

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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- 19 Abstract
- 20

In the first part of this study, a unified chromatography (UC) analysis method, which is 21 similar to supercritical fluid chromatography (SFC) but with wide mobile phase 22 gradients of pressurized CO₂ and solvent, was developed to analyse short-chain 23 peptides, with UV and mass spectrometry (MS) detection. In this second part, the 24 method is compared to a reference reversed-phase ultra-high-performance liquid 25 chromatography (RP-UHPLC) method, based on the analysis of 43 peptides, including 26 10 linear peptides and 33 cyclic ones. 27 First, the orthogonality between the two methods was examined, based on the 28 retention patterns. As the UC method was developed on a polar stationary phase 29 (Ascentis Express OH5), the elution orders and selectivities were expected to be 30 31 significantly different from RPLC on a non-polar stationary phase (ACQUITY CSH C18). Secondly, the success rate of the methods was examined, based on successful 32 33 retention / elution of the peptides and the absence of observed co-elutions between the main peak and impurities. A successful analysis was obtained for 81% of the 34 35 peptides in UC and 67% in RPLC. Thirdly, the performance of the methods for the intended application of impurity profiling of peptide drug candidates was assessed, 36 based on the comparison of peak purities, the number of impurities detected and the 37 thorough examination of impurity profiles. Excellent complementarity of the two 38 methods for the specific task of impurity profiling, and for the separation of isomeric 39 species was observed, with only one isomeric pair in this set remaining unresolved. 40 The method sensitivity was however better with RPLC than UC. Finally, the 41 operational costs in terms of solvent cost per analysis were the same between the two 42 methods. 43 44 45

46 Keywords

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

- 50 **1. Introduction**
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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 59 reviews [3,4]. In addition, pharmaceutical peptides of biological or synthetic origin 60 under 5 000 Da and commercialized in northern countries (Europe, Japan and North 61 62 America) for therapeutic and diagnostic applications are all freely listed in the PepTherDia database (http://peptherdia.herokuapp.com/) [5]. This database currently 63 comprises around 100 compounds information such as physicochemical and 64 pharmacokinetic properties. Examining this database, it appears that short-chain 65 peptides (between 300 Da and 2000 Da) stand for 75% of commercialized 66 pharmaceutical peptides. Among them, about a third (a quarter of the total) has a 67 molecular weight below 1000 Da. Until the 1990s, most of the therapeutic peptides 68 entering in clinical study were short-chain peptides [4]. Then, as the synthesis and 69 manufacturing technologies improved [6,7], peptides with higher molecular weights 70 71 were more often described. Short-chain peptides however continued to be developed in a steady fashion. 72

Reversed-phase liquid chromatography (RPLC) is the most employed method for 73 peptide analysis [8–11] because of its robustness, high-resolution power, short 74 analysis times and compatibility to mass spectrometry (MS). Different authors 75 described RPLC as a flexible and versatile technique, which could also explain its 76 77 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, 78 especially with isobaric compounds are isomeric species are frequent in such 79 mixtures. Complementary techniques are always desirable to obtain a more complete 80 picture of a sample [13]. Besides, solubilization of the peptides in adequate dilution 81 solvent and carryover effects have been reported as significant issues in RPLC 82 83 [14,15]. In addition, retention in RPLC is mostly governed by hydrophobic interactions

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

96 Looking for complementary methods that could cover a wide polarity range for short-chain and long-chain peptides, and could possibly be transferred to preparative 97 scale when needed, Unified Chromatography (UC) appears as an interesting option. 98 Indeed, UC mobile phase gradients may start with a non-polar composition comprising 99 large proportions of pressurized carbon dioxide (usual working range of supercritical 100 fluid chromatography – SFC), and end with a rather polar composition containing large 101 proportions of liquid solvents (usual working range of enhanced fluidity liquid 102 chromatography – EFLC), possibly ending in pure-liquid solvent conditions (LC). This 103 promises the possible analysis of non-polar and polar analytes in a single run [22], or 104 makes it possible to propose a single and generic method to analyze both non-polar 105 and polar peptides. Although carbon-dioxide mobile phases are usually perceived as 106 being incapable to elute polar analytes, SFC, EFLC and UC have all been 107 demonstrated to be useful for this purpose [23-27]. Some examples of peptides and 108 proteins separations by SFC, EFLC or UC appeared in literature recently [28-30]. 109 However, most of the peptides appearing in previous works had molecular weights 110 111 above 1000 Da, which set different issues in terms of solubility and multiple conformations than short-chain peptides [31]. Further information on the topic of 112 biomolecules analysis with SFC, EFLC and UC can be found in a recent review [32]. 113 In the first part of this series of papers [33], a UC method with UV and ESI-MS 114 detection was developed for the purpose of determining the purity of peptide drug 115 candidates, with molecular weights below 1000 Da. The method was developed on a 116 117 modern instrument included reduced dead volumes, and with a stationary phase

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

- 133
- 134 **2. Material and methods**
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2.1. Chemicals and solvents

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

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

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

For UC analysis, gradient-grade MeOH was purchased from VWR (Fontenay-154 sous-bois, France) and CO₂ with a purity of 99,995% was provided by Air Liquide 155 (Bagneux, France). Water was provided by Purelab flex from Veolia (Wissous, 156 France). Ammonium hydroxide solution was obtained from Sigma-Aldrich (Merck, 157 France). For RPLC analysis, HPLC-grade acetonitrile (ACN) was purchased from 158 Merck (VWR international SAS, France), water was obtained from a Milli-Q Purification 159 160 System from Millipore (Millipore SAS, France) and trifluoroacetic acid (TFA) for spectroscopy was obtained from Merck (VWR International SAS, France). 161

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2.2. UC-MS Instrument

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

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2.3. UC-MS method

The UC-MS method development was previously described [33]. The stationary 173 phase was Ascentis Express OH5 (150 x 4.6 mm; 2.7 µm) from Sigma Aldrich. The 174 mobile phase was a CO₂-cosolvent gradient, with a cosolvent composed of MeOH 175 containing 2% H₂O (v/v) and 20 mM NH₄OH. Two different methods were used. The 176 first one, referred to as "generic gradient", is the gradient optimized in the first part of 177 this series. The co-solvent gradient starts at 5% and reaches 80% in 10 min, together 178 179 with a reverse flow-rate gradient from 3.0 to 1.5 ml/min (see details in Table 1a). Total run duration is 15 min. A second gradient, referred to as "focus gradient", was 180 developed in the course of this study, to enhance the resolution of impurities eluting 181 close to the target peptide. The co-solvent gradient is adjusted individually for each 182 target peptide based on its elution composition Ce, corresponding to the proportion of 183 co-solvent necessary to elute the analyte with the generic gradient. A slow gradient is 184 185 then conducted with a co-solvent proportion varying from $C_e - 5\%$ to $C_e + 5\%$ in 10

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

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

198 When the proportion of co-solvent is low (at the beginning of UC gradient), it is useful to introduce an additional flow of liquid prior to entering the MS. It is however 199 200 unnecessary when the proportion of co-solvent reaches high values (at the end of UC gradient). Consequently, the MS make-up flow-rate for "large gradient" also applied a 201 202 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 analyzed with a focus gradient were eluted with a large proportion of co-solvent. The 204 composition of make-up fluid was identical to that of the chromatographic co-solvent 205 (MeOH comprising 2% H₂O and 20 mM NH₄OH). 206

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2.4. UHPLC-MS Instrument

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

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

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2.5. RP-UHPLC-MS method

The RP-UHPLC-MS method serving here as reference has been routinely used at 225 Servier Research Laboratories since 2016 as a generic method to analyze peptides of 226 varied sizes and polarities. The stationary phase was ACQUITY CSH C18 (100 × 2.1 227 228 mm, 1.7 μ m) from Waters. Mobile phase A was H₂O / ACN / TFA 100/1/0.1 (v/v/v) and mobile phase B was ACN / H₂O / TFA 100/1/0.1 (v/v/v). Similarly to the UC methods, 229 230 two different gradient programs were used: a large gradient referred to as "RPLC generic gradient" and a slower gradient focused on the target peptide, individually 231 232 adapted to each peptide, referred to as "RPLC focus gradient". In the generic gradient, the B solvent proportion was increased from 0 to 60% in 11 min (see details in Table 233 234 1c). The total analysis time was 14.3 min. In focus gradient, the B solvent proportion was slowly increased from C_e -3% to C_e +3. Details are provided in Table 1d. 235 236 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 blank and peptide analysis. The lower injected volume was necessary to maintain 238 comparable ratios of injected volume to column volume between the UC and RPLC 239 methods (0.3 vs. 0.4%). 240

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

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2.6. Design of experiment for UC robustness assessment

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To assess the method robustness, a Design of Experiments (DoE) with
 Doehlert matrix was carried out. The DoE was prepared and evaluated with Ellistat
 software version 6.5 2021/01 (Ellistat, Chavanod, France). Three parameters were

considered: back-pressure (ranging from 111.3 to 128.7 bar), column oven

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

- 257
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- 259 3. Results & Discussion
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3.1. Orthogonality of the two methods

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

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

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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.

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

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

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

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

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3.2. <u>General chromatographic achievement</u>

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

(i) First, the peptide needed to be retained in the chromatographic system and 321 eluted within the gradient time frame. In other words, a measurable peak 322 should be observed for the target peptide. In addition, because impurities 323 are expected to be eluted both before and after the main peak, the fact that 324 the main peak elutes with sufficient but not excessive retention should be 325 favorable to the observation of impurities. An elution of the main peptide 326 occurring 1 min after the dead time and initial baseline disturbances was 327 considered as "sufficient" retention. Indeed, the major disturbance caused 328 by DMSO elution occurred at about 2 min in UC, and some baseline 329 disturbances were observed before 2 min in RPLC, thus "sufficient retention" 330 was considered at 2 min in RPLC and 3 min in UC. As no peptide was totally 331 retained on the column, no excessive retention was defined with this set of 332 333 analytes.

- 334 (ii) Secondly, the target peptide should show no co-elution with impurities,
 335 based on the examination of UV and MS spectra.
- (iii) Finally, the target peak should not be eluted in a chromatographic area
 where some baseline disturbances occur. Indeed, the presence of additives
 in the mobile phase, adsorbing on the stationary phase and causing
 breakthrough waves when the stationary phase is saturated, is responsible
 for baseline disturbances. In the first part of this study [33], the UC generic
 gradient had been particularly optimized to minimize the impact of such
 baseline disturbances on impurity profiling.
- The overall achievement considering these three criteria was controlled a first time based on the generic gradient analyses. In cases of failure, a "focus gradient", individually tailored to each peptide, was applied and the achievement was assessed a

346 second time based on this second, supposedly more resolutive analysis.

The results are presented in Figure 2.

In UC with the generic gradient, all target peptides were eluted and were sufficiently retained to ensure adequate measurement of peak purity. A "successful analysis" was obtained for 81% of the peptides (35 out of 43). The 8 failed cases all resulted from co-elution of impurities. Consequently, they were re-analyzed with tailored focus gradients. After this second analysis, only 3 peptides (7% of the cases) remained as "unsuccessful", as co-eluted impurities could still be detected. In real-life operation, the gradient program would be further optimized to achieve complete resolution of impurities [37], but naturally this would require time and efforts, while theobjective here was to compare the methods based on minimal effort.

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

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

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

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3.3. <u>Performance for impurity profiling</u>

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

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

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3.3.1. Purity assessment

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

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3.3.2. Detection of impurities

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

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

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

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

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

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

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

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3.3.3. Impurity profiling

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

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

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

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

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3.4. <u>Resolution of isomers</u>

The separation of isomeric species is of interest as isomeric impurities are often 513 present in synthesized peptides. In the set of 43 peptides that could be analyzed with 514 both UC and RPLC methods, no isomeric pairs were present for the linear peptides, 515 but 10 isomeric pairs or trios were present for cyclic peptides. When enough retention 516 could be obtained in RPLC, the resolution of isomeric pairs was generally good with 517 518 RPLC method. In UC, where all peptides were eluted in an adequate retention time, some isomeric pairs were well resolved while others remained co-eluted. Because the 519 520 set of compounds was very small for this comparison, it is impossible to draw any conclusion as to the reason for success or failure of isomers separation in one or the 521 522 other method. Sample chromatograms are presented in Figure 6, to illustrate the different capabilities for isomer separation. in Figure 6a, a case where UC offered 523 524 superior resolution, is observed with positional isomers: CP 18791 was -R₁-R₂-R₃-R₄-

R₅- while CP 18787 was -R₁-R₄-R₃-R₂-R₅-, *i.e.* the positions of R₂ and R₄ were 525 exchanged. In Figure 6b a case where RPLC offered superior resolution is observed 526 with a diastereomeric pair, where CP 19846 was -R₁-(D-R₂)-R₃-R₄-R₅- and CP 19850 527 was -R₁-(L-R₂)-R₃-R₄-R₅-, i.e. one amino acid had opposite absolute configuration in 528 the two peptides (D/L). Figure 6 also shows that the two techniques provided 529 comparable chromatographic quality, as previously mentioned when comparing 530 significant features. Finally, only one isomeric pair could not be resolved with any of 531 the two methods, again supporting the high complementarity of the UC and RPLC 532 533 methods.

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3.5. Robustness

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

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

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3.6. Operational cost comparison

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

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4. Conclusions and perspectives

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Orthogonality between the UC and RPLC methods on this set of peptides is 582 583 somewhat less significant than was previously observed when small chemical drugs were compared between SFC and RPLC. However, the UC method is particularly 584 interesting for the cases when RPLC retention is too low, which occurred frequently for 585 586 these small, rather polar molecules. In the present test set, 19% of the samples could not be analyzed with the RPLC method while all of them had adequate retention in 587 UC. When adequate retention could be obtained, less effort was required with UC than 588 with RPLC to obtain satisfying results, as the generic gradient was most successful 589 while the RPLC method often required a second experiment with focus gradient. In 590 terms of capability for impurity profiling, the RPLC method seems superior as it was 591 592 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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- 777 Figure captions
- 778

Figure 1. Comparison of the values of elution composition obtained with the LC
method and the UC method, based on the set of 43 peptides and according to Eq. (1).
Blue dashes are linear peptides; orange circles are cyclic peptides. The central grey
line is the correlation line; the grey interrupted lines are the 95% confidence limits
(indicating that 95% of the points in a random set of analytes should be comprised
within these lines) and delimit the retention space covered by the two techniques.
Figure 2. Cases of failures observed among the 43 peptides analyzed with both

787 gradient methods (generic and focus) in UC and RPLC

788

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.

793

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

800

Figure 5. Comparison of retention times obtained for the API (red square) and

impurities. Yellow circles are impurities observed with both RPLC and UC methods,

green triangles are impurities observed with only one method: green triangles at the

top of the figure were seen with UC only, green triangles at the right of the figure wereseen with RPLC only.

- **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
- 808 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

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



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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.



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

(a) linear peptide

(b) cyclic peptide



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



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	Cosolvent	Flow rate
(min)	proportion (%)	(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% (H₂O/ACN/TFA)	B% (ACN/H₂O/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 ₂ O/ACN/TFA)	B% (ACN/H ₂ 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

Method	UC	RPLC
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%