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Sub/supercritical fluid chromatography versus liquid chromatography for peptide analysis

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ABSTRACT

The aim of this study was to evaluate the potential of ultra-high performance supercritical fluid chromatography (UHPSFC) for peptide analysis by comparing its analytical performance to several chromatographic approaches based on reversed-phase liquid chromatography (RPLC), hydrophilic interaction liquid chromatography (HILIC) and mixed-mode liquid chromatography. First, the retention behavior of synthetic peptides with 3 to 30 amino acids and different isoelectric points (acid, neutral, and basic) was evaluated. For all the tested conditions (13 peptides in 8 conditions), only 4 results were not exploitable (not retained or not eluted), confirming that all the tested chromatographic conditions can be successfully applied when analyzing a wide range of diverse peptides. Average tailing factor were quite comparable across all chromatographic modes, while the best peak capacity values were obtained under mixed-mode LC conditions. Selectivity for each chromatographic mode was also evaluated for six closely related peptides having minor modifications on their structures. The LC-based chromatographic modes confirmed their superior selectivity over UHPSFC. By contrast, when analyzing short peptides (di- or tripetides), UHPSFC was the only technique allowing to simultaneously separate highly polar and less polar peptides within the same run confirming its unique versatility. In addition, the sensitivity of each chromatographic approach was accessed by for two representative peptides by both UV and MS detection. With UV detection, limit of detection (LOD) values were comparable among the different chromatographic modes, ranging from 0.5 to 2 μg mL^{-1}. However, major differences were found when employing MS detection (LOD values ranged from 0.05 to 5 μg mL^{-1}). The best results were obtained under HILIC conditions, followed by SFC, and finally mixed-mode LC and RPLC modes.

1. Introduction

The interest of pharmaceutical companies towards the development of new peptides as efficient therapeutic agents has increased significantly over the years. These molecules represent an intermediate point between small and large molecules in terms of properties, having the potential to be used against a multitude of diseases as well as diagnostic targets [1–3]. Because of their growing attractiveness, more chemists are becoming involved in their synthesis, which can be often challenging due to their higher complexity than synthetic small active pharmaceutical ingredients [1,4]. A great focus was put, therefore, on tools such as online databases (e.g. PepTherDia) containing information on peptide drugs already available on the market [5]. The challenge with synthetic peptides is not only limited to their production, but also to their analytical characterization [6–8]. Powerful and efficient techniques are, thus, required to establish purity levels and separate impurities from the desired compound(s), at an analytical as well as preparative scale [9–11].

In this context, ultra-high performance liquid chromatography (UHPLC) has proved to be very helpful, throughout the use of different modes such as reversed phase liquid chromatography (RPLC) [12–14], hydrophilic interaction chromatography (HILIC) [15–17] and mixed-mode liquid chromatography [18–20]. Nonetheless, a margin of improvement in the context of peptide analysis is still present, thus pushing analytical laboratories to explore new approaches. Among many, ultra-high performance super/subcritical fluid chromatography (UHPSFC) has regained attractiveness as an

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interesting alternative [21,22]. While peptide analysis employing SFC has already been the subject of preliminary research in the past 20–30 years [23,24], we are currently observing a resurgence of new methods involving CO_2-based mobile phases for the separation and characterization of therapeutic peptides, with a stronger focus in establishing its real potential [11,25–28]. An increasing number of articles seems to demonstrate an unexplored potential for UHPSFC specifically for synthetic peptides, at an analytical and semi-preparative scale [28–30]. Additional advantages of UHPSFC derive from its easiness to be hyphenated to several detectors, such as ultraviolet (UV) and mass spectrometers (MS), demonstrating comparable performance to those of UHPLC-UV-MS [25,26]. More recently, a comparison of UHPSFC *vs* UHPLC in reversed-phase mode has been reported, together with an assessment of an unorthodox additive (methanesulfonic acid, MSA) under UHPSFC conditions, using a set of synthetic and commercially available peptides [25]. The authors concluded that UHPSFC offers an interesting complementarity in the separation profile to RPLC, while generating comparable chromatographic performance (e.g., separation efficiency and peak shape, etc.).

Previous works have successfully established UHPSFC as a valid alternative to RPLC in the context of peptide analysis. Nonetheless, understanding its performance against other chromatographic modalities commonly used, such as HILIC or mixed-mode LC, would be highly beneficial to better position UHPSFC within collection of peptide separation techniques. Hence, in this work we studied the performance of UHPSFC against various LC-based techniques, including RPLC, HILIC and mixed-mode LC. To do so, a set of 13 synthetic peptides with different molecular weights and isoelectric points (acidic, neutral and basic) have been used to understand their retention behavior under all various chromatographic techniques considered. Subsequently, the selectivity achievable with UHPSFC was also investigated, using a set of six structurally related synthetic peptides simulating minor changes (oxidation, deamidation, amino acid inversion, etc.), commonly observed with this category of biomolecules. The applicability of the various chromatographic modes was also assessed using another set of di- and tripeptides with different polarity. Lastly, the sensitivity of both UV and MS detection was evaluated for synthetic peptides when combining these detectors to different chromatographic approaches.

2. Materials and methods

2.1. Chemicals, reagents and sample preparation procedures

Methanol (MeOH) and acetonitrile (ACN) of OPTIMA LC-MS grade and water (H_2O) of UHPLC grade were purchased from Fischer Scientific (Loughborough, UK). Carbon dioxide (CO_2) of 4.5 grade (99.995% purity) was purchased from PanGas (Dagmerstellen, Switzerland). Ammonia solution at 25% of MS grade, trifluoroacetic acid (TFA) of MS grade, formic acid (FA), MSA (≥99.5% purity), hydrogen peroxide solution at 30%, and methionine were purchased from Sigma-Aldrich (Buchs, Switzerland). Synthetic peptides at a purity level of ≥ 95% have been purchased from GenScript Biotech (Leiden, Netherlands). Their name and chemical properties in terms of amino acid sequence, molecular weight, number of amino acids, isoelectric point (pI) and GRAVY number (a measure of peptides hydrophobicity) are listed in Table 1. These values were calculated using on-line ExPASy tools [31].

For the first part of the study (namely retention behavior in Table 1), stock solutions at 1.0 mg mL^{-1} were prepared for all synthetic peptides in pure H_2O. Further dilutions to a final concentration of 200 ng mL^{-1} were performed with pure ACN (for HILIC and SFC analyses) or pure H_2O (for RPLC and mixed-mode LC analyses). In the specific part of the work related to selectivity evaluation (namely selectivity study in Table 1), the oxidation of reference peptide was performed by adding 0.2% *v/v* hydrogen peroxide to the peptide stock solution prepared at 300 ng mL^{-1} in H_2O/ACN 35:65 *v/v*. After a 3 days incubation at room temperature, the oxidation process was stopped by adding 0.6 mg of methionine to the solution. Stock solutions for the other five peptides were prepared at 1.0 mg mL^{-1} in H_2O (for RPLC and mixed-mode LC conditions) and in H_2O/ACN 80:20 *v/v* (for HILIC and SFC conditions) Then, two solutions containing the six peptides at 40 ng mL^{-1} each were prepared by dilution with pure ACN (for HILIC and SFC analyses) and pure H_2O (for RPLC and mixed-mode LC analyses). Concerning the di-/tripeptides, stock solutions at 1.0 mg.mL^{-1} were prepared in pure H_2O (for RPLC and mixed-mode LC analyses) and in H_2O/ACN 80:20 *v/v* (for HILIC and SFC conditions). Then, further dilutions to a final concentration of 500 ng.mL^{-1} were performed with pure ACN (for HILIC and SFC analyses) or pure H_2O (for RPLC and mixed-mode LC analyses). Finally, for the sensitivity study, stock solutions of 3-mer A and 6-mer B were prepared at 2.0 mg mL^{-1} in pure H_2O. Then, solutions containing the mixture of both peptides were prepared at concentrations of 50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1, and 0.05 ng mL^{-1} either by diluting with pure ACN (for HILIC and SFC analyses) or pure H_2O (for RPLC and mixed-mode LC analyses).

2.2. Chromatographic and MS instrumentation and conditions

All UHPSFC-UV-MS analyses were performed on a Waters Acquity UPC^2 system (Milford, MA, USA) equipped with a binary solvent manager delivery pump, a sample manager autosampler which included a 10 µL loop for partial loop injection, a column oven with active preheater, a PDA detector with an 8.4 µL flow-cell and a two-step (active and passive) backpressure regulator (BPR). The UHPSFC-UV system was hyphenated to a Waters QDa single quadrupole mass spectrometer, fitted with a Z-spray ESI source, via a "*pre-BPR splitter with make-up pump*" SFC-MS interface provided by Waters [32]. Make-up solvent was delivered *via* a Waters Acquity isocratic solvent manager (ISM) module, at a flow-rate of 0.1 mL min^{-1}. Pure MeOH was chosen as the make-up solvent. The autosampler temperature was fixed at 10°C. Empower v3.0 (Waters, Milford, MA, USA) was used to control the UHPSFC-UV and UHPSFC-UV-MS instruments.

UHPLC-UV and UHPLC-MS analyses were performed on a Waters Acquity UPLC system, equipped with a binary solvent manager delivery pump, a sample manager autosampler with a 2 µL loop for partial loop injection, a column oven with active preheater and a PDA detector with a 500 nL flow-cell. For MS hyphenation, the previously mentioned Waters QDa single quadrupole was employed. Autosampler temperature was fixed at 10°C. Empower v3.0 was also used to control the UHPLC-UV and UHPLC-UV-MS instruments. For UHPSFC-MS and UHPLC-MS analyses, the same ionization conditions were used, consisting in a capillary voltage of +1.5 kV, cone voltage of 15 V, and desolvation temperature at 500°C. Nitrogen (N_2) was used as both desolvation and cone gas. All MS analyses were performed in ESI positive mode, recording SIR masses at 377.29 and 367.23 Da for 3-mer A and 6-mer B, respectively.

2.3. Analytical conditions

For the first part of the study (retention behavior) as well as for the sensitivity study, the following chromatographic conditions were selected to perform the analyses in each chromatographic mode. For the retention behavior and selectivity evaluations, chromatograms were obtained at 210 and 280 nm for the UHPSFC and UHPLC experiments, respectively. For the sensitivity study, the UV data were recorded at 214 nm, using the "absorbance-MBF" mode

Table 1
List of synthetic peptides used for each part of this study.

Retention behavior					
Name	Amino acid sequence	MW (DA)	Number of amino acids	pI(predicted)	GRAVY number
3-mer A	WDG	376.36	3	3.10	NA
3-mer N	WHG	398.42	3	7.80	NA
3-mer B	WKG	389.45	3	10.10	NA
6-mer A	WGDTAQ	678.68	6	3.80	-1.20
6-mer N	WHGSAT	657.68	6	6.74	-0.70
6-mer B	WRGSPM	732.86	6	9.75	-1.05
9-mer A	WGDTQAEMS	1024.07	9	3.67	-1.07
9-mer N	WHGSHATSM	1013.10	9	6.92	-0.70
9-mer B	WRGSHATPM	1042.18	9	9.76	-0.93
18-mer A	WGDQSEMAWDMNTQAEWG	2142.26	18	3.43	-1.23
18-mer N	WGHTQASMWSATSPMHGW	2058.28	18	6.92	-0.63
18-mer B	WRGTAHSPMKWHQICAWN	2209.53	18	9.51	-0.86
30-mer B	WGHTQASMWSATSPMHGWGHTQASMWSATS	3522.00	30	10.06	-0.94
Selectivity study					
Name	Amino acid sequence	MW (DA)	Number of amino acids	pI (predicted)	GRAVY number
Reference	KEHWNMWSHL	1367.55	10	6.92	-1.42
Deaminated	KEHWDMWSHL	1368.53	10	5.99	-1.42
K truncation	EHWNMWSHL	1239.37	9	5.99	-1.14
L replacement	KEHWNMWSHP	1351.50	10	6.92	-1.96
AA inversion	KEWHNMWSHL	1367.55	10	6.92	-1.42
Dipeptide 1	VL	230.31	2	5.49	NA
Dipeptide 2	VD	232.24	2	3.80	NA
Dipeptide 3	VR	273.34	2	9.72	NA
Tripeptide 1	VLA	301.39	3	5.49	NA
Tripeptide 2	VRK	401.51	3	11.00	NA
Tripeptide 3	VDE	361.35	3	3.67	NA
Sensitivity study					
Name	Amino acid sequence	MW (DA)	Number of amino acids	pI (predicted)	GRAVY number
3-mer A	WDG	376.36	3	3.10	NA
6-mer B	WRGSPM	732.86	6	9.75	-1.05

A, Ala; G, Gly; I, Ile; L, Leu; M, Met; P, Pro; W, Trp; V, Val; N, Asn; Q, Gln; S, Ser; T, Thr; D, Asp; E, Glu; C, Cys; R, Arg; H, His; K, Lys.

for both UHPSFC-UV and UHPLC-UV analyses. On the UHPLC system, the original mixing chamber of 50 µL was modified into a mixing chamber having a volume of 250µL, as it is well known that sensitivity can be strongly impacted when using TFA in the mobile phase [33].

2.3.1. SFC with acidic additive

A 100 × 3 mm I.D. Waters Torus 2-PIC stationary phase with 1.7 µm fully porous particles was chosen. A mixture of MeOH/H_2O 95:5 *v/v* containing 0.05% MSA was employed as organic modifier and was mixed with carbon dioxide. An optimized gradient consisting in a 7 min gradient from 40 to 85% of organic modifier, then a return to initial conditions in 0.1 min and an isocratic hold for 4.9 min for a total run time of 12 min was employed. Injection volume, flow rate, and column temperature were set at 2 µL, 0.9 mL min^{-1}, and 55 °C, respectively. The backpressure regulator (BPR) was set at 103 bar (1500 psi). Pure ACN and a mixture of ACN/H_2O 50:50 *v/v* were used, respectively, as weak and strong needle washes.

2.3.2. SFC with basic additive

A 100 × 3 mm I.D. Waters Torus DIOL stationary phase with 1.7 µm fully porous particles was chosen. A mixture of MeOH/H_2O 95:5 *v/v* containing 0.2% NH_4OH was employed as organic modifier and was mixed with carbon dioxide. An optimized gradient of 7 min from 50 to 75% of organic modifier, then a return to initial conditions in 0.1 min and an isocratic hold for 4.9 min for a total run time of 12 min was employed. Injection volume, flow rate, and column temperature were set at 2 µL, 0.9 mL min^{-1} and 55°C, respectively. Backpressure regulator (BPR) was set at 103 bar (1500 psi). Pure ACN and a mixture of ACN/H_2O 50:50 *v/v* were used, respectively, as weak and strong needle washes.

2.3.3. RPLC in acidic conditions

A 100 × 2.1 mm I.D. Waters Acquity UPLC BEH C18 stationary phase with 1.7 µm fully porous particles was chosen. Mobile phase A consisted in H_2O with 0.1% *v/v* TFA, while mobile phase B was ACN with 0.1% *v/v* TFA. An optimized gradient consisting in a 7 min gradient from 10 to 35% B, then a return to initial conditions in 0.1 min and an isocratic hold for 4.9 min for a total run time of 12 min was employed. Injection volume, flow rate and column temperature were set at 1 µL, 0.3 mL min^{-1} and 60°C, respectively. Pure ACN and a mixture of ACN/H_2O 90:10 *v/v* were used, respectively, as strong and weak needle washes.

2.3.4. RPLC in basic conditions

A 100 × 2.1 mm I.D. Waters Acquity UPLC BEH C18 stationary phase with 1.7 µm fully porous particles was chosen. Mobile phase A consisted in H_2O with 10 mM ammonium hydroxide adjusted at pH=9 with formic acid, while mobile phase B was pure ACN. An optimized gradient consisting in a 7 min gradient from 2 to 65% B, then a return to initial conditions in 0.1 min and an isocratic hold for 4.9 min for a total run time of 12 min was employed. Injection volume, flow rate and column temperature were set at 1 µL, 0.3 mL min^{-1} and 60°C, respectively. Pure ACN and a mixture

of ACN/H_2O 90:10 *v/v* were used, respectively, as strong and weak needle washes.

2.3.5. HILIC with bare hybrid silica column

A 100 × 2.1 mm I.D. Waters Acquity BEH HILIC stationary phase with 1.7 μm fully porous particles was chosen. Mobile phase A consisted in H_2O with 0.1% *v/v* TFA, while mobile phase B was ACN with 0.1% *v/v* TFA. An optimized gradient consisting in a 7 min gradient from 95 to 70% B, then a return to initial conditions in 0.1 min and an isocratic hold for 4.9 min for a total run time of 12 min was employed. Injection volume, flow rate and column temperature were set at 1 μL, 0.3 mL.min^{-1} and 45°C, respectively. Pure ACN and a mixture of ACN/H_2O 50:50 *v/v* were used, respectively, as weak and strong needle washes.

2.3.6. HILIC with amide column

A 100 × 2.1 mm I.D. Waters Acquity BEH amide stationary phase with 1.7 μm fully porous particles was chosen. Mobile phase A consisted in H_2O with 0.1% *v/v* TFA, while mobile phase B was ACN with 0.1% *v/v* TFA. An optimized gradient consisting in a 7 min gradient from 85 to 65% B, then a return to initial conditions in 0.1 min and an isocratic hold for 4.9 min for a total run time of 12 min was employed. Injection volume, flow rate and column temperature were set at 1 μL, 0.3 mL.min^{-1} and 60°C, respectively. Pure ACN and a mixture of ACN/H_2O 50:50 *v/v* were used, respectively, as weak and strong needle washes.

2.3.7. Mixed-mode RPLC/AEX with acidic additive

A 100 × 2.1 mm I.D. Waters Atlantis Premier BEH C18 AX stationary phase with 1.7 μm fully porous particles was chosen. Mobile phase A consisted in H_2O with 0.1% *v/v* TFA, while mobile phase B was ACN with 0.1% *v/v* TFA. An optimized gradient consisting in a 7 min gradient from 5 to 45% B, then a return to initial conditions in 0.1 min and an isocratic hold for 4.9 min for a total run time of 12 min was employed. Injection volume, flow rate and column temperature were set at 1 μL, 0.3 mL.min-1 and 60°C, respectively. Pure ACN and a mixture of ACN/H2O 90:10 *v/v* were used, respectively, as strong and weak needle washes.

2.3.8. Mixed-mode RPLC/AEX with basic additive

A 100 × 2.1 mm I.D. Waters Acquity UPLC BEH C_{18} stationary phase with 1.7 μm fully porous particles was chosen. Mobile phase A consisted in H_2O with 10 mM ammonium hydroxide at pH=9, while mobile phase B was pure ACN. An optimized gradient consisting in a 7 min gradient from 2 to 45% B, then a return to initial conditions in 0.1 min and an isocratic hold for 4.9 min for a total run time of 12 min was employed. Injection volume, flow rate and column temperature were set at 1 μL, 0.3 mL.min^{-1} and 60°C, respectively. Pure ACN and a mixture of ACN/H_2O 90:10 *v/v* were used, respectively, as strong and weak needle washes.

For the selectivity study performed on the mixture of six synthetic decapeptides, the previously described conditions were kept, except for the elution conditions that need to be adjusted to maximize selectivity in all chromatographic modes. For RPLC with acidic or basic conditions, HILIC with both bare hybrid silica and amide columns, and mixed-mode LC with basic conditions, isocratic conditions with 23, 18, 89, 81, and 20% B, were employed, respectively. Analysis time was always set at 7 min whatever the chromatographic modes, except for RPLC with basic conditions, which required an analysis time of 10 min to elute the most retained peptide. For mixed-mode LC with acidic conditions, an optimized gradient from 17 to 22% B in 7 min was selected. No modification was applied to the SFC conditions for these analyses (use of the generic gradient conditions previously described).

Concerning the work on di-/tri-peptides, the following generic gradients were selected. For SFC with acidic additive, a 7 min gradient from 25 to 60% was applied. For SFC with basic additive, a 7 min gradient from 25 to 70% was considered. Concerning RPLC and mixed-mode LC with both acidic and basic conditions, a 7 min gradient from 2 to 50% B was selected. Finally, in HILIC, with both bare hybrid silica and amide columns, a 7 min gradient from 95 to 60% B was applied.

3. Results and discussion

3.1. Retention behavior for different chromatographic modes

In a first instance, the retention behavior of 13 model peptides was compared using the various chromatographic modes (i.e. RPLC, mixed-mode LC, HILIC and SFC). Model peptides were chosen in order to present different sizes (number of amino acids (AAs) between 3 and 30) and isoelectric points (pI between 4 and 10, corresponding either to acidic, neutral or basic peptide), see Table 1. To have reliable and comparable data, the chromatographic conditions in all modes were adjusted. The gradient time was systematically equal to 7 min since the column lengths were identical, and flow rate was adjusted to maintain a comparable mobile phase linear velocity whatever the column internal diameter. In addition, the initial and final compositions of the gradient were adjusted to have no peak eluted before 1 minute (sufficient apparent retention factor under gradient conditions, considering a delay time of 20 s and 29 s for the UHPLC and UHPSFC systems, respectively) and have as many peptides as possible eluted before 7 min. All the corresponding retention times have been reported in Table S1 of the supplementary material. As shown, all the 13 model peptides were eluted whatever the chromatographic mode, except in a very few cases (only four values were missing among the 104 expected).

Fig. 1 illustrates the elution range for the model peptides under all chromatographic conditions herein tested. The reference method for peptides analysis (RPLC under acidic pH conditions) allows the elution of peptides with a gradient from 10 to 35 %ACN, mostly based on their increasing sizes, meaning that hydrophobic interactions increase with the number of AAs in the sequence. A significant retention difference between peptides composed of 3 to 9 AAs, and the ones containing 18 or 30 AAs is also visible, with the latter eluting towards the end of the gradient. This observation is perfectly in line with the results obtained by Gilar *et al.*[34], but also Dwivedi *et al.* [35]. Indeed, the latter shows that only the presence of a few amino acids, namely arginine, histidine, and lysine (positively charged amino acids) in the amino acid sequence reduces retention when using 0.1%TFA in the mobile phase, while all the other amino acids increase retention or have negligible effect. Interestingly, when moving from acidic to basic (pH 9) mobile phase conditions, the selectivity was modified, and retention was also quite different (elution of the 13 peptides requires a gradient from 2 to 65 %ACN). When considering the gradient range employed at pH 9, small acidic peptides are less retained due to their overall negative surface charge, while the more retained peptides (long neutral or basic peptides) require higher proportions of ACN in the mobile phase. Importantly, it was not possible to elute the largest peptide composed of 30 AAs under these conditions. This difference in chromatographic behavior is related to i) the different peptides ionization state at basic vs. acidic pH, and ii) the absence of ion pairing reagent in the mobile phase at basic pH (ammonia instead of TFA). This experimental behaviour can again be confirmed by the work of Dwivedi *et al.* [35], as only the presence of aspartic acid and glutamic acid in the peptide sequence (negative retention coefficients for these two individual amino acids) reduces the retention, while all the other amino acids contribute to retention increase.

In addition to RPLC with a C18-based column, a mixed-mode stationary phase (composed of alkyl chains and anion exchanger group) was also utilized under acidic and basic conditions. With

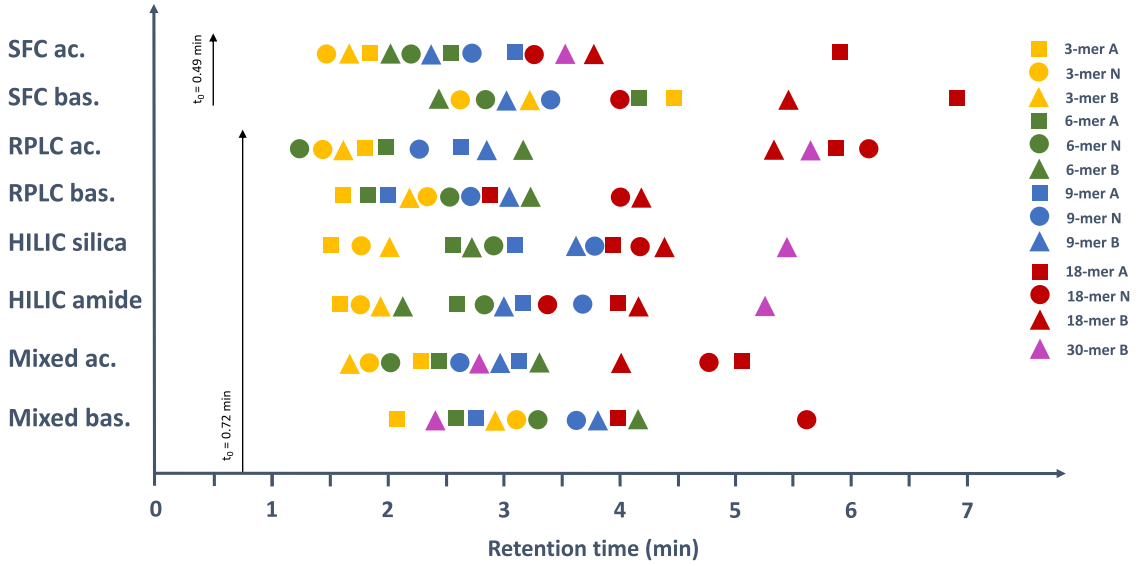

Fig. 1. Graphical representation of the retention times obtained from the analysis of 13 peptides with the different chromatographic modes. The square, round and triangle symbols have been used to designate peptides with acidic, neutral and basic isoelectric points, respectively. Yellow, green, blue, red, and purple colors have been attributed to 3-mer, 6-mer, 9-mer, 18-mer and 30-mer peptides, respectively.

this column, retention was comparable to C18 material (retention ranges were equal to 5-45 %ACN at acidic pH and 2–45% ACN at basic pH), but the elution order was modified due to the addition of anion exchange capability. In particular, the 30-mer B peptide was much less retained on the mixed-mode column whatever the pH. This behavior could be attributed to: i) the presence of positively charged groups at the surface of the column, creating electrostatic repulsions, and ii) difference in pore size (130 Å for the regular C18 material *vs.* 95 Å on the mixed-mode column) which could be responsible from partial exclusion of large peptide from the pores. Surprisingly, the 18-mer B peptide was not eluted under basic conditions while the 30-mer B was easily eluted.

Next, we have also evaluated the possibilities offered by HILIC for the analysis of various model peptides. Two different stationary phases were tested under HILIC conditions, namely a bare hybrid silica and amide chemistry. The mobile phase remains identical and TFA was used to have acceptable retention and peak shapes under HILIC conditions. Here, the gradient ranged from 95 to 70% ACN and 85 to 65 %ACN on the bare silica and amide columns, respectively. This means that the bare silica was less retentive for the less hydrophilic peptides. For this part of the work, a publication of Gilar and Jaworski was considered [36]. Based on the retention coefficient calculated in this previous work, it is logical that the amide column was more retentive than the bare silica, when predicting the retention times of the model peptides. On the bare silica column, all the peptides can be eluted thanks to the addition of TFA in the mobile phase, and the elution order is strictly based on the size of the peptides. In other words, when increasing the size of the peptides, they can interact more strongly with the water layer immobilized at the surface of the stationary phase. In general, the acidic peptides are eluted first followed by the neutral and basic ones. This behavior is obviously related to the presence of negative charges at the surface of the stationary phase (residual silanols) and to electrostatic repulsion. On the amide stationary phase, the selectivity is modified, due to the more limited number of residual silanols. Even if the separation is still based on peptide size, the basic peptides often elute before the neutral peptides and sometimes even before the acidic peptides of the same size. With the HILIC amide column chemistry, it is interesting to notice that the predicted retention order based on the coefficients of Gilar and Jaworski was different from the retention order experimentally observed in our study. This difference can be easily attributed to the different mobile phase conditions (0.1%TFA in the present work, and 10 mM ammonium formate pH 4.5 in the publication of Gilar and Jaworski). Despite this slight modification of selectivity between bare silica and amide chemistries, the elution order remains highly comparable between the two HILIC conditions, mostly due to the presence of TFA in the mobile phase, which contributes to limit the ionic interactions under HILIC conditions.

Finally, we have also explored the possibilities offered by UH-PSFC for the analysis of the same peptides. Here, the gradient conditions ranged from 40 to 85% organic cosolvent with acidic additive (MSA), and from 50 to 75% cosolvent with basic additive (NH_4OH). Based on Fig. 1, it appears that the selectivity was very strongly modified between these two conditions. Indeed, under SFC conditions with acidic additive, the peptides were mostly eluted based on their size, but the 30-mer was eluted much earlier than expected. The acidic peptides were always more retained compared to the neutral and basic peptides of the same size. This behavior could be explained by the fact that the selected column contains a basic ligand (2-PIC) and therefore, the acidic peptides are more retained due to the existence of ionic interactions. Indeed, despite the use of MSA in the mobile phase, the apparent pH is probably higher under SFC conditions compared to RPLC, as already demonstrated [25] and therefore, ionic interactions can still exist. Under SFC conditions with basic additive, both the chemistry of the column (Diol) and the nature of mobile phase additive were modified, based on conditions described in a very recent publication where peptides were successfully analyzed in SFC [37]. Due to these multiple changes, a significant selectivity alteration was observed, as highlighted in Fig. 1. Indeed, the peptides were now not anymore separated based on their size, but the elution order was rather diverse (this is not something that can be easily predicted as retention coefficients for all individual amino acids have never been published in SFC). As an example, the 3-mer A peptide was among the most retained ones, while it elutes much earlier with the acidic additive. Under these conditions, the 9-mer A and 30-mer B peptides were not eluted, while the 18-mer A was strongly retained. Based on this observation, it is clear that SFC with basic additive is much less adapted to the analysis of a wide range of diverse peptides.

Table 2
Average tailing factor, average peak capacity and maximal pressure drop calculated on the data obtained from the analysis of 13 peptides with each chromatographic mode.

Chromatographic mode	Tailing factor	Peak capacity	ΔP max (bar)
SFC ac.	1.43	141	354
SFC bas.	1.52	71	346
RPLC ac.	1.38	145	411
RPLC bas.	1.28	203	386
HILIC silica	2.26	87	206
HILIC amide	1.28	130	198
Mixed ac.	1.41	230	385
Mixed bas.	1.46	215	430

SFC ac., SFC with acidic additive; SFC bas., SFC with basic additive; RPLC ac., RPLC with acidic additive; RPLC bas., RPLC with basic additive; HILIC silica, HILIC with bare silica column; HILIC amide, HILIC with amide column; Mixed ac., mixed-mode LC with acidic additive; Mixed bas., mixed-mode LC with basic additive.

3.2. Comparison of other figures of merit for peptide separation by various chromatographic modes

After addressing the differences in the retention profile of the model peptides under different chromatographic techniques, we have moved to understand how peak shapes (tailing factor and peak capacity) and the maximal pressure drop varied across all chromatographic techniques and conditions (Table 2). As shown, the average tailing factor values were comparable for all the chromatographic modes (values ranged from 1.28 to 1.52), and only the HILIC conditions with bare hybrid silica provide a much higher tailing factor (2.26). This was probably due to the coexistence of two retention mechanisms, including both hydrophilic partitioning and strong ionic interactions with free silanols. In terms of peak capacity (maximum number of resolvable peaks), the RPLC with basic conditions and the mixed-mode LC under both acidic and basic conditions offer values beyond 200, which is remarkable for a gradient time of only 7 min. Then, RPLC with acidic conditions, SFC with acidic conditions and HILIC with amide stationary phase provide peak capacities comprised between 130 and 145, which is still fully acceptable. Only two chromatographic strategies provide much broader peaks, namely SFC with basic conditions and HILIC with bare hybrid silica. Here, peak capacities were equal to 71 and 87, respectively. Pressure drops observed during the gradient separations was about 200 for HILIC conditions and 350-400 bar for the other chromatographic modes. The low pressure observed under HILIC conditions is obviously related to the highly organic mobile phase. It is important to notice that the pressure drop observed in SFC was comparable to the one obtained in RPLC and mixed-mode LC. This was due to the use of high proportion of cosolvent in the mobile phase, and the need to generate a backpressure throughout the system (approx. 100 bar).

To better emphasize the potential of SFC for peptides analysis, Fig. 2 highlights the peak shapes observed for a few representative peptides of different sizes and pI. As illustrated, the peaks are symmetrical and quite narrow whatever the peptide when using SFC with acidic additive. On the other hand, peaks were broader when considering a basic additive, as already shown in Table 2, but the overall performance remained acceptable, and a different selectivity was obtained between the two conditions.

Lastly, it is also important to consider that the sample diluent needs to be adjusted depending on the chromatographic mode, to obtain suitable peak shapes and avoid peak distortion. In RPLC and mixed-mode LC, the peptides were dissolved in pure H_2O, while the peptides were dissolved in ACN/H_2O 90:10 *v/v* in HILIC and SFC, in line with previously published works [38]. Whatever the sample diluent applied, no solubility issue was noticed for all the peptides at the tested concentrations.

3.3. Selectivity of closely related peptides in different chromatographic modes

Another important chromatographic property worthy of investigation is the separation selectivity for closely related peptides. During the several synthetic steps needed to obtain a therapeutic peptide, minor modifications of the amino acidic chain can occur with the generation of unwanted species presenting only few changes on selected amino acids. Therefore, a decapeptide was considered as reference, with an isoelectric point of 6.92, together with 5 different modified peptides presenting minor variations in the sequence, as illustrated in Fig. 3 and Table 1. Deamidation is a very common modification that can take place during the long-term storage and affects most peptides and proteins. It occurs generally on asparagine while it is less common on glutamine. Herein, asparagine contained on the decapeptide sequence was replaced by as aspartic acid to mimic, in part, what would happen after deamidation. An Asn-Asp substituted analog was then used to represent this process. Next, oxidation of methionine (and tryptophan to a less extend) is also a widely reported chemical degradation pathway of peptides and proteins, but it is less prevalent than deamidation. It occurs because of extended/improper storage conditions (buffer concentration, pH, excipients), thus used as an indicator of chemical instability. In the selected peptide sequence, the thioether group of methionine can be chemically oxidized into sulfoxide using hydrogen peroxide, as mentioned in the material and method section. Besides these two modifications, we have also obtained an additional peptide where the terminal lysine was truncated. In another sample, the terminal leucine was replaced with proline. Finally, two amino acids (tryptophan and histidine) were inverted in the sequence. These six different peptides have therefore some very minor modifications in the sequence and could be helpful to challenge the chromatographic methods.

The conditions in all the different chromatographic modes were optimized as much as possible to obtain the best possible selectivity and resolution. Table 3 summarized the minimum resolution, number of peaks which are baseline resolved and average tailing factor.

This table clearly highlights the superiority of RPLC and mixed-mode LC, both under acidic and basic conditions. These four different chromatographic methods allow the baseline separation of the six closely related peptides, with a minimum resolution varying from 2.43 (mixed-mode LC with basic conditions) and 4.00 (mixed-mode LC with acidic conditions) using either isocratic conditions or narrow gradient range. In addition, the average tailing factor was very good and comprised between 1.25 and 1.42 for RPLC (acidic and basic conditions) and mixed-mode LC under basic conditions. These values were slightly worse (average tailing fac-

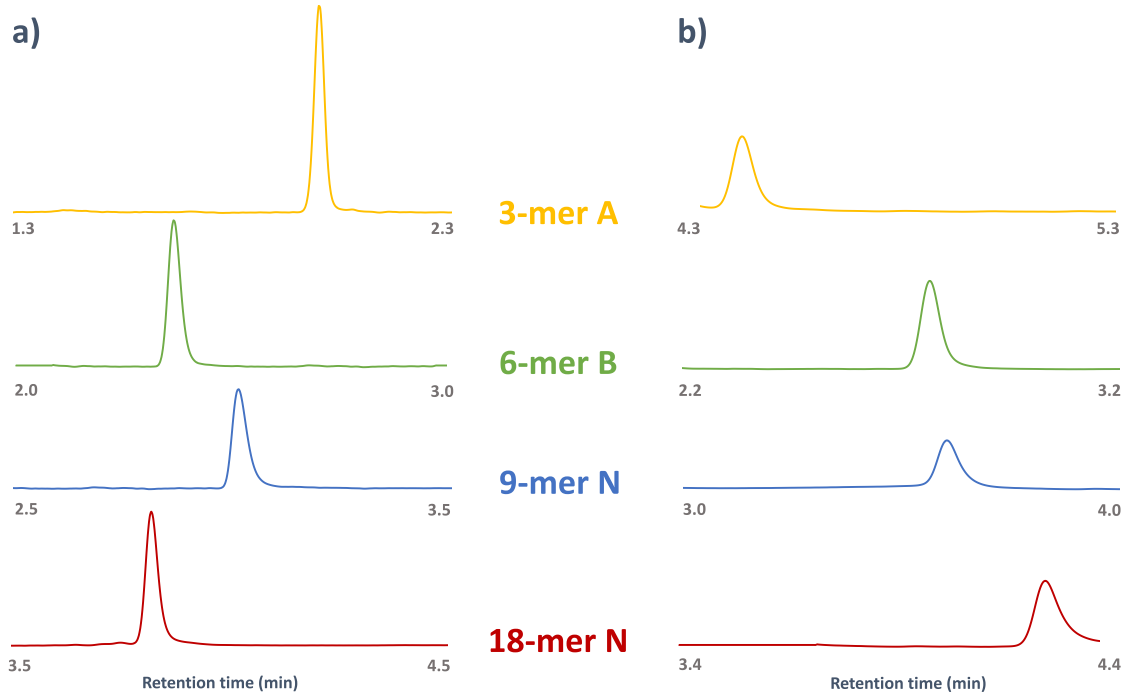

Fig. 2. Chromatograms obtained for the analysis of 3-mer A, 6-mer B, 9-mer N and 18-mer N peptides by a) UHPSFC with acidic additive; b) UHPSFC with basic additive.

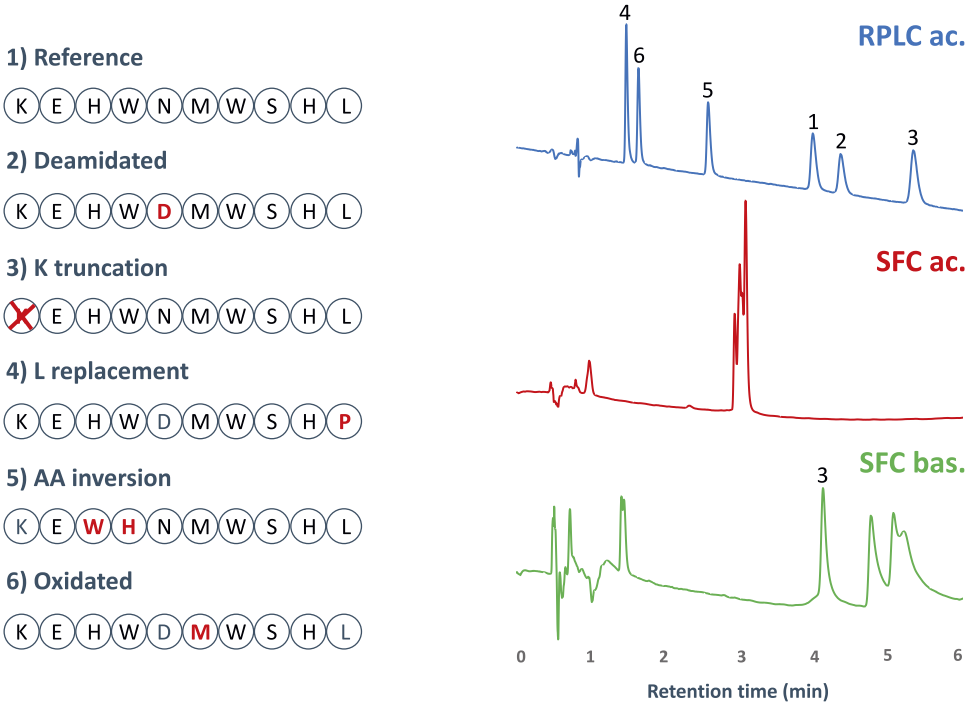

Fig. 3. Chromatograms obtained for the analysis of 1) reference, 2) deamidated, 3) K truncation, 4) L replacement, 5) AA inversion, 6) oxidated peptides by RPLC with acidic additive; using SFC with acidic additive and SFC with basic additive. K, Lys; E, Glu; H, His; W, Trp; N, Asn; M, Met; S, Ser; L, Leu; D, Asp; P, Pro.

tor of 1.89) for mixed-mode LC under acidic conditions, but still acceptable. To better visualize the separation obtained under RPLC with acidic conditions, the corresponding chromatogram was reported in Fig. 3. The lower resolution (Rs of 2.70) was obtained between the reference peptide and its deamidated form. However, it is important to notice that these two peaks were very well separated in RPLC or mixed-mode LC with basic conditions (Rs higher than 10), while the use of mixed-mode LC column under acidic conditions offers a resolution of 4.6 for these two peaks.

HILIC mode was performed in both cases (bare hybrid silica and amide stationary phases) under isocratic conditions, to optimize selectivity. However, as shown in Table 3, the selectivity and overall resolution remain clearly lower than what can be observed in RPLC and mixed-mode LC. Indeed, even if the average tailing fac-

Table 3
Minimum resolution, number of separated peaks and tailing factor average obtained from the analysis of 6 peptides with each chromatographic mode here discussed.

Chromatographic mode	Minimum resolution	Number of separated peaks	Tailing factor
SFC ac.	0.00	0	1.96*
SFC bas.	0.00	1	2.89*
RPLC ac.	2.74	6	1.42
RPLC bas.	2.78	6	1.25
HILIC silica	0.00	4	1.57
HILIC amide	0.00	3	1.19
Mixed ac.	4.00	6	1.89
Mixed bas.	2.43	6	1.28

SFC ac., SFC with acidic additive; SFC bas., SFC with basic additive; RPLC ac., RPLC with acidic additive; RPLC bas., RPLC with basic additive; HILIC silica, HILIC with bare silica column; HILIC amide, HILIC with amide column; Mixed ac., mixed-mode LC with acidic additive; Mixed bas., mixed-mode LC with basic additive.
* values calculated on individual injections.

tor remains very good (1.19 on the HILIC amide and 1.57 on the bare hybrid silica), only 3 to 4 peptides were baseline resolved with the amide and bare hybrid silica, respectively. Under HILIC conditions, the most difficult peaks to separate correspond to the reference peptide and the version where two AAs were inverted in the sequence. The two species perfectly coeluted whatever the column chemistry. On the HILIC amide, the deamidated peptide also coeluted with the reference peptide, while it was perfectly resolved (Rs of 2.87) on the bare hybrid silica. As expected, major differences in selectivity were observed in HILIC vs. RPLC and mixed-mode LC. This is why HILIC can sometimes be a good alternative to achieve sufficient selectivity for certain peptides. As an example, oxidized peptide and peptide with Lys-Pro replacement was replaced were strongly retained in HILIC, while they were quickly eluted in RPLC. Similarly, the peptide with lysine truncation was the first eluted under HILIC conditions on the two columns, while it was more retained in RPLC and mixed-mode LC.

Finally, the chromatograms obtained under SFC conditions were reported in Fig. 3. As shown, all the peaks coeluted under SFC conditions with acidic conditions. Selectivity was slightly better under SFC with basic conditions, with a few peptides partially separated, but the overall performance remained far from what can be achieved with the other chromatographic modes. In addition, average tailing factor values (calculated from individual injections) for the two SFC conditions were much larger and comprised between 1.96 and 2.89 in acidic and basic conditions, respectively. Obviously, the observed behavior can be considered as peptide dependent, since this conclusion is different from the ones previously reported in the literature [25]. However, one of the reasons for the poor separation might be related to the insurgence of peak broadening and distortion when shallow gradients isocratic conditions were used. Therefore, the best possible separation was obtained with the generic gradient already employed in the first part of this work. This seems to indicate the limits of SFC for the analysis of closely related peptides with narrow gradient conditions but does not preclude the use of SFC for a wide range of diverse peptides under generic conditions.

3.4. Analysis of short peptides with various chromatographic modes

Next, we have also tried to evaluate the applicability of the various chromatographic modes for the analysis of small peptides, including di- and tri-peptides. For this purpose, we have considered three different dipeptides composed of either two neutral AAs, one neutral and one acidic AA, or one neutral and one basic AA. The tripeptides were composed either of three neutral AAs, one neutral and two acidic AAs or one neutral and two basic AAs. When analyzing these different peptides under RPLC and mixed-mode LC conditions in acidic and basic conditions using an identical gradient, the same elution pattern was observed. The retention was always suitable for the neutral peptides, but when adding one or several charged AAs, the retention was too low, even with only 2%ACN in the mobile phase. This behavior is illustrated in Fig. 4. The tripeptide Val-Leu-Ala was well retained and adequately analyzed under RPLC and mixed-mode LC conditions, while the Val-Arg-Lys peptide was not sufficiently retained, and peak shape was strongly distorted.

The exact reversed behavior was observed under HILIC conditions when applying a gradient from 95 to 60%ACN, since the retention was mostly based on hydrophilic partitioning. Therefore, the charged peptides (such as Val-Arg-lys) were eluted as very sharp peaks with a sufficient retention, while several peaks were observed for the neutral tripeptides at a retention time very close to the column dead time (see Fig. 3).

Finally, when using SFC conditions (both with acidic and basic additives) and a gradient from 25 to 60%MeOH, it appeared that both the neutral and charged peptides were sufficiently retained and eluted as sharp peaks, as illustrated in Fig. 4. The versatility of SFC allows to simultaneously analyze highly polar and less polar peptides, as already demonstrated in the past with hydrosoluble and liposoluble vitamins [39] or with hydrophilic and lipophilic metabolites [40]. The use of a gradient profile called unified chromatography, which enables the transition from a supercritical to liquid state mobile phase, seems to provide SFC with superior performance in analyzing short di- and tri-peptides against all LC techniques here considered.

3.5. UV and MS sensitivity of peptides analyzed under various chromatographic modes

In addition to the chromatographic performance, it is also important to evaluate the sensitivity with both UV and MS detectors when analyzing peptides under the various chromatographic modes evaluated in this work. For this purpose, we have analyzed two peptides that can be eluted in all chromatographic modes with suitable peak shapes, namely the 3-mer A and the 6-mer B. These two peptides were injected at various concentrations in the eight different chromatographic modes and limits of detection (LOD) were calculated based on S/N ratio of 3. To have comparable results between chromatographic modes, the same UV settings were used (wavelength, time constant, data acquisition rate). These parameters can be found in Section 2.2. In addition, it is important to mention that injected volumes were equal to 1 µL in RPLC, mixed-mode LC and HILIC, where a column of 100 × 2.1 mm was used. On the other hand, the injected volume was increased to 2 µL in SFC as the column has a two-fold larger volume (100 × 3.0

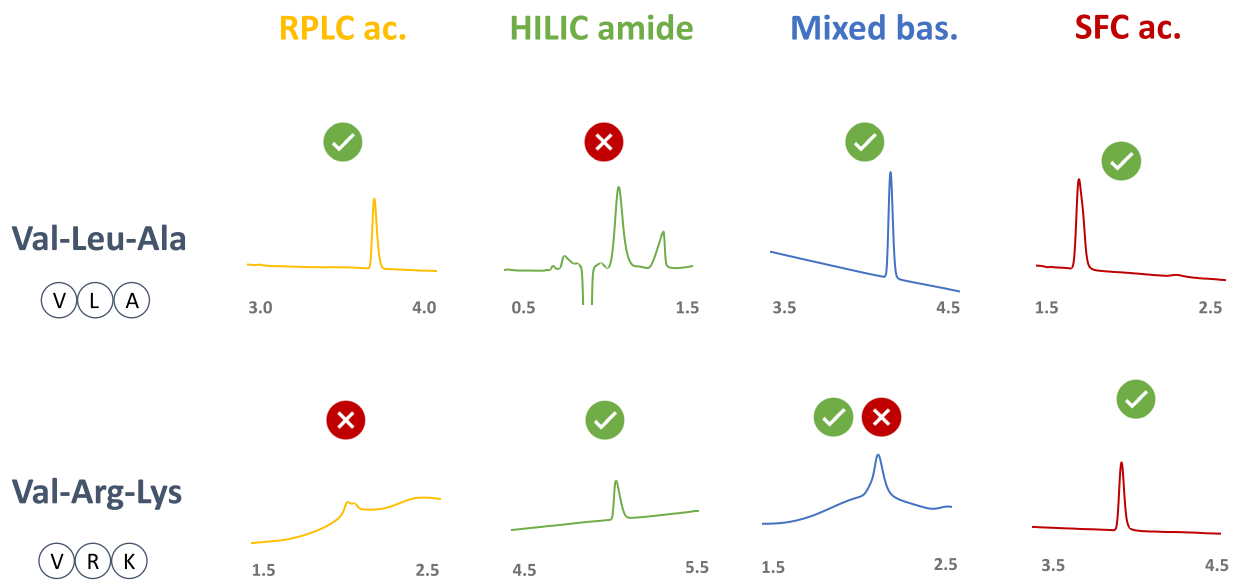

Fig. 4. Chromatograms obtained for the analysis of Val-Leu-Ala and Val-Arg-Lys tripeptides using different chromatographic modes.

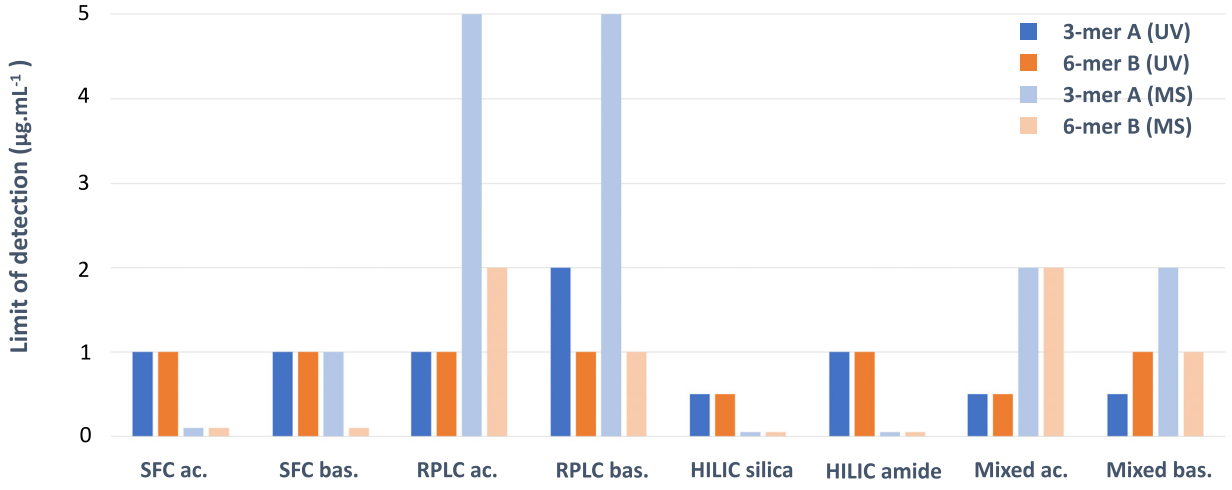

Fig. 5. Histogram displaying the limits of detection for 3-mer A and 6-mer B peptides as a measure of the sensitivity of UV and MS detectors when coupled with the different chromatographic modes.

mm). Therefore, the dilution factor due to the column is expected to be the same in all cases.

With UV detection, the LOD values were comprised between 0.5 and 2 µg.mL^{-1} for the 3-mer A using the different chromatographic modes, while they were comprised between 0.5 and 1 µg mL^{-1} for the 6-mer B, as reported in Fig. 5. This means that the differences between the chromatographic modes and between the two peptides were minor. However, to obtain such very low LOD, the mixing chamber of the UHPLC system was modified (250 µL vs. 50 µL on the original configuration) to avoid any issues related to the use of TFA as mobile phase additive. Surprisingly, the LOD observed in SFC (with basic and acidic additive) were already low (only 1 µg mL^{-1}), without any modification on the instrument. These results are clearly not in line with the historically poor SFC sensitivity, which is known to be one of the main limitations of the technique, especially with the older generation SFC systems [41]. Indeed, as reported in the literature, the reduced SFC sensitivity is mostly due to higher background noise due to pressure fluctuations from the backpressure regulator and refractive index changes [42]. In the present work, a modern UHPSFC system was used allowing a better control of the backpressure (less than 2 bar backpressure variation during the run) and therefore lower background noise. In addition, when analyzing peptides in SFC, high percentages of modifier were constantly used. Under such highly organic conditions, the mobile phase compressibility is extremely limited and therefore, the refractive index change with pressure remains minimal. In other words, the variation of pressure only has a limited impact on background noise under the conditions employed in this work, which are quite far from being purely supercritical.

With MS detection, the LOD values were much more diverse and ranged from 0.05 to 5 µg mL^{-1} for the 3-mer A peptide, while they were comprised between 0.05 and 2 µg mL^{-1}for the 6-mer B. Interestingly, HILIC conditions offer the best sensitivity (0.05 µg mL^{-1}whatever the peptides and the stationary phase, and despite the use of 0.1 %TFA in the mobile phase). This excellent sensitivity has already been described in HILIC for small molecules, but not yet for peptides, at least to the best of our knowledge. It has been attributed to an improvement of the desolvation process in electrospray when using a highly organic mobile phase, as well as to a modification of ionization state of the compound in pres-

ence of high proportion of ACN [43,44]. Beside HILIC, SFC also offers some very good LOD (always equal to 0.1 µg mL^{-1}, except for the 3-mer A when using basic additive). Here again, the good SFC-MS sensitivity has already been described elsewhere and is due to the absence of water (or minimal presence) in the mobile phase, thus improving the performance of the ESI process [45,46]. Finally, for RPLC and mixed-mode LC, the sensitivity was always equal or worse than with UV detection. The LOD values ranged from 2 to 5 µg $mL^{-1}$for the 3-mer A peptide, while they were comprised between 1 and 2 µg $mL^{-1}$for the 6-mer B. Obviously, the presence of TFA is known to be a strong contributor to signal suppression in electrospray ionization mode, but was required to obtain suitable peak shapes. In addition, the elution composition for the two selected peptides was always below 20-30% of ACN whatever the chromatographic mode (RPLC or mixed-mode LC), then the mobile phase was highly aqueous, which may further decrease MS sensitivity.

In conclusion, it appears that SFC (either 0.05% MSA or 0.2% NH_4OH as mobile phase additive) offers a very good sensitivity compared to other chromatographic modes, when analyzing peptides.

4. Conclusion

In this study, several chromatographic approaches for peptides analysis were investigated at different levels. First, the retention behavior of 13 synthetic peptides with different properties in terms of sizes and isoelectric points was studied with each chromatographic mode. All the peptides were eluted with at least one set of chromatographic conditions for each mode, allowing to obtain data on retention time and peak shape. Concerning peak shapes, average tailing factors were quite comparable among the various conditions, except for HILIC with bare silica, which provided the highest average tailing factor value (Tf = 2.26). The best results in terms of peak capacity were obtained under RPLC with basic conditions and mixed-mode LC conditions with both acidic and basic conditions ($P > 200$ for 7 minutes analysis time), followed by RPLC and SFC both under acidic conditions and HILIC with amide column ($P > 100$). The broadest peaks were observed with SFC using basic additive and HILIC with bare silica column ($P < 100$). In conclusion, it can be stated that the overall qualitative performance of SFC (in terms of retention capability and peak shape) was comparable to that obtained with the other chromatographic modes tested in this study.

However, this comparability changed when evaluating the selective performance of each chromatographic mode on closely related peptides. The best results (6 out of 6 separated peaks) were obtained by RPLC and mixed-mode LC, no matter the nature of the additive used. HILIC showed intermediate selective performance (3 out of 6 and 4 out of 6 separated peaks by HILIC with bare silica and amide columns, respectively) while SFC was the worst one (0 out of 6 and 1 out of 6 separated peaks for SFC with acidic and basic additives, respectively). However, when analyzing short peptides, the high versatility of SFC came out, enabling it to be the sole technique capable to simultaneously analyze peptides with wide differences in terms of polarity.

Concerning the sensitivity of each mode, the LOD values for the UV detection ranged from 0.5 to 2 µg.$mL^{-1}$for the peptides considered. The results obtained by SFC were comparable to those obtained with the other chromatographic modes. However, with MS detection, the observed LOD range was much wider, going from 0.05 to 5 µg.mL^{-1}. HILIC presented the lowest LOD values, followed by SFC, which outperformed RPLC and mixed-mode LC for this aspect.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Riccardo Deidda: Writing – original draft, Writing – review & editing, Conceptualization, Formal analysis, Visualization. **Gioacchino Luca Losacco:** Writing – original draft, Writing – review & editing. **Cedric Schelling:** Writing – review & editing, Formal analysis. **Erik L. Regalado:** Writing – review & editing, Funding acquisition. **Jean-Luc Veuthey:** Writing – review & editing, Conceptualization, Supervision, Funding acquisition, Project administration. **Davy Guillarme:** Writing – original draft, Writing – review & editing, Conceptualization, Supervision, Funding acquisition, Project administration.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.463282.

References

[1] D.J. Craik, D.P. Fairlie, S. Liras, D. Price, The future of peptide-based drugs, Chem. Biol. Drug Des. 81 (2013) 136–147, doi:10.1111/cbdd.12055.
[2] A. Henninot, J.C. Collins, J.M. Nuss, The current state of peptide drug discovery: back to the future? J. Med. Chem. 61 (2018) 1382–1414, doi:10.1021/acs.jmedchem.7b00318.
[3] N. Tsomaia, Peptide therapeutics: targeting the undruggable space, Eur. J. Med. Chem. 94 (2015) 459–470, doi:10.1016/j.ejmech.2015.01.014.
[4] M. Tyagi, F. Begnini, V. Poongavanam, B.C. Doak, J. Kihlberg, Drug synthesis beyond the rule of 5, Chem. Eur. J. 26 (2020) 49–88, doi:10.1002/chem.201902716.
[5] V. D'Aloisio, P. Dognini, G.A. Hutcheon, C.R. Coxon, PepTherDia: database and structural composition analysis of approved peptide therapeutics and diagnostics, Drug Discov. Today 26 (2021) 1409–1419, doi:10.1016/j.drudis.2021.02.019.
[6] Z. Lian, N. Wang, Y. Tian, L. Huang, Characterization of synthetic peptide therapeutics using liquid chromatography- mass spectrometry: challenges, solutions, pitfalls, and future perspectives, J. Am. Soc. Mass Spectrom. 32 (2021) 1852–1860, doi:10.1021/jasms.0c00479.
[7] B.K. Prabhala, O. Mirza, P. Højrup, P.R. Hansen, Characterization of synthetic peptides by mass spectrometry, in: G. Houen (Ed.), Peptide Antibodies. Methods in molecular biology, Humana Press, New York, 2015, doi:10.1007/978-1-4939-2999-3_9.
[8] G.L. Adams, P.S. Pall, S.M. Grauer, X. Zhou, J.E. Ballard, M. Vavrek, R.L. Kraus, P. Morissette, N. Li, S. Colarusso, E. Bianchi, A. Palani, R. Klein, C.T. John, D. Wang, M. Tudor, A.F. Nolting, M. Biba, T. Nowak, A.A. Makarov, M. Reibarkh, A.V. Buevich, W. Zhong, E.L. Regalado, X. Wang, Q. Gao, A. Shahripour, Y. Zhu, D. de Simone, T. Frattarelli, N.M. Pasquini, P. Magotti, R. Iaccarino, Y. Li, K. Solly, K.-J. Lee, W. Wang, F. Chen, H. Zeng, J. Wang, H. Regan, R.P. Amin, C.P. Regan, C.S. Burgey, D.A. Henze, C. Sun, D.M. Tellers, Development of ProTx-II analogues as highly selective peptide blockers of Na_v 1.7 for treatment of pain, J. Med. Chem. 65 (2022) 485–496, doi:10.1021/acs.jmedchem.1c01570.
[9] R. Bennett, G.F. Pirrone, T. Nowak, D. Mukherjee, V. Shchurik, C. Mapelli, J.L. Hickey, E.L. Regalado, I. Mangion, A.A. Makarov, Ultra-high throughput SPE-MALDI workflow: Blueprint for efficient purification and screening of peptide libraries, Anal. Chim. Acta 1142 (2021) 10–18, doi:10.1016/j.aca.2020.10.045.
[10] A.A. Makarov, G.F. Pirrone, V. Shchurik, E.L. Regalado, I. Mangion, Liposome artificial membrane permeability assay by MALDI-hydrogen-deuterium exchange mass spectrometry for peptides and small proteins, Anal. Chim. Acta 1099 (2020) 111–118, doi:10.1016/j.aca.2019.09.063.
[11] J. Liu, A.A. Makarov, R. Bennett, I.A.H. Ahmad, J. DaSilva, M. Reibarkh, I. Mangion, B.F. Mann, R.L. Regalado, Chaotropic effects in sub/supercritical fluid chromatography via ammonium hydroxide in water-rich modifiers: enabling separation of peptides and highly polar pharmaceuticals at the preparative scale, Anal. Chem. 91 (2019) 13907–13915, doi:10.1021/acs.analchem.9b03408.
[12] L. Zang, T. Carlage, D. Murphy, R. Frenkel, P. Bryngelson, M. Madsen, Y. Lyubarskaya, Residual metals cause variability in methionine oxidation measurements in protein pharmaceuticals using LC-UV/MS peptide mapping, J. Chromatogr. B 895-896 (2012) 71–76, doi:10.1016/j.jchromb.2012.03.016.
[13] C.X. Cai, N.A. Schneck, V.B. Ivleva, K. Gulla, Y. Zhang, D. Gowetski, Q.P. Lei, Quantification of residual hydrophobic fusion peptide with monomer and dimer forms using reversed-phase liquid chromatography, J. Chromatogr. B 1144 (2020) 122073, doi:10.1016/j.jchromb.2020.122073.
[14] Y. Yang, R.I. Boysen, J. Chowdhury, A. Alam, M.T.W. Hearn, Analysis of peptides and protein digests by reversed phase high performance liquid

chromatography-electrospray ionisation mass spectrometry using neutral pH elution conditions, Anal. Chim. Acta 872 (2015) 84–94, doi:10.1016/j.aca.2015.02.055.
[15] A. D'Attoma, S. Heinisch, On-line comprehensive two dimensional separations of charged compounds using reverse-phase high performance liquid chromatography and hydrophilic interaction chromatography. Part II: application to the separation of peptides, J. Chromatogr. A 1306 (2013) 27–36, doi:10.1016/j.chroma.2013.07.048.
[16] S. Chapel, F. Rouvière, S. Heinisch, Pushing the limits of resolving power and analysis time in on-line comprehensive hydrophilic interaction x reversed phase liquid chromatography for the analysis of complex peptide samples, J. Chromatogr. A 1615 (2020) 460753, doi:10.1016/j.chroma.2019.460753.
[17] W. Liu, Y. Cao, Y. Ren, X. Xu, L. He, R. Xia, P. Tu, Y. Wang, Y. Song, J. Li, Simultaneously quantitative analysis of peptides and chemical components in Carvus and Cucumis polypeptide injection (Songmeile®) using reversed phase liquid chromatography-hydrophilic interaction liquid chromatography-tandem mass spectrometry, J. Chromatogr. A 1617 (2020) 460827, doi:10.1016/j.chroma.2019.460827.
[18] P. Kozlik, J. Vaclova, K. Kalikova, Mixed-mode hydrophilic interaction/ion-exchange liquid chromatography- separation potential in peptide analysis, Microchem. J. 165 (2021) 106158, doi:10.1016/j.microc.2021.106158.
[19] S. Ray, M. Takafuji, H. Ihara, Chromatographic evaluation of a newly designed peptide-silica stationary phase in reverse phase liquid chromatography and hydrophilic interaction liquid chromatography: mixed mode behavior, J. Chromatogr. A 1266 (2012) 43–52, doi:10.1016/j.chroma.2012.10.004.
[20] F. Progent, M. Taverna, A. Banco, A. Tchapla, C. Smadja, Chromatographic behavior of peptides on a mixed-mode stationary phase with an embedded charged group by capillary electrochromatography and high-performance liquid chromatography, J. Chromatogr. A 1136 (2006) 221–225, doi:10.1016/j.chroma.2006.09.095.
[21] G.L. Losacco, J.-L. Veuthey, D. Guillarme, Metamorphosis of supercritical fluid chromatography: A viable tool for the analysis of polar compounds? Trends Analyt. Chem. 141 (2021) 116304, doi:10.1016/j.trac.2021.116304.
[22] J. Molineau, M. Hideux, C. West, Chromatographic analysis of biomolecules with pressurized carbon dioxide mobile phases- A review, J. Pharm. Biomed. Anal. 193 (2021) 113736, doi:10.1016/j.jpba.2020.113736.
[23] J. Zheng, J.D. Pinkston, P.H. Zoutendam, L.T. Taylor, Feasibility of supercritical fluid chromatography/mass spectrometry of polypeptides with up to 40-Mers, Anal. Chem. 78 (2006) 1535–1545, doi:10.1021/ac052025s.
[24] J. Zhang, K.B. Thurbide, Direct analysis of gramicidin double helices using packed column supercritical fluid chromatography, J. Chromatogr. A 1101 (2006) 286–292, doi:10.1016/j.chroma.2005.10.008.
[25] G.L. Losacco, J. Oliviera DaSilva, J. Liu, E.L. Regalado, J.-L. Veuthey, D. Guillarme, Expanding the range of sub/supercritical fluid chromatography: advantageous use of methanesulfonic acid in water-rich modifiers for peptide analysis, J. Chromatogr. A 1642 (2021) 462048, doi:10.1016/j.chroma.2021.462048.
[26] K. Govender, T. Naicker, S. Baijnath, A.A. Chuturgoon, N.S. Abdul, T. Docrat, H.G. Kruger, T. Govender, Sub/supercritical fluid chromatography employing water-rich modifier enables the purification of biosynthesized human insulin, J. Chromatogr. B 1155 (2020) 122126, doi:10.1016/j.jchromb.2020.122126.
[27] N.M. Schiavone, R. Bennett, M.B. Hicks, G.F. Pirrone, E.L. Regalado, I. Mangion, A.A. Makarov, J. Chromatogr. B 1110-1111 (2019) 94–100, doi:10.1016/j.jchromb.2019.02.012.
[28] V. Spelling, M. Stefansson, Evaluation of chromatographic parameters in supercritical fluid chromatography of amino acids as model polar analytes and extended to polypeptide separations, J. Chromatogr. A 1633 (2020) 461646, doi:10.1016/j.chroma.2020.461646.
[29] M. Enmark, E. Glenne, M. Leśko, A. Langborg Weinmann, T. Leek, K. Kaczmarski, M. Klarqvist, J. Samuelsson, T. Fornstedt, Investigation of robustness for supercritical fluid chromatography separation of peptides: isocratic vs gradient mode, J. Chromatogr. A 1568 (2018) 177–187, doi:10.1016/j.chroma.2018.07.029.
[30] M. Ventura, Advantageous use of SFC for separation of crude therapeutic peptides and peptide libraries, J. Pharm. Biomed. Anal. 185 (2020) 113227, doi:10.1016/j.jpba.2020.113227.
[31] E. Gasteiger, A. Gattiker, C. Hoogland, I. Ivanyi, R.D. Appel, A. Bairoch, ExPASy: the proteomics server for in-depth protein knowledge and analysis, Nucleic Acids Res 31 (2003) 3784–3788, doi:10.1093/nar/gkg563.
[32] D. Guillarme, V. Desfontaine, S. Heinisch, J.-L. Veuthey, What are the current solutions for interfacing supercritical fluid chromatography and mass spectrometry, J. Chromatogr. B 1083 (2018) 160–170, doi:10.1016/j.jchromb.2018.03.010.
[33] K. Choikhet, B. Glatz, G. Rozing, The physicochemical causes of baseline disturbances in HPLC, LC GC Europe 16 (2) (2003) 96–105 http://gazanaliz.ru/manuals/Varian/service/pif/6_training/Reading/Baseline_Ripple_I.pdf. (accessed 14 April 2022).
[34] M. Gilar, H. Xie, A. Jaworski, Utility of retention prediction model for investigation of peptide separation selectivity in reversed-phase liquid chromatography: impact of concentration of trifluoroacetic acid, column temperature, gradient slope and type of stationary phase, Anal. Chem. 82 (1) (2010) 265–275, doi:10.1021/ac901931c.
[35] R.C. Dwivedi, V. Spicer, M. Harder, M. Antonovici, W. Ens, K.G. Standing, J.A. Wilkins, O.V. Krokhin, Practical implementation of 2D HPLC scheme with accurate peptide retention prediction in both dimensions for high-throughput bottom-up proteomics, Anal. Chem. 80 (18) (2008) 7036–7042, doi:10.1021/ac800984n.
[36] M. Gilar, A. Jaworski, Retention, Retention behavior of peptides in hydrophilic-interaction chromatography, J. Chromatogr. A 1218 (2011) 8890–8896, doi:10.1016/j.chroma.2011.04.005.
[37] J. Molineau, M. Hideux, P. Hennig, S. Bertin, F. Mauge, E. Lesellier, C. West, Analysis of short-chain bioactive peptides by unified chromatography-electrospray ionization mass spectrometry. Part II. Comparison to reversed-phase ultra-high performance liquid chromatography, J. Chromatogr. A 1663 (2022) 462771, doi:10.1016/j.chroma.2021.462771.
[38] V. Desfontaine, A. Tarafder, J. Hill, J. Fairchild, A.G.-G. Perrenoud, J.-L. Veuthey, D. Guillarme, A systematic investigation of sample diluents in modern supercritical fluid chromatography, J. Chromatogr. A 1511 (2017) 122–131, doi:10.1016/j.chroma.2017.06.075.
[39] K. Taguchi, E. Fukusaki, T. Bamba, Simultaneous analysis for water- and fat-soluble vitamins by a novel single chromatography technique unifying supercritical fluid chromatography and liquid chromatography, J. Chromatogr. A 1362 (2014) 270–277, doi:10.1016/j.chroma.2014.08.003.
[40] V. Desfontaine, G.L. Losacco, Y. Gagnebin, J. Pezzati, W.P. Farrell, V. González-Ruiz, S. Rudaz, J.-L. Veuthey, D. Guillarme, Applicability of supercritical fluid chromatography- mass spectrometry to metabolomics. I- optimization of separation conditions for the simultaneous analysis of hydrophilic and lipophilic substances, J. Chromatogr. A 1562 (2018) 96–107, doi:10.1016/j.chroma.2018.05.055.
[41] R. Helmy, M. Biba, J. Zang, B. Mao, K. Fogelman, V. Vlachos, P. Hosek, C.J. Welch, Improving sensitivity in chiral supercritical fluid chromatography for analysis of active pharmaceutical ingredients, Chirality 19 (2007) 787–792, doi:10.1002/chir.20451.
[42] T.A. Berger, B.K. Berger, Minimizing UV noise in supercritical fluid chromatography. I. Improving back pressure regulator pressure noise, J. Chromatogr. A 1218 (2011) 2320–2326, doi:10.1016/j.chroma.2011.02.030.
[43] A. Periat, J. Boccard, J.-L. Veuthey, S. Rudaz, D. Guillarme, Systematic comparison of sensitivity between hydrophilic interaction liquid chromatography and reversed phase liquid chromatography coupled with mass spectrometry, J. Chromatogr. A 1312 (2013) 49–57, doi:10.1016/j.chroma.2013.08.097.
[44] A. Periat, I. Kohler, A. Burgey, S. Bieri, F. Versace, C. Staub, D. Guillarme, Hydrophilic interaction chromatography versus reversed phase liquid chromatography coupled to mass spectrometry: Effect of electrospray ionization source geometry on sensitivity, J. Chromatogr. A 1356 (2014) 211–220, doi:10.1016/j.chroma.2014.06.066.
[45] A.G.-G. Perrenoud, J.-L. Veuthey, D. Guillarme, Coupling state-of-the-art supercritical fluid chromatography and mass spectrometry: from hyphenation interface optimization to high- sensitivity analysis of pharmaceutical compounds, J. Chromatogr. A 1339 (2014) 174–184, doi:10.1016/j.chroma.2014.03.006.
[46] G.L. Losacco, J.-L. Veuthey, D. Guillarme, Supercritical fluid chromatography-Mass spectrometry: recent evolution and current trends, Trends in Analyt. Chem. 118 (2019) 731–738, doi:10.1016/j.trac.2019.07.005.