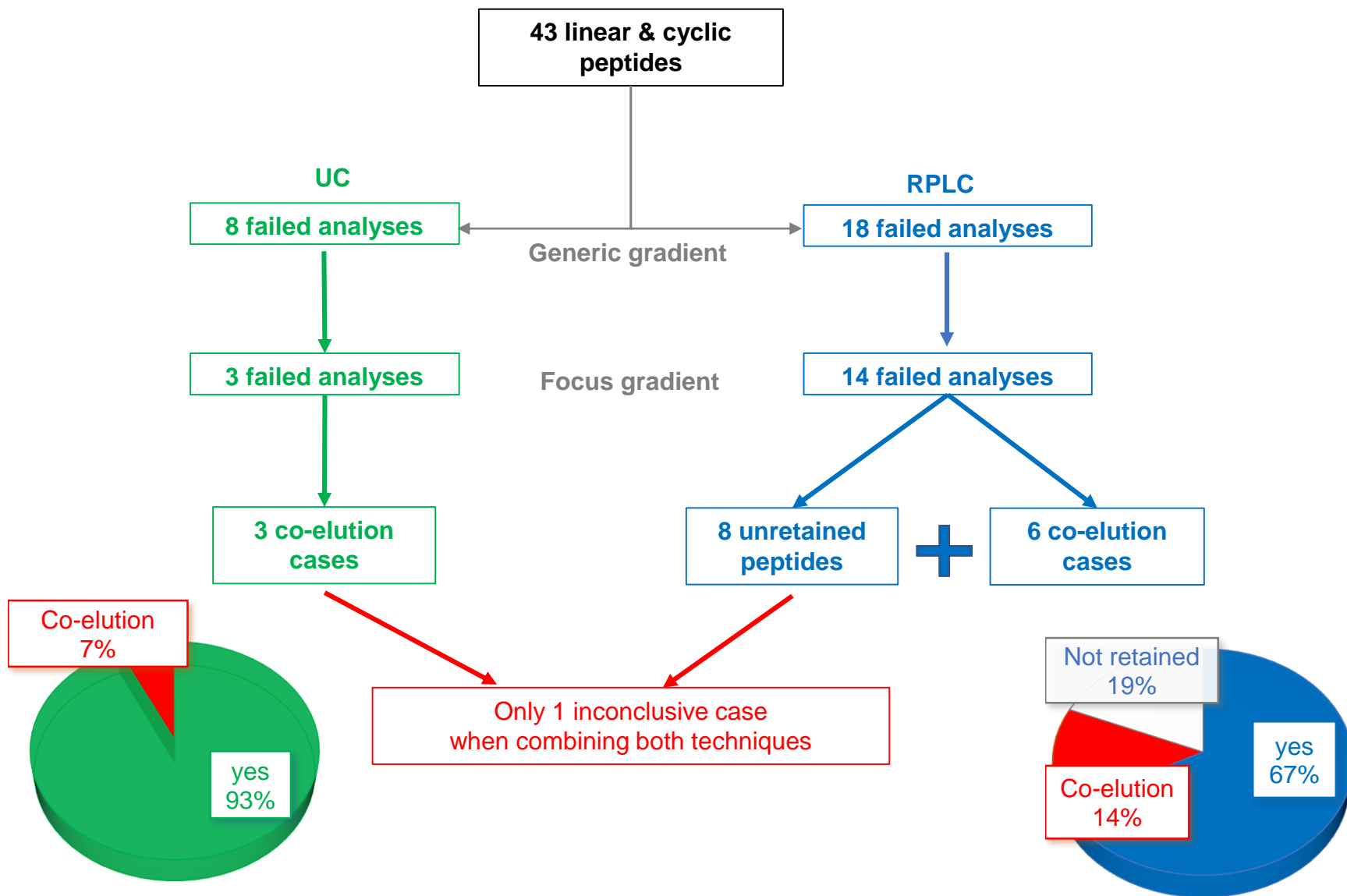
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807 gradient conditions (a) cyclic pentapeptides better resolved with UC (b) cyclic  
808 pentapeptides better resolved with RPLC.

809 **Figure 7.** Design of experiment to assess the variation of retention factor in UC  
810 conditions. (a) Effect of back-pressure vs. final gradient composition. (b) Effect of oven

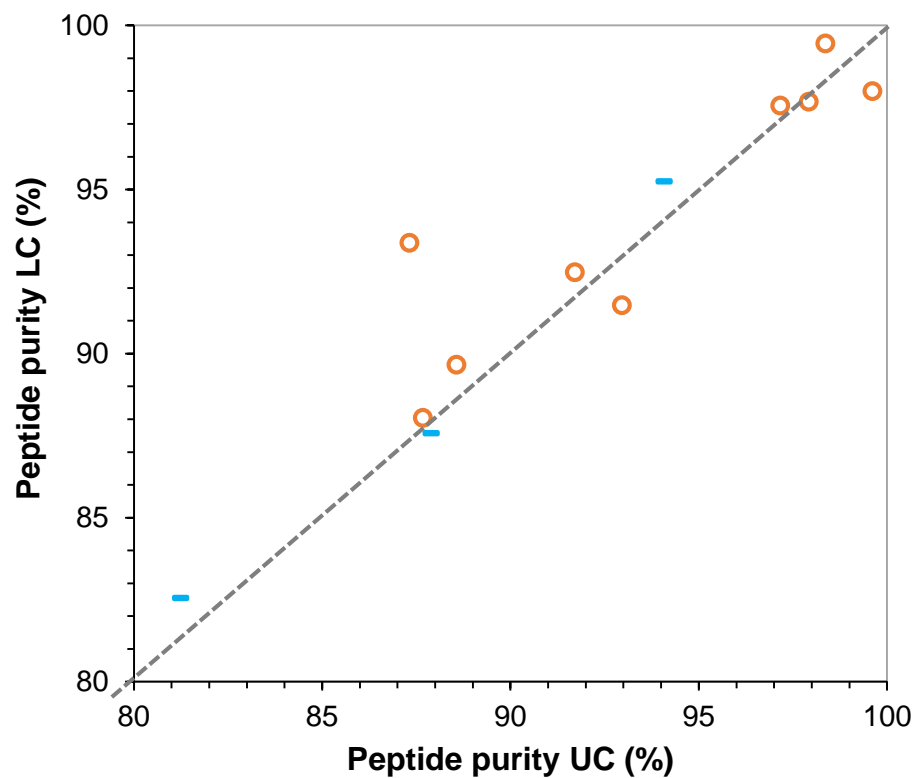
811 temperature vs. final gradient composition. The stars indicate the set conditions in  
812 generic UC gradient.  
813



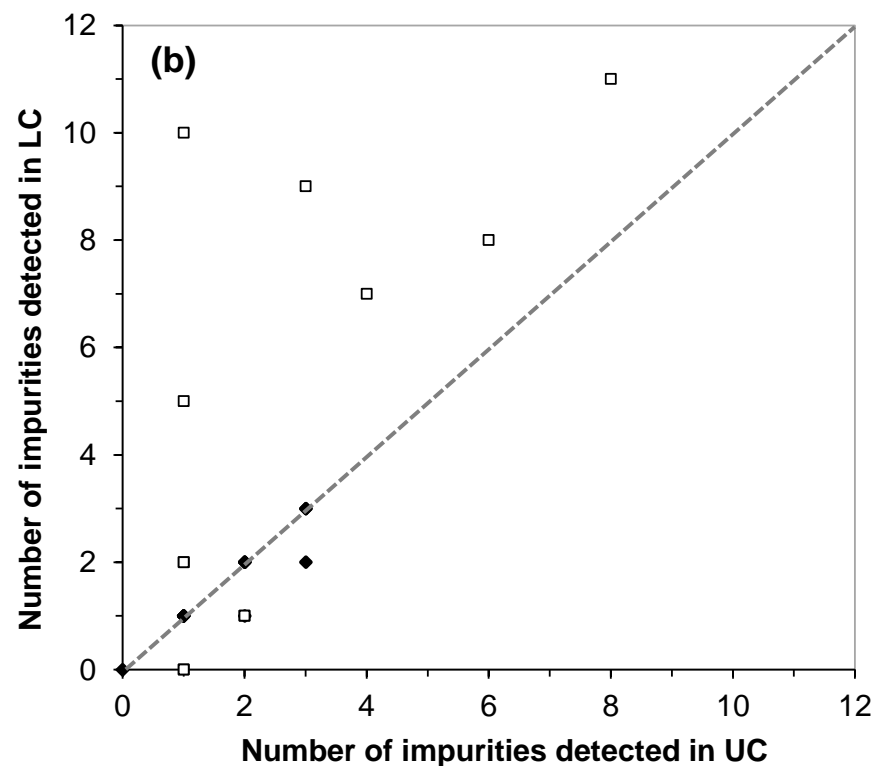
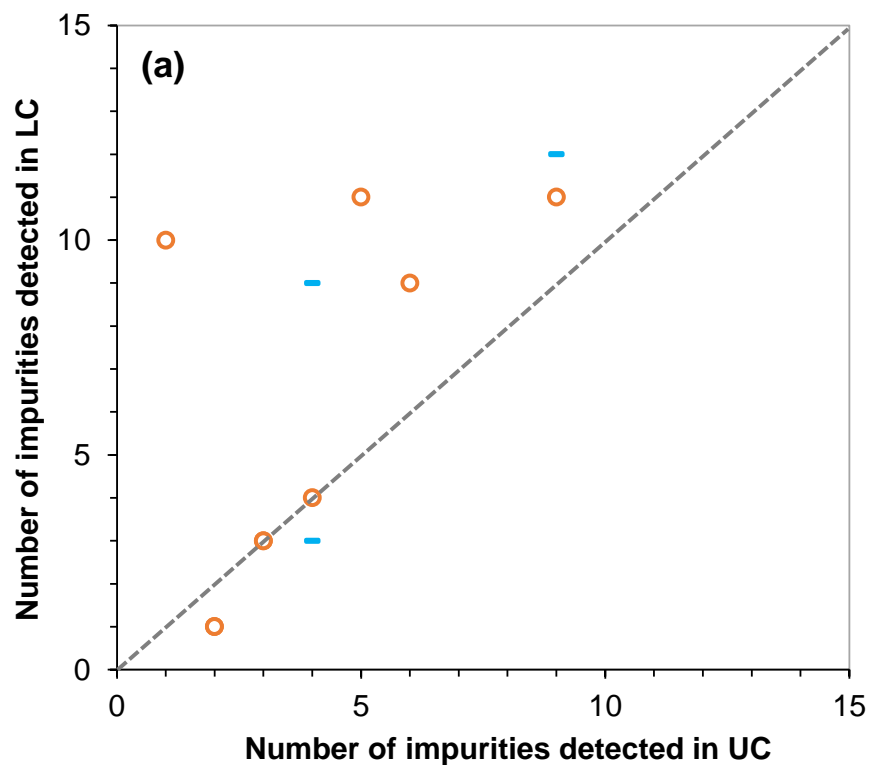
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**Figure 2.** Cases of failures observed among the 43 peptides analysed with both gradient methods in UC and RPLC.

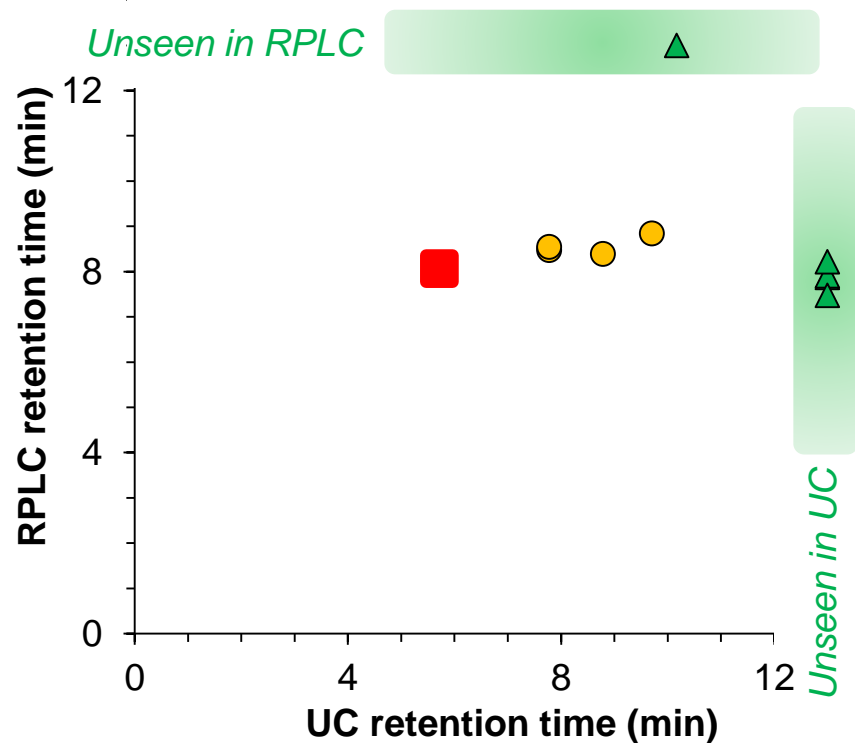


**Figure 3.** Comparison of peptide purity measured with both generic gradient methods, for 12 peptides that were successfully analyzed with both methods. Blue dashes are linear peptides; orange circles are cyclic peptides. The interrupted grey line is the first bisector, indicating identical values measured with both methods.

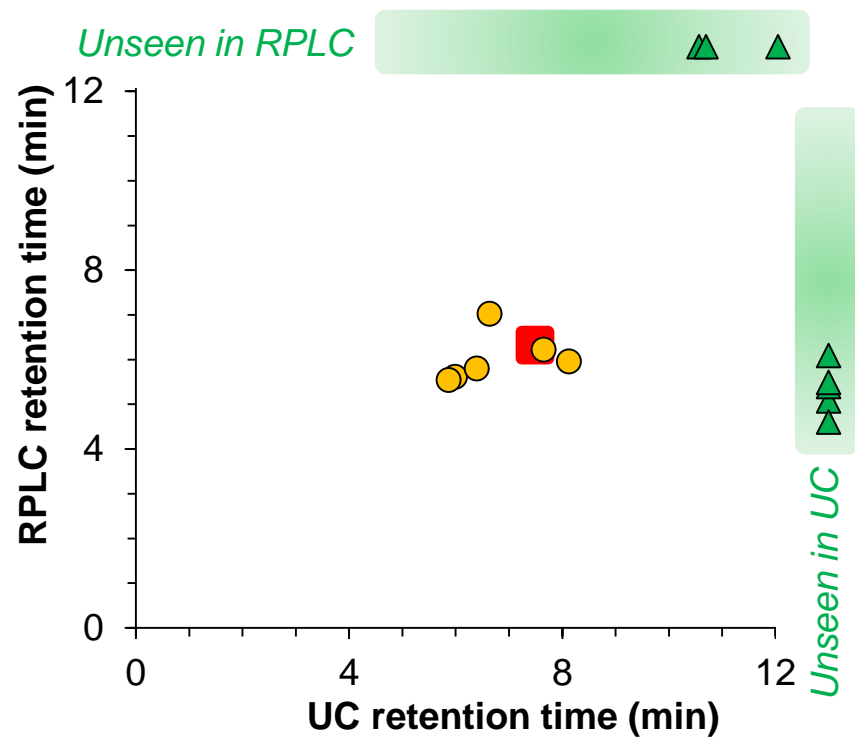


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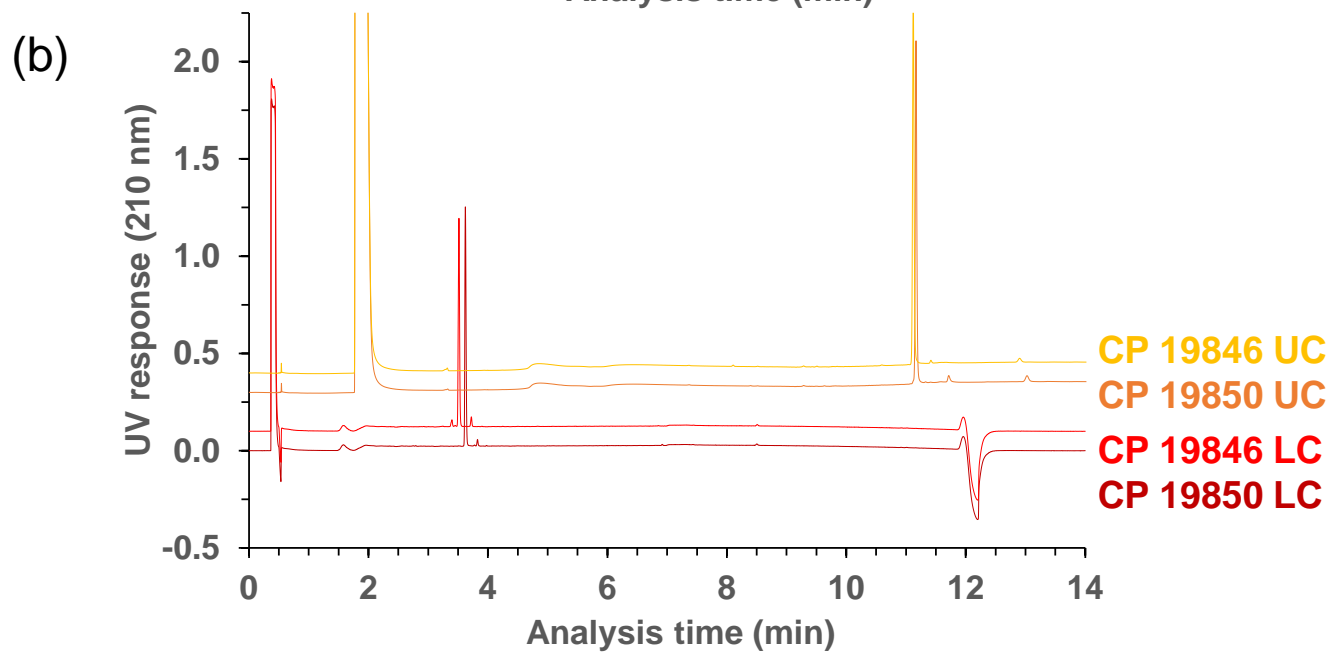
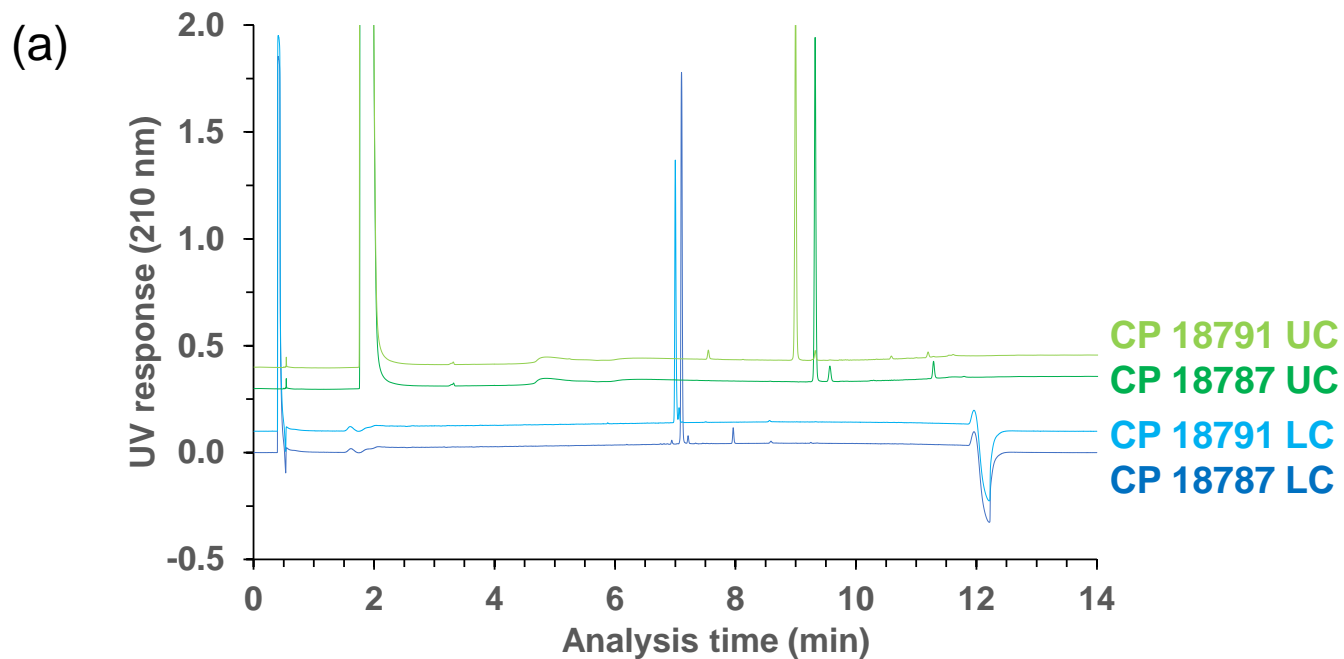
(a) linear peptide



(b) cyclic peptide

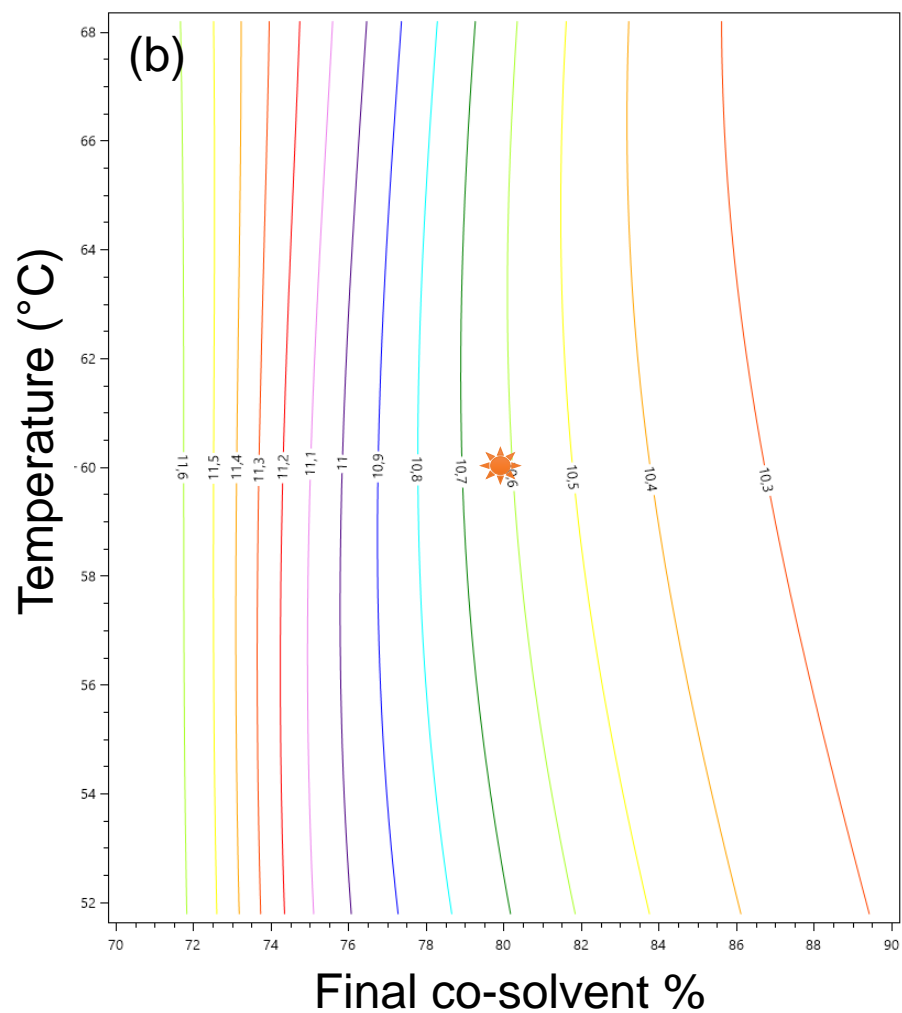
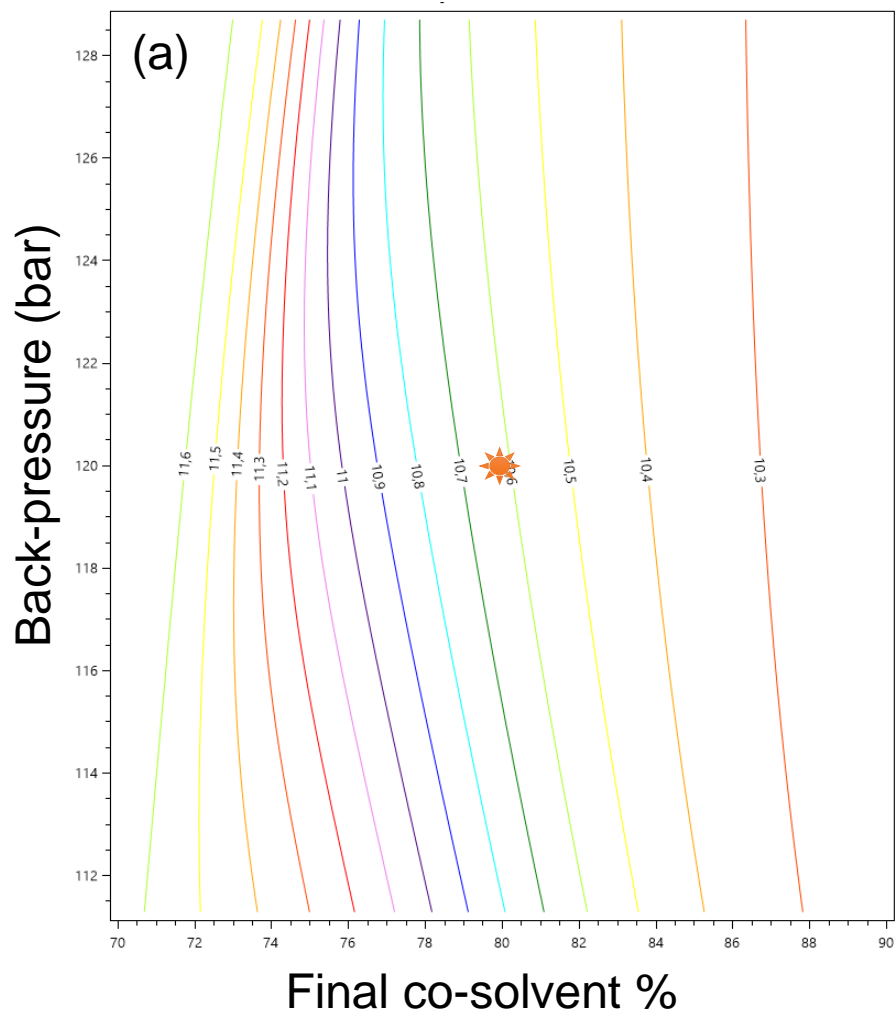


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**Figure 6.** Chromatograms for two pairs of isomers in UC and RPLC under generic gradient conditions (a) cyclic pentapeptides better resolved with UC (b) cyclic pentapeptides better resolved with RPLC.





**Figure 7.** Design of experiment to assess the variation of retention factor in UC conditions. (a) Effect of back-pressure vs. final gradient composition. (b) Effect of oven temperature vs. final gradient composition. The stars indicate the set conditions in generic UC gradient.

**Table 1.**

Four gradient methods employed in this study.

(a) UC Generic gradient

Gradient time (min)	Cosolvent proportion (%)	Flow rate (ml/min)
0	5	3
0.5	5	3
10.5	80	1.5
13.5	80	1.5
14	5	1.5
15	5	3

(b) UC Focus gradient

Gradient time (min)	Cosolvent proportions (%)	Flow rate (ml/min)
0	5	1.5
0.5	5	1.5
1.5	$C_e - 5\%$	1.5
11.5	$C_e + 5\%$	1.5
11.6	100	1.5
14.6	100	1.5
14.7	5	1.5
15	5	1.5

(c) RPLC Generic gradient

Gradient time (min)	A% (H <sub>2</sub> O/ACN/TFA)	B% (ACN/H <sub>2</sub> O/TFA)
0	100	0
0.6	100	0
11.5	40	60
11.6	100	0
14.3	100	0

(d) RPLC Focus gradient

Gradient time (min)	A% (H <sub>2</sub> O/ACN/TFA)	B% (ACN/H <sub>2</sub> O/TFA)
0	$100 - (C_e - 3\%)$	$C_e - 3\%$
0.6	$100 - C_e$	$C_e$
11.4	$100 - (C_e + 3\%)$	$C_e + 3\%$
11.5	$100 - (C_e - 3\%)$	$C_e - 3\%$
14.3	$100 - (C_e - 3\%)$	$C_e - 3\%$

**Table 2.**

Determination of peak capacity with the two generic methods based on 12 peptides well eluted with both methods

<b>Method</b>	<b>UC</b>	<b>RPLC</b>
Gradient time (min)	10	10.9
Average peak width at 50% height	0.032	0.021
Peak capacity	189	314
RSD%	30.4%	29.9%