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Expanding the range of sub/supercritical fluid chromatography: Advantageous use of methanesulfonic acid in water-rich modifiers for peptide analysis.

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ABSTRACT

The aim of this work was to expand the applicability range of UHPSFC to series of synthetic and commercialized peptides. Initially, a screening of different column chemistries available for UHPSFC analysis was performed, in combination with additives of either basic or acidic nature. The combination of an acidic additive (13 mM TFA) with a basic stationary phase (Torus DEA and 2-PIC) was found to be the best for a series of six synthetic peptides possessing either acidic, neutral or basic isoelectric points. Secondly, methanesulfonic acid (MSA) was evaluated as a potential replacement for TFA. Due to its stronger acidity, MSA gave better performance than TFA at the same concentration level. Furthermore, the use of reduced percentages of MSA, such as 8 mM, yielded similar results to those observed with 15 mM of MSA. The optimized UHPSFC method was, then, used to compare the performance of UHPSFC against RP-UHPLC for peptides with different pI and with increasing peptide chain length. UHPSFC was found to give a slightly better separation of the peptides according to their pI values, in few cases orthogonal to that observed in UHPLC. On the other hand, UHPSFC produced a much better separation of peptides with an increased amino acidic chain compared to UHPLC. Subsequently, UHPSFC-MS was systematically compared to UHPLC-MS using a set of linear and cyclic peptides commercially available. The optimized UHPSFC method was able to generate at least similar, and in some cases even better performance to UHPLC with the advantage of providing complementary information to that given by UHPLC analysis. Finally, the analytical UHPSFC method was transferred to a semipreparative scale using a proprietary cyclic peptide, demonstrating excellent purity and high yield in less than 15 min.

1. Introduction

Peptides and peptide-like drugs are compounds which typically generated a lot of interest within the pharmaceutical industry. Their presence in several key biological processes makes them an interesting class of molecules from which new drugs could be developed [1,2]. There have been several developments in the use of peptides as therapeutic agents: originally, they were simply used in replacement therapies, when patients lacked a specific peptide in their organism [3,4]. A classic example of this strategy is the administration of insulin to patients suffering from type 1 diabetes [3]. Subsequently, synthetic analogs of different peptides already present in the human body came along [5,6]. However, peptides present several issues as drugs, mainly related to their pharmacokinetic properties [7,8], because of their low bioavailability due to their size, up to 5000 – 6000 Da for peptides with an amino acidic sequence of 40–50 amino acids, as well as an facile metabolism [9]. To improve their properties, modern synthetic peptides have started to differ, from a structural point of view, from their biological precursors, including new functional groups in their structure (i.e. polymers and fatty acids) introduced to develop a better bioavailability via their oral formulation [10,11].

The analytical strategy to characterize this class of molecules has revolved on the use of ultra-high performance liquid

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chromatography (UHPLC) as the preferred technique, mainly in reversed-phase mode (RPLC) [12–14]. Its ease of use, high throughput capacity and ability to couple with ultraviolet (UV) detector and, more importantly, mass spectrometry (MS) made it a popular technique for peptide analysis [15–17]. Despite the advantages of UHPLC-UV-MS, a demand for greener, faster and complementary analytical techniques is always present [18]. Among them, one of the most promising strategies is ultra-high performance supercritical fluid chromatography (UHPSFC). Thanks to the development of dedicated sub-2 µm stationary phases, as well as the release of chromatographic systems able to withstand the backpressures generated by these columns, UHPSFC has shown a great potential as a complementary alternative to UHPLC. This was possible thanks to the use of a mobile phase consisting in a mixture of supercritical carbon dioxide with polar organic modifier [19]. Moreover, it presents an easy hyphenation to various detectors such as UV and MS [20] and can provide fast analyses as the mobile phase presents low viscosity, enabling higher flow-rates without experiencing high backpressures. Finally, a high degree of orthogonality exists between UHPSFC and UHPLC, especially with the RPLC mode [21].

The analysis of peptides in UHPSFC is described in the literature, and there have already been studies demonstrating the use of UHPSFC for their analysis [22–25]. However, a systematic comparison between UHPSFC and UHPLC has not been made until now. This is probably because UHPSFC is difficult to use for the analysis of highly polar compounds having high molecular weight (partial elution from the column, solubility issues, distorted peaks…). Nonetheless, in the last 2–3 years a new trend appears in UHPSFC, consisting in the use of gradient profiles reaching percentages of organic modifier up to 90–100% [26–28]. Furthermore, the addition of water, up to 5–7% in the organic co-solvent has enabled UHPSFC to give improved performance in the analysis of polar and ionized metabolites, as it increases the elution strength of the mobile phase [28,29]. These new trends in UHPSFC could, therefore, reinvigorate its applicability for the analysis of peptides.

The aim of this study was to evaluate the performance of UHPSFC, coupled to different detectors (UV and MS), for the analysis of a series of synthetic and therapeutic peptides. Different chromatographic aspects, such as retention, selectivity and peak shape, as well as compatibility with MS detection and, finally, scale-up to the preparative scale, have been investigated. The impact of peptide isoelectric point, hydrophobicity and amino acids chain length, on the UHPSFC separation was assessed. A systematic comparison to UHPLC in the RPLC mode was also performed with the goal of highlighting possible advantages and disadvantages of the newly developed method.

2. Materials and methods

2.1. Chemicals, reagents and sample preparation procedures

For all experiments performed at the University of Geneva, 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 level) was purchased from PanGas (Dagmerstellen, Switzerland). Metanil yellow and methyl orange, lysine, arginine, aspartic acid, glutamic acid, ammonia solution at 25% of MS grade, trifluoroacetic acid (TFA) of MS grade and methanesulfonic acid (MSA) at a purity level of 99.5% or higher were purchased from Sigma-Aldrich (Buchs, Switzerland). Synthetic peptides 1N, 2N, 1B, 2B, 1A, 2A, 6mer, 9mer, 12mer, 15mer, 18mer and 21mer at a purity level of $\geq$ 95% have been purchased from GenScript Biotech (Leiden, Netherlands). More information regarding their amino acid sequences, molecular weights as well as predicted isoelectric points (pI) and GRAVY numbers are provided in Table 1. GRAVY number is a measure of the grade of hydrophilicity of a protein/peptide based on its hydropathy index, a value which varies between −2 to 2 for most proteins; the higher the hydropathy index, the higher the hydrophobicity. GRAVY number and pI values were obtained using the ProtParam tool available on the proteomic server ExPASy [30,31]. Commercial pharmaceutical formulations of liraglutide, leuprorelin, glucagon, cyclosporin A, eptifibatide and linaclotide (Table 1) have been purchased from the hospital pharmacy at the Geneva University Hospitals (HUG, Geneva, Switzerland).

For all purification experiments, methanol (HPLC Grade) and water (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Methanesulfonic acid (MSA), 99% extra pure was purchased from ARCŌS Organics (Morris Plains, NJ, USA). The cyclic peptide was obtained in-house (Merck & Co., Inc., Kenilworth, NJ, USA). Bone dry-grade CO2 was obtained from Air Gas (New Hampshire, USA).

Details regarding the sample preparation and stress procedures used in this study can be found in the supplementary material.

Table 1
List of synthetic and commercial peptides used in this study.

Name	Sequence	MW (Da)	Number of amino acids	pI (predicted)	GRAVY number
Peptide 1N	Trp-Asn-Ser-Val-Lys-Tyr-Asp-Ile-Ser-Tyr-His-Thr	1512	12	6.74	−0.93
Peptide 2N	Ala-Tyr-His-Asp-Gln-Trp-Lys-Tyr-His-Phe-Cys	1497	11	6.95	−1.24
Peptide 1B	Trp-Gln-Ser-Thr-Tyr-His-Asp-Lys-Phe-Ala-Trp-Arg-Tyr	1788	13	8.50	−1.53
Peptide 2B	Phe-Lys-Asn-Ser-Tyr-His-Gln-Ile-Arg-Trp-Val-Tyr-Asn-Phe	1902	14	9.70	−0.86
Peptide 1A	Phe-Asn-Glu-Cys-Tyr-Arg-Ser-Asp-Ala-Tyr-Ser-Asn-Thr-Phe	1717	14	4.37	−0.96
Peptide 2A	Tyr-Asn-Ser-Phe-Asp-Glu-Trp-Lys-Cys-Thr-Phe-Ser-Trp	1713	13	4.37	−0.90
Peptide 6mer	Leu-Trp-His-Gly-Ser-Asn	713	6	6.74	−0.83
Peptide 9mer	Leu-Trp-His-Gly-Ser-Asn-Lys-Trp-Asp	1142	9	6.74	−1.48
Peptide 12mer	Leu-Trp-His-Gly-Ser-Asn-Lys-Trp-Asp-Asn-Gly-Gln	1441	12	6.74	−1.73
Peptide 15mer	Leu-Trp-His-Gly-Ser-Asn-Lys-Trp-Asp-Asn-Gly-Gln-Trp-Ser-Asn	1829	15	6.74	−1.73
Peptide 18mer	Leu-Trp-His-Gly-Ser-Asn-Lys-Trp-Asp-Asn-Gly-Gln-Trp-Ser-Asn-Gly-Thr-Gln	2115	18	6.74	−1.69
Peptide 21mer	Leu-Trp-His-Gly-Ser-Asn-Lys-Trp-Asp-Asn-Gly-Gln-Trp-Ser-Asn-Gly-Thr-Gln-Ala-Asn-Ser	2387	21	6.74	−1.57
Liraglutide	His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys(γ-Glu-palmitoyl)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly	3751	32	4.96	−0.36
Leuprorelin	pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt	1209	9	8.75	−0.52
Glucagon	His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr	3483	29	6.75	−0.99
Cyclosporin A	Abu-Sar-Leu-Val-Leu-Ala-dAla-Leu-Leu-Val-Bmt	1203	11	NA	NA
Eptifibatide	Cys-hArg-Gly-Asp-Trp-Pro-Cys	832	7	3.80	−0.20
Linaclotide	Cys-Cys-Glu-Tyr-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys-Tyr	1527	14	4.00	0.32

2.2. Chromatographic and MS instrumentations and conditions

At University of Geneva, for UHPSFC analyses, five different columns have been initially employed, namely Torus 2-PIC, Torus DEA, Torus DIOL, BEH silica (Waters, Milford, MA, USA), all packed with 1.7 µm fully porous silica particles, and Nucleoshell HILIC (Macherey-Nagel, Düren, Germany), packed with 2.7 µm superficially porous silica particles. All columns possess the same geometry of 100 × 3.0 mm I.D. A generic gradient was developed for the analysis of synthetic peptides, from 10 to 100% organic modifier in the mobile phase over 7 min, followed by an isocratic hold at 100% of co-solvent for 1 min, then a return to initial conditions in 0.1 min, and a final isocratic step with 10% of organic modifier for 2.9 min, giving a total run time of 11 min (section 3.1.). Organic modifier employed at this stage was a mixture of MeOH/H_2O 95:5 *v/v* containing either 13 mM (0.1%) TFA, 15 mM (0.1%) MSA or 52 mM (0.2%) NH_4OH. Flow-rate was fixed at 0.7 mL.min^{-1}. Following this preliminary step, an optimized method for the analysis of synthetic peptides was developed and used in the second part of the study (section 3.2), based on the Torus 2-PIC stationary phase with mixture of MeOH/H_2O 95:5 *v/v* + 8 mM MSA as the co-solvent. The optimized method follows a different gradient profile, starting from 30 to 80% organic modifier over 5 min, then an isocratic step at 80% of co-solvent for 0.5 min, followed by a return to initial conditions in 0.1 min and a second isocratic step of 1.9 min for a total analysis time of 7.5 min. Flow-rate was fixed, in this case at 0.9 mL.min^{-1}. For the commercially available peptides (i.e. liraglutide, leuprorelin, glucagon, linaclotide and eptifibatide), a modified version of the optimized gradient was employed: start at 35% co-solvent, reaching 90% in 5 min, then an isocratic step at 90% of co-solvent for 0.5 min, followed by a return to initial conditions in 0.1 min and a second isocratic step under these conditions of 1.9 min for a total analysis time of 7.5 min. For cyclosporin A, a third gradient was chosen, starting from 2 to 40% over 5 min, with an isocratic step at 40% of co-solvent for 0.5 min, then return to initial conditions in 0.1 min and a second isocratic step for 1.9 min, giving a total run time of 7.5 min.

Under UHPLC conditions, a 50 × 2.1 mm I.D. BEH C_{18} stationary phase packed with 1.7 µm fully porous particles (Waters) was used. Mobile phase A was H_2O + 13 mM TFA, while mobile phase B was ACN + 13 mM TFA. An optimized gradient was employed for all synthetic and therapeutic peptides (with the only exception of cyclosporin A), consisting in a 5 min gradient from 5 to 65%B, a hold up for two minutes at 65% B, then a return to initial conditions in 0.1 min and an isocratic hold for 1.9 min at 5% for a total run time of 9 min. For cyclosporin A the gradient time and total run time were the same, however the highest percentage of B reached during the gradient was 95%. In all these conditions, the flow-rate was fixed at 0.4 mL.min^{-1}.

The column screening consisted of eight different stationary phases, namely Chiralpak IC and Chiralcel OZ (both of geometry of 100 × 4.6 mm I.D. – 3.0 µm fully porous particles); Chiralcel OJ, Chiralpak IG and DCpak P4VP (all with geometry of 150 × 4.6 mm I.D. – fully porous particle sizes of 3.0 µm for Chiralcel OJ and of 5.0 µm Chiralpak IG and DCpak P4VP) from Chiral Technologies (West Chester, PA, USA); CELERIS 4EP from Regis Technologies (Morton Grove, IL, USA) and Torus DEA and Torus 2-PIC from Waters Corp. (Milford, MA, USA), all with the geometry of 250 × 4.6 mm I.D. and packed with 5.0 µm fully porous particles. SFC screenings were carried out on the diverse set of columns described in the above section by gradient elution at a flow rate of 2 mL.min^{-1} with the backpressure regulator (BPR) set at 103 bar (1500 psi). The SFC eluents consisted of CO_2 and organic modifier, which consisted of MeOH/H_2O 95/5 *v/v* + 8 mM MSA. The mobile phases were programmed as follows: 35% B at 0 min, linear gradient from 35% to 90% B in 5 min, a hold at 90% B for 0.5 min, then return to 35% B in 0.1 min and finally hold at 35% B for 1.9 min. The PDA scans from 190 to 400 nm and the chromatogram is extracted at 210 nm. The MS scans the mass range of 100 to 1200 with a sampling frequency of 2 Hz, cone voltages of 10 and 50 V in ESI (+) and a cone voltage of 10 V in ESI (-). Preparative SFC purification was performed on a Waters Torus 2-PIC 30.0 mm x 250 mm, 5 µm column with a mobile phase of 35% MeOH/H_2O 95/5 *v/v* + 8 mM MSA / CO_2. The flow rate was 140 mL.min^{-1}, mobile phase and column oven temperature at 35 °C, back pressure regulator set to 103 bar (1500 psi), UV detection at 210 nm. The sample was prepared at 20 mg/mL in methanol with a load of 1 mL.

SFC analysis of the cyclic peptide was carried out on a Waters Torus 2-PIC 4.6 mm I.D. x 250 mm 5 µm column at a flow rate of 2 mL.min^{-1} with the backpressure regulator (BPR) set at 100 bar; The SFC eluent solvent was 40% MeOH/H_2O 95/5 *v/v* + 8 mM MSA / CO_2. The PDA scans from 190 to 400 nm and the chromatogram was extracted at 210 nm.

All information regarding the chromatographic and MS instruments conditions, as well as on the software employed for data treatment can be found in the supplementary material.

3. Results and discussion

3.1. Development of the UHPSFC chromatographic method

3.1.1. Impact of the additive nature on the stationary phase performance

To ensure the elution of peptides using a CO_2-based mobile phase, various parameters have to be considered. Firstly, the addition of water in the co-solvent seems necessary to ensure acceptable peak shapes as well as elution within reasonable time [25,32–34]. Secondly, additives are needed to further reduce the tailing factor and peak widths [23,24,35]. To choose the most appropriate stationary phase, a screening of the several chemistries available was often needed. Overall, analytical conditions for peptide analysis under SFC can be summarized as follows: a mixture of methanol and water as the organic co-solvent, in combination with an additive (in most cases TFA). However, the application of such conditions is mostly limited to the analysis of peptides with relatively short amino acidic sequences (often 10–12 or less) [22–24,32]. Therefore, the goal of the present study was to find conditions suitable for a wider range of peptides, through the screening of different stationary phase chemistries, in combination with the use of acidic and basic additives. Such a screening strategy was firstly applied to a series of synthetic peptides described in Table 1 (peptides 1 N, 2 N, 1B, 2B, 1A and 2A). These peptides all possess a sequence of a length between 11 – 14 amino acids and with a molecular weight ranging from 1500 to 1900 Da. Furthermore, these different peptides possess either an acidic (pI < 7), neutral (pI ≈ 7), or basic nature (pI > 7) and they all possess an important polar character (GRAVY number between −1 and −2). Indeed, compounds with these properties have always been challenging to analyze under UHPSFC conditions, as they are strongly retained on the (polar) stationary phase, and poorly soluble in mobile phases with a predominant presence of supercritical CO_2. Each stationary phase (i.e. Torus 2-PIC, Torus DEA, Nucleoshell HILIC, Torus DIOL, BEH silica) was tested with the same organic modifier composition (MeOH/H_2O 95:5) in which either 13 mM (0.1%) of TFA or 52 mM (0.2%) of NH_4OH was added. In Fig. 1a, a table summarizing the data is presented. Stationary phases with a "basic" nature (having one or more positively charged functional groups) are those providing the best results, yielding complete elution of all synthetic peptides with good peak shape, as illustrated in Fig. 1b for peptides 1B and 2A on the Torus 2-PIC. Between the Torus 2-PIC and Torus DEA, no major differences were observed,

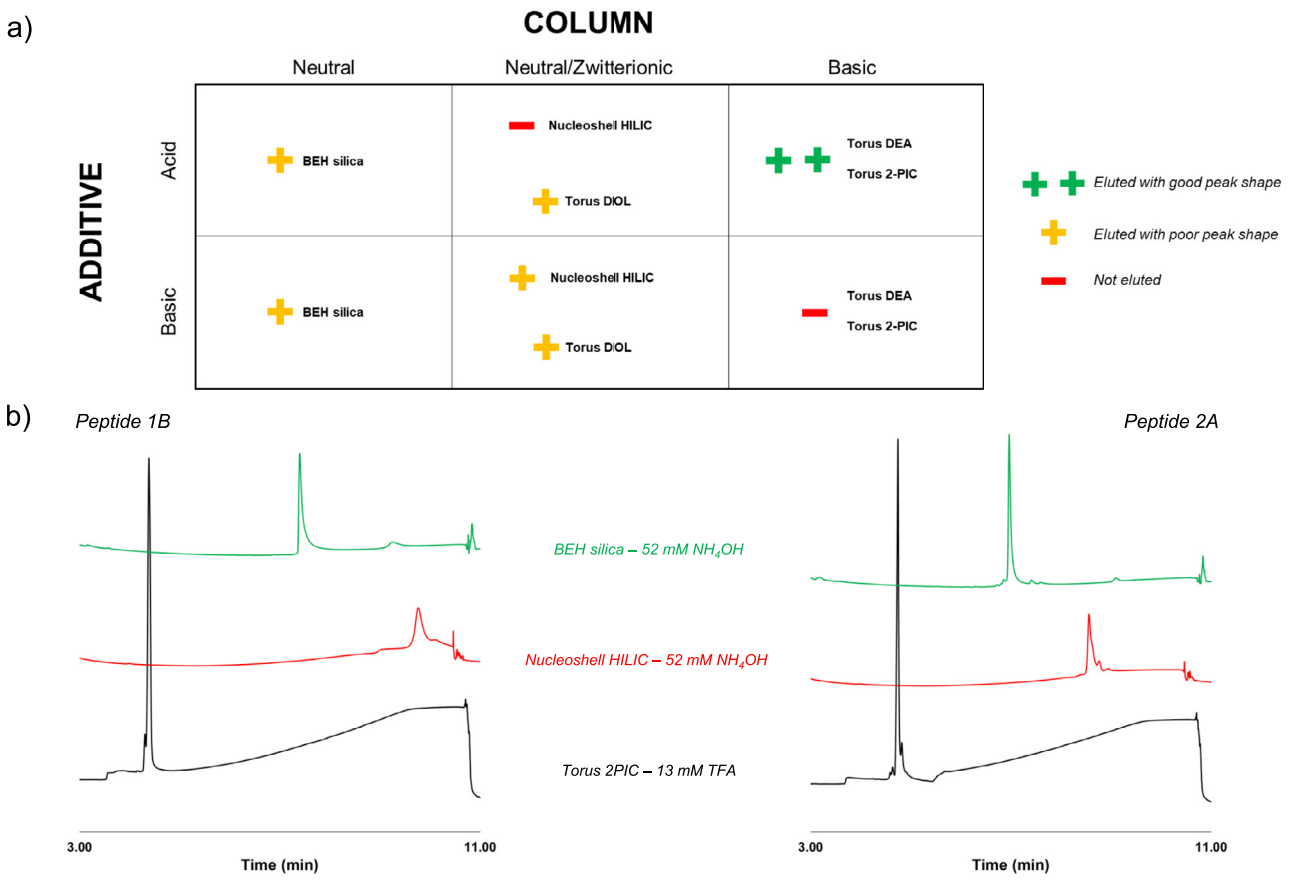

Fig. 1. a) A classification of the combination between the nature of the additive and the properties of the stationary phase chemistries evaluated in this study, on the quality of the chromatographic separation and elution for a series of synthetic peptides. b) Chromatograms, for peptides 1B and 2A, obtained on a "neutral", "zwitterionic" and "basic" stationary phase using the best combination between stationary phase and nature of the additive in the mobile phase.

but the Torus 2-PIC gave a slightly faster elution. As expected, these columns gave good results only when an acidic additive was used. The addition of 52 mM NH_4OH in the mobile phase provided a severe loss of performance on the two "basic" stationary phases (i.e. Torus 2-PIC and Torus DEA) (Fig. S1). The combination of a bare silica (BEH silica) stationary phase with acidic additive such as TFA, or even basic additives (52 mM NH_4OH) provided inferior performance to those witnessed on the Torus 2-PIC/DEA columns (Fig. 1a-b). With acidic peptides (peptide 2A), the BEH silica gave comparable peak shapes to that observed on the Torus 2-PIC (Fig. 1b), but did not for peptides with higher isoelectric points (peptide 1B – Fig. 1b). Finally, the two remaining columns employed in this study, namely the Torus DIOL (neutral) and Nucleoshell HILIC (zwitterionic), were those offering the worst performance overall. More specifically, the use of a zwitterionic stationary phase performed rather poorly with 13 mM TFA, while the addition 52 mM NH_4OH ensured the proper elution of peptides, but with extremely poor peak shapes as shown for peptides 1B and 2A (Fig. 1b). In conclusion, the combination of a column having basic properties (Torus 2-PIC) with an acidic additive (13 mM TFA) provided the best performance for all peptides and was kept for further evaluation.

3.1.2. Evaluation of MSA as a replacement of TFA

The use of TFA is quite widespread in the literature for peptide analysis, regardless of the considered chromatographic technique (UHPLC or UHPSFC). This additive, however, presents issues when coupling the chromatographic method to a MS detector, mostly due to its tendency to cause ion suppression in the ionization source. Moreover, its use does not always guarantee, in the case of UHPSFC, good chromatographic performance with peptides. The use of alternative additives that would either improve the MS compatibility or the chromatographic performance without sacrificing even further the MS sensibility is desirable. In this context, a recent article on the use of UHPSFC for the analysis of amino acids describes the use of a different additive, namely methanesulfonic acid (MSA), in substitution to TFA [28]. The use of MSA is not new in UHPSFC [36], and in this paper [28] the authors have highlighted a major improvement of the chromatographic performance in UHPSFC for the analysis of underivatized amino acids, in comparison with TFA. Moreover, authors have shown a compatibility of MSA-based mobile phases with MS detection. Therefore, it was decided to evaluate MSA instead of TFA for analyzing the same set of synthetic peptides previously used (i.e. 1N, 2N, 1B, 2B, 1A and 2A) on the Torus 2-PIC column. In Fig. 2, a comparison of 13 mM TFA vs. 15 mM MSA for peptides with acidic, neutral and basic pI is shown. It is immediately visible how 15 mM MSA largely improves the quality of the separation under UHPSFC conditions, improving both peak widths and peak shapes. Moreover, a higher number of impurities, which were not detected with 13 mM TFA, are now visible with 15 mM MSA. In order to make the mobile phase even more MS friendly, lower percentages of MSA (8 mM and 4 mM) have been assessed on the same set of peptides (Fig. 3). The reduced percentage of MSA did not negatively impact the performance of the chromatographic method overall, and 8 mM MSA gave similar results to those observed with 15 mM of MSA. A further reduction to 4 mM MSA was still sufficient to ensure the proper elution of the peptides, but peaks widths were slightly larger, and selectivity

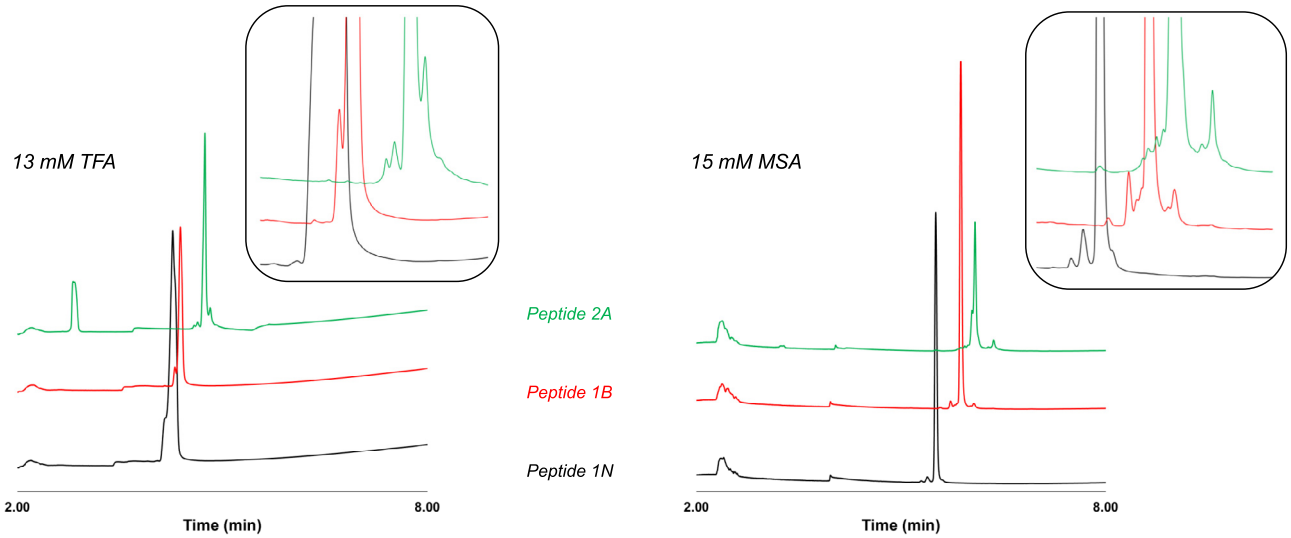

Fig. 2. Chromatograms, relative to peptides 1N, 1B and 2A, obtained on the Torus 2-PIC column with 13 mM TFA (left) or 15 mM MSA (right) as additives in the organic co-solvent.

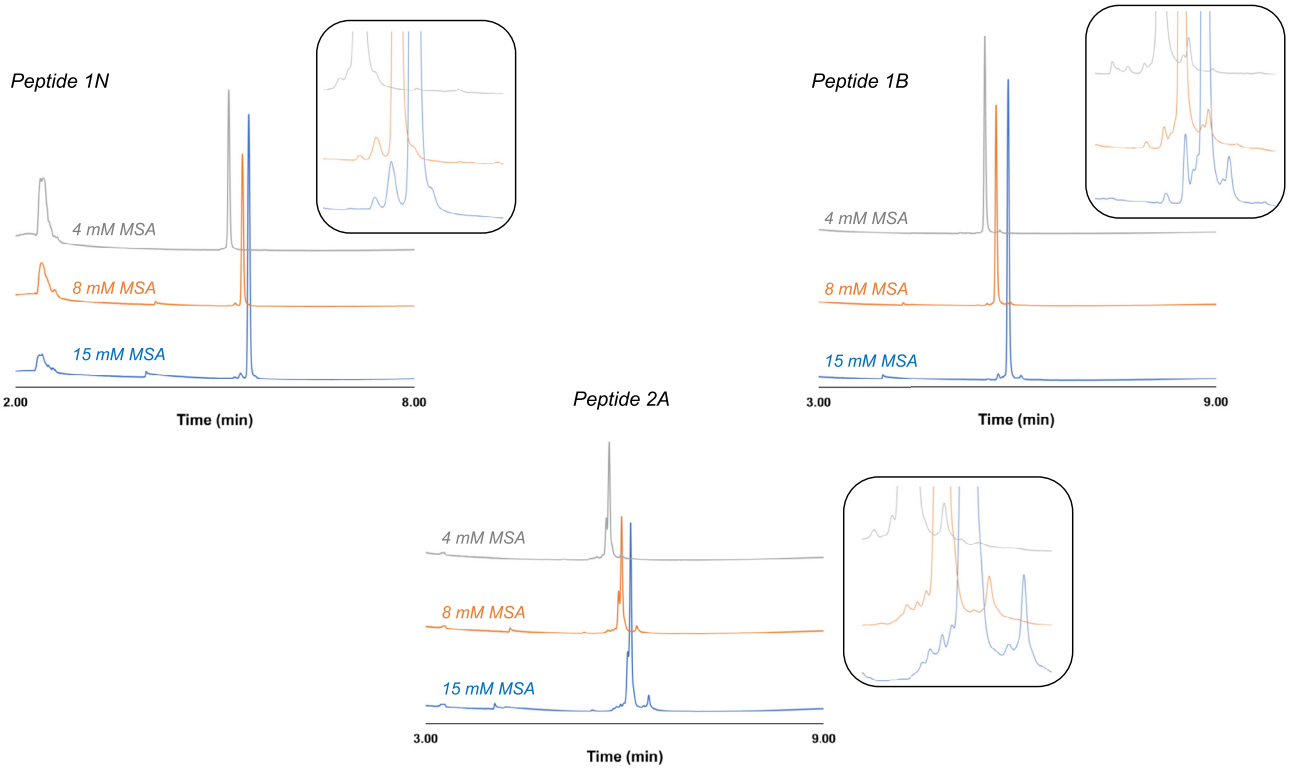

Fig. 3. A comparison of chromatograms obtained by using different percentages of MSA (4 mM – 8 mM – 15 mM) in the organic co-solvent on a series of three peptides (1N – 1B – 2A) on the Torus 2-PIC stationary phase.

was reduced compared to 15 mM and 8 mM MSA. Consequently, it was decided that 8 mM MSA was the best compromise for the UHPSFC method.

The chemical properties of this additive could explain the better chromatographic performance obtained for peptide analysis in comparison to TFA. Indeed, MSA is a strong organic acid with a very low pK_a value ($pK_a \approx -1.9$), in comparison with TFA (pKa $\approx$ 0.5). This important difference in the acidity scale might generate, some potential changes in the apparent mobile phase pH (pH_{app}). Due to the peculiar nature of the mobile phase generally employed in UHPSFC, consisting in a mixture of supercritical CO_2 with a polar organic modifier (generally methanol), a straightforward discussion of the mobile phase pH is almost impossible. However, in a recent article [37], the prediction of the pH_{app} in UHPSFC mobile phases was made thanks to the use of colorimetric pH indicators. In this work, the authors discovered that UHPSFC mobile phases possessed an average pH of 4–5, reaching lower values with the employment of acidic additives, such as TFA. Using the same strategy, an evaluation of the mobile phase acidity, with 8 mM MSA and 7 mM TFA, was carried out (Fig. 4), using 50% of supercritical CO_2 and 50% of co-solvent as the mobile phase. A reference solution without any additive in the co-

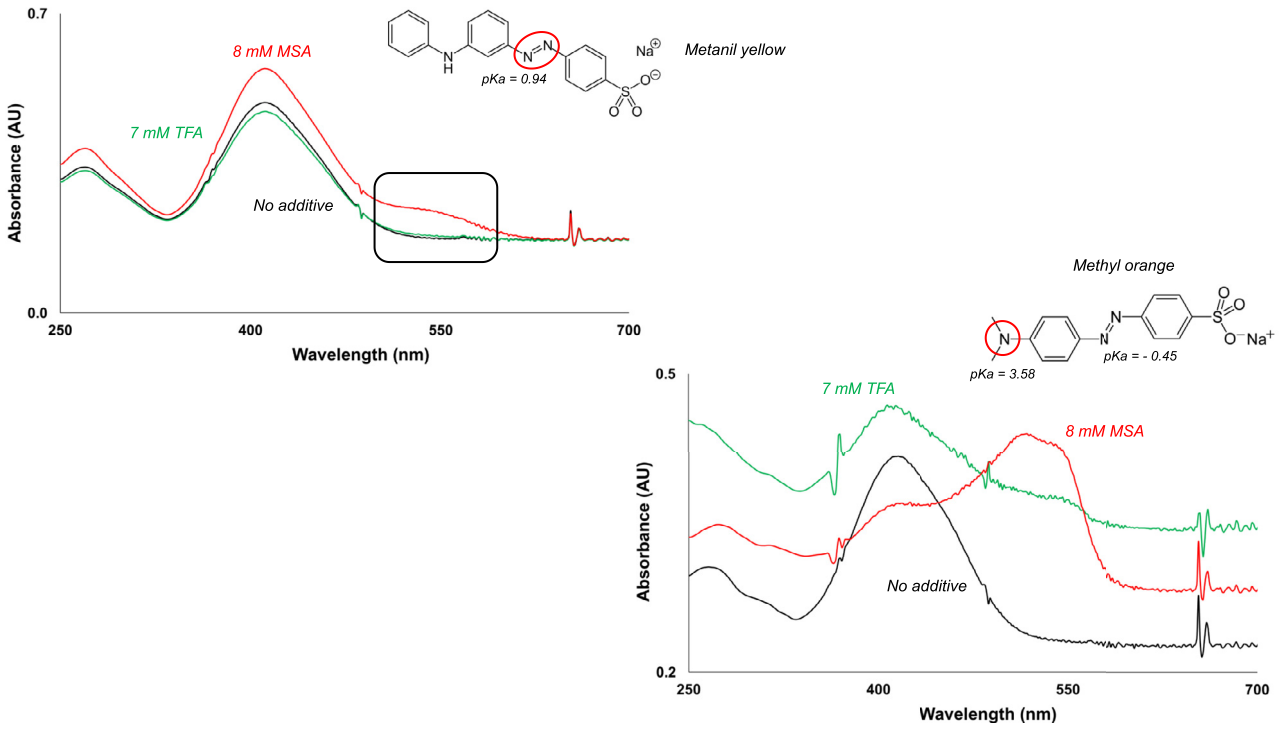

Fig. 4. UV spectra recorded for metanil yellow (left) and methyl orange (right) using 50/50 CO_2:B as the mobile phase, with B being: MeOH:H_2O 95/5 *v/v* (black trait), MeOH:H_2O 95/5 *v/v* + 8 mM MSA (red trait) and MeOH:H_2O 95/5 *v/v* + 7 mM TFA (green trait). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

solvent was also considered. The UV spectra recorded for two pH indicators, namely methyl orange ($pK_a \approx 3.6$) and metanil yellow ($pK_a \approx 0.9$) indicated that both additives were differently affected by the mobile phase acidity. This difference was already visible when methyl orange was used. Indeed, while no difference was observed, in the UV spectra, between the co-solvent without additive and with 7 mM TFA, a shift of the maximum absorbance towards higher wavelength was observed with 8 mM MSA (Fig. 4). This indicates a possible change in the protonation site present in the structure of the pH indicator. Surprisingly, a slight variation of the UV spectra was also observed for metanil yellow, a molecule with a much lower pK_a value (Fig. 4). It becomes therefore clear that MSA can generate a more acidic environment than TFA. The mobile phase pH_{app} seems to have a key role when considering the performance of UHPSFC for peptide analysis. The acidic conditions generated by 8 mM MSA can be sufficient to protonate all tested peptides, as their free carboxyl group at one end of the peptide chain (a weak acid) should be present in its protonated (neutral) form, while the free primary amine at the N-terminus should be increasingly present in its protonated form. The use of a "basic" column would also translate into a protonated stationary phase, under such pH conditions. Protonated molecules, such as the investigated peptides, would therefore experience an electrostatic repulsion with the stationary phase possessing the same net charge, which seems to drastically improve peak shape and peak width (Fig. 2).

An interesting phenomenon was highlighted in Fig. 3: peptides showed a faster elution at lower MSA concentration. This phenomenon did not seem to affect either peak shape or peak width, but solely the retention. This trend is not similar with TFA (Fig. S2). In this case, the reduction of TFA concentration from 13 mM to 7 mM generated an increase in retention. This behavior is due to the ion pairing behavior of TFA. With MSA, however, the situation needs to be further clarified. As above-mentioned, MSA is a strong acid, which generates an acidic environment able to protonate all peptides and the basic groups at the surface of the stationary phase employed in UHPSFC. The increase of MSA concentration would translate in a further increase of the mobile phase acidity, but it also means that a higher number of methanesulfonate anions ($H_3C\text{-}SO_3^-$) should be present, allowing ion-pairing behavior of the MSA anion with the positively charged compounds. The positive charge present on the peptide is, therefore, better shielded, thus reducing the electrostatic repulsion with the positively charged stationary phase, explaining the higher retention. To confirm this hypothesis, a test with 4 amino acids, two of them having a basic functional group (lysine and arginine) and two with acidic functional groups in their structure (glutamic and aspartic acid), was performed on the Torus 2-PIC using 8 mM and 15 mM of MSA and also using TFA. While peptides containing lysine and arginine have experienced a noteworthy reduction of their retention time with lower MSA concentration, the two acidic amino acids showed no significant retention time variation when switching from 15 mM MSA to 8 mM of MSA (Table S1). Higher percentages of TFA, on the other hand, always producing decreasing retention (Table S1).

3.2. Comparison of UHPSFC-UV vs. UHPLC-UV for peptides analysis

3.2.1. Influence of peptide isoelectric point on selectivity

Following the first part of the study, an investigation of how UHPSFC might provide practical advantages over UHPLC (under RPLC conditions) for the analysis of peptides was performed. For this purpose, the six previously described synthetic peptides (i.e. 1N, 2N, 1B, 2B, 1A and 2A) possessing either acidic, neutral or basic isoelectric points were evaluated under UHPSFC and UHPLC conditions. Fig. 5 shows the corresponding chromatograms obtained with the two chromatographic techniques. Some trends become immediately visible. Firstly, the elution order is not the same: in

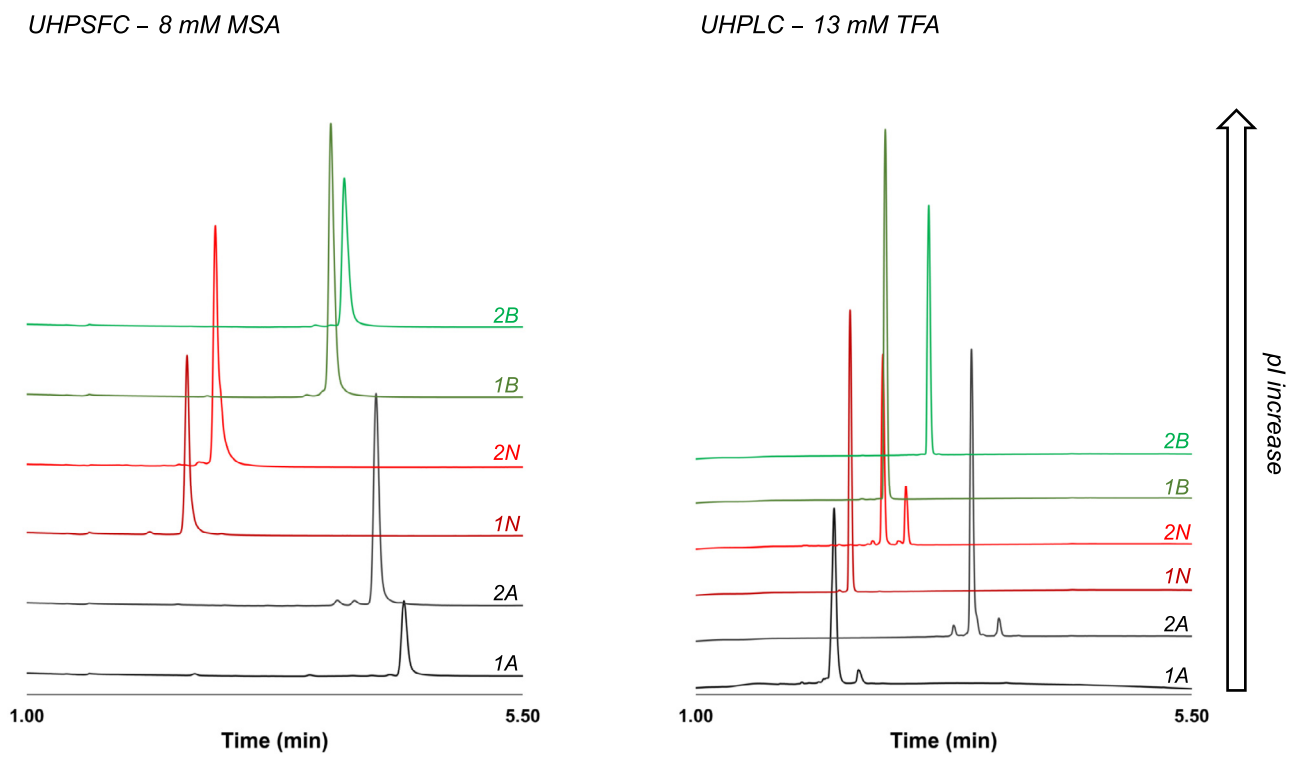

Fig. 5. Chromatograms obtained under UHPSFC-UV (left) and in UHPLC-UV (right) for the set of 6 synthetic peptides with increasing isoelectric point values (from bottom to the top: peptide 1A, 2A, 1N, 2N, 1B, 2B).

UHPLC acidic peptides show divergent retention, as seen with peptide 1A and 2A, being respectively the first and last eluted peptides among those tested. Neutral peptides, in UHPLC, are followed by basic peptides, but the separation can become hard to achieve (peptides 2N and 1B). While in UHPLC it was not always possible to obtain separate elution windows between peptides according to their pI value, as seen in the case of peptides 1A and 2A, with UHPSFC this was obtained (Fig. 5). Indeed, in UHPSFC peptides retention appears grouped according to pI: acidic, neutral and basic peptides possess their own elution windows, allowing a clear separation between these three groups for this example. The elution order is also different to the UHPLC one: neutral peptides are the least retained ones by the stationary phase, then the basic ones are eluted before those with an acidic pI. In reversed-phase UHPLC conditions, peptides are generally retained as hydrophobicity becomes higher, especially when TFA is employed in the mobile phase. In UHPSFC, the acidic environment protonates basic peptides to a higher degree compared to acidic peptides, but the presence of a positively charged stationary phase causes a stronger electrostatic repulsion phenomenon (as described in the previous section) with the basic peptides, thus reducing their retention. To clarify, however, why neutral peptides (1N and 2N) were even less retained under UHPSFC conditions compared to acidic and, more importantly, basic peptides, the influence of the chain length needs to be considered. A more detailed elucidation is given in the next section (3.2.2).

In summary, while the retention generally appears to follow the increase of pI in UHPLC, the retention behavior is different in UHPSFC. In the present example, the separation between peptides having different pI in UHPLC was challenging in some cases, as shown with peptides 2N and 1B On the other hand, UHPSFC was able to provide a satisfactory resolution (Fig. 5). While these results were all confirmed with the peptides at disposal, additional work needs to be performed with different samples.

3.2.2. Influence of peptide chain length on selectivity

Next to the impact of isoelectric point on retention and selectivity under UHPSFC and UHPLC, we also investigated the length of their amino acidic sequence. For this purpose, a new series of six synthetic peptides was employed (Table 1): peptide 6mer, 9mer, 12mer, 15mer, 18mer and 21mer. These peptides all share the same isoelectric point, to rule out the influence of this parameter. These peptides were then injected under the same optimized UHPSFC and UHPLC conditions used in section 3.2.1. Under UHPLC conditions, the elution of peptides with an amino acidic chain length comprised between 9 and 21 amino acids does not follow any order, as shown in Fig. 6. In addition, the selectivity between these different peptides was quite limited under these conditions and most of the peaks eluted within a narrow retention time window. In UHPSFC, the separation is much better, and peptides retention increases linearly with the sequence length (Fig. 6), without sacrificing the chromatographic resolution. The explanation of this retention behavior is quite obvious. Together with the increase in peptide length, there is also an increase in the number of polar groups on the molecule (amide bonding in particular), thus generating a higher retention on the polar stationary phase. Moreover, the electrostatic repulsion phenomenon would become less important as the positive charge on the peptide could be more delocalized when the peptide surface increases. In UHPLC, on the other hand, the apolar C18 stationary phase was not able to discriminate between shorter and longer peptides, even when using TFA as an ion pairing agent. This suggests that the lipophilicity of the peptides does not increase significantly with the increase of the length of their amino acidic chain for the samples taken into consideration, thus reducing chromatographic selectivity.

In section 3.2.1, it was highlighted that neutral peptides presented lower retention under UHPSFC conditions compared to basic ones. According to the electrostatic repulsion hypothesis, the opposite elution order would have been expected as basic peptides

should have a higher positive charge density compared to neutral peptides. However, an important parameter was left out from the discussion: peptides 1 N and 2 N have an amino acid chain length with 3 amino acids less compared to peptides 1B and 2B As it was just described, shorter peptides are less retained under UHPSFC conditions. This phenomenon could, therefore, influences the unexpected elution order previously observed between neutral and basic peptides, in combination with the different pI values possessed by these samples.

3.3. Application to the analysis of commercially available peptides

3.3.1. Analysis of linear and cyclic peptides

Various commercial therapeutic peptides (both linear and cyclic ones) were analyzed using the developed UHPSFC and the reference UHPLC methods. Furthermore, a MS detector was hyphenated to evaluate its performance with the developed UHPSFC method in comparison with the UHPLC one. Three linear (i.e. liraglutide, leuprorelin and glucagon) and cyclic (i.e. linaclotide, eptifibatide and cyclosporin A) therapeutic peptides have been employed in this part (Table 1). In addition, three different stressing procedures (i.e. acidic, basic or oxidative) were performed. Four samples for each peptide (control sample + 3 stressed sample) were, therefore evaluated in UHPSFC and UHPLC conditions. Chromatograms of control and stressed samples for each peptide with the two chromatographic techniques are shown in Fig. S3 for UHPSFC and Fig. S4 for UHPLC. All linear and cyclic peptides were eluted under UHPSFC conditions, while under UHPLC conditions, cyclosporine A could not be eluted under the generic conditions, even after a modification of the gradient profile to reach up to 95% ACN in the mobile phase. This result is not surprising, since cyclosporin A is a highly lipophilic cyclic peptide. In UHPSFC, a lower percentage of co-solvent in the gradient allowed the successful analysis of this particular sample. This result confirms the flexibility of UHPSFC at analyzing samples with a wide range of polarities on a single stationary and mobile phase.

A closer look to specific samples is shown in Figs. 7–8. In Fig. 7, a comparison between UHPLC and UHPSFC for control and stressed samples of leuprorelin is given (sequence of 9 amino acids). Both techniques provided a comparable chromatographic profile for the control sample, as well as the one stressed under acidic conditions, with impurity 1 ($[M + H]^+ = m/z$ 1101 under UHPLC-MS conditions, $[M + 2H]^{2+} = m/z$ 551 for UHPSFC-MS) always eluting prior to the main peak. The situation slightly varies with the basic conditions (Fig. 7). In this case, UHPSFC offered a better selectivity between impurities 2 ($[M + H]^+ = m/z$ 777), 1 ($[M + H]^+ = m/z$ 1101 under UHPLC-MS conditions, $[M + 2H]^{2+} = m/z$ 551 for UHPSFC-MS) and 3 ($[M + H]^+ = m/z$ 1194 under UHPLC-MS conditions, $[M + 2H]^{2+} = m/z$ 598 for UHPSFC-MS. Interestingly, in UHPSFC conditions, the elution order of impurities 1, 2 and 3 as well as leuprorelin was proportional to the molecular weights of the impurities. However, the chromatographic profile obtained after an oxidative stress was better resolved with the UHPLC method (Fig. 7), where a larger number of impurities was observed. The two new impurities 4 ($[M + H]^+ = m/z$ 1228 for UHPLC-MS, $[M + 2H]^{2+} = m/z$ 615 for UHPSFC-MS) and 5 ($[M + H]^+ = m/z$ 1245 for UHPLC-MS, $[M + 2H]^{2+} = m/z$ 622 for UHPSFC-MS) were eluted in opposite order by both methods.

Similar results were found with a second linear peptide, glucagon (Fig. 8). This 29 amino acid peptide possesses one of the longest amino acidic sequence among all samples tested in this work, as well as a relatively low GRAVY number, indicating a high polarity. Nonetheless, this peptide was eluted under UHPSFC conditions with a satisfactory peak shape using high amount of co-solvent (around 85% MeOH). Again, control as well as acidic stressed samples gave comparable profiles with both chromatographic techniques (Fig. 8). Impurities obtained after the addition of 0.1 M NaOH and hydrogen peroxide followed the same trends

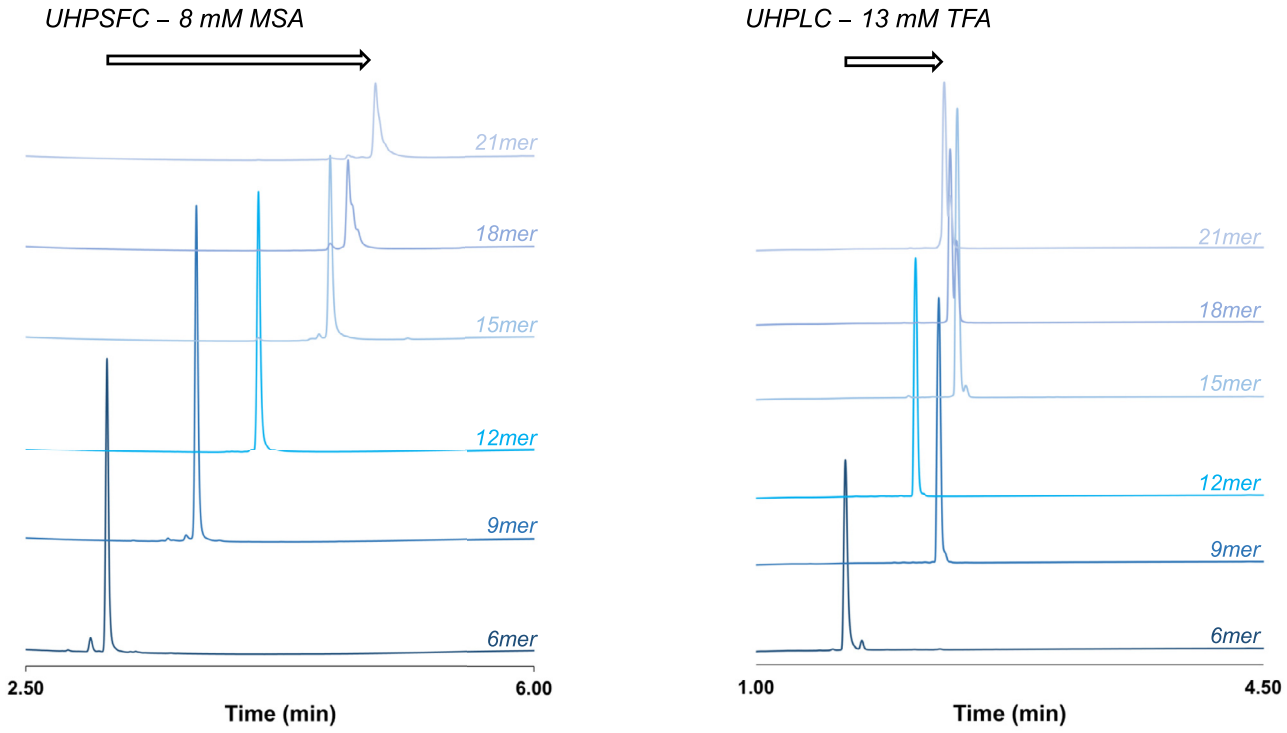

Fig. 6. Chromatograms obtained under UHPSFC-UV (left) and in UHPLC-UV (right), for the set of 6 synthetic peptides with increasing amino acidic chain length (from bottom to the top: peptide 6mer, 9mer, 12mer, 15mer, 18mer, 21mer).

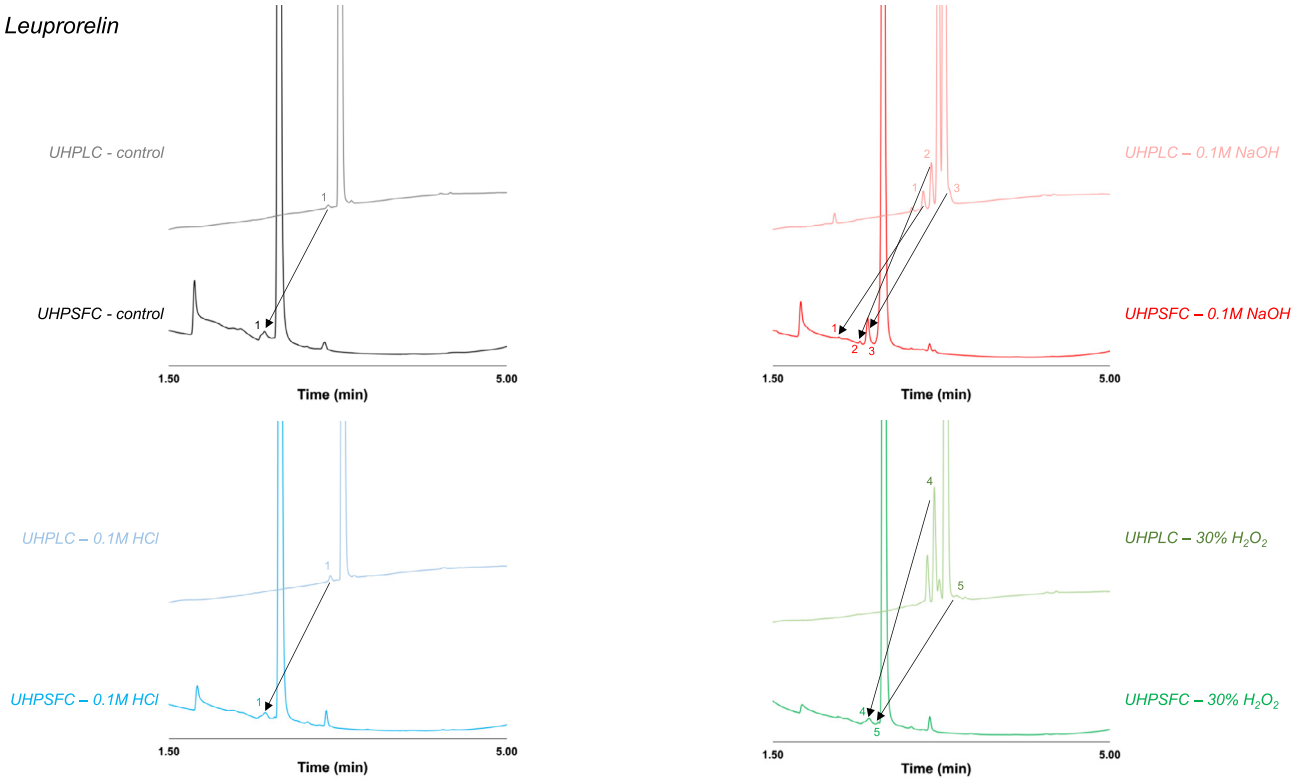

Fig. 7. Chromatograms obtained for leuprorelin and leuprorelin + impurities after exposure to different stress conditions in UHPSFC-UV-MS and UHPLC-UV-MS.

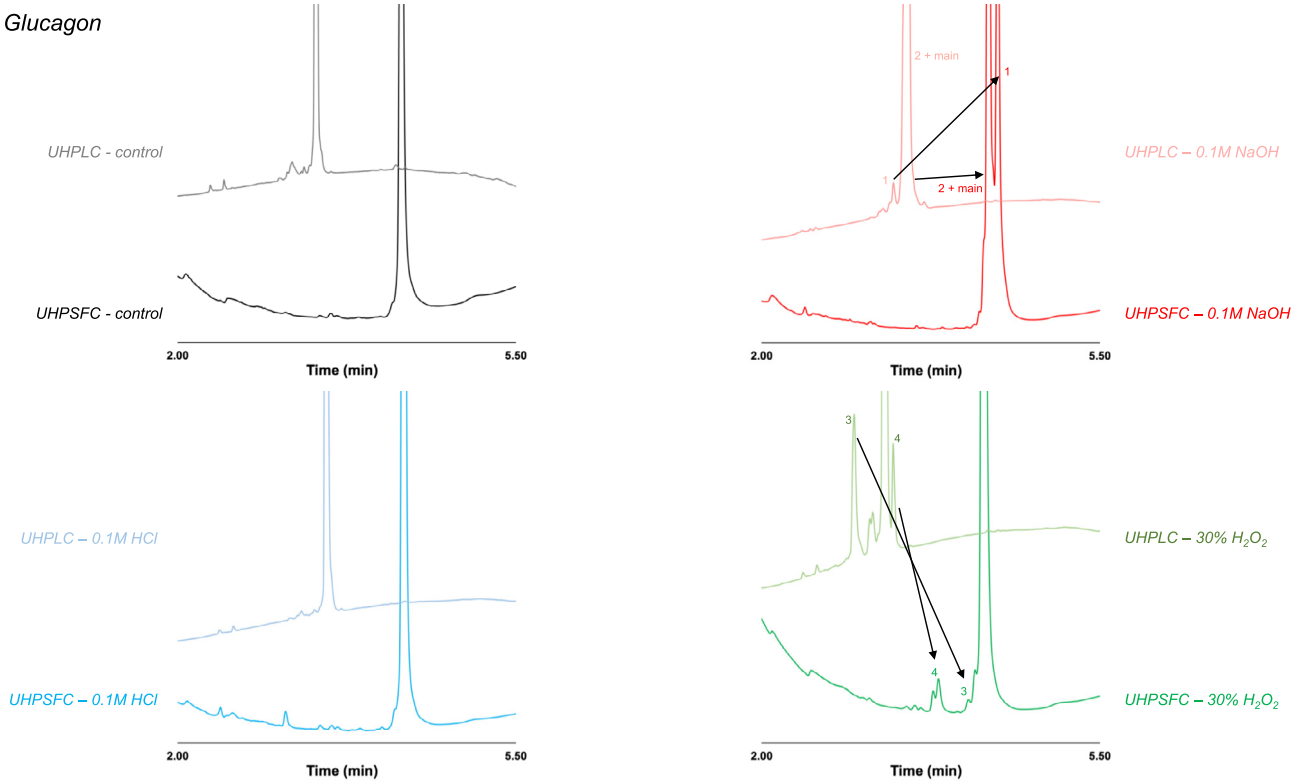

Fig. 8. Chromatograms obtained for glucagon and glucagon + impurities after exposure to different stress conditions in UHPSFC-UV-MS and UHPLC-UV-MS.

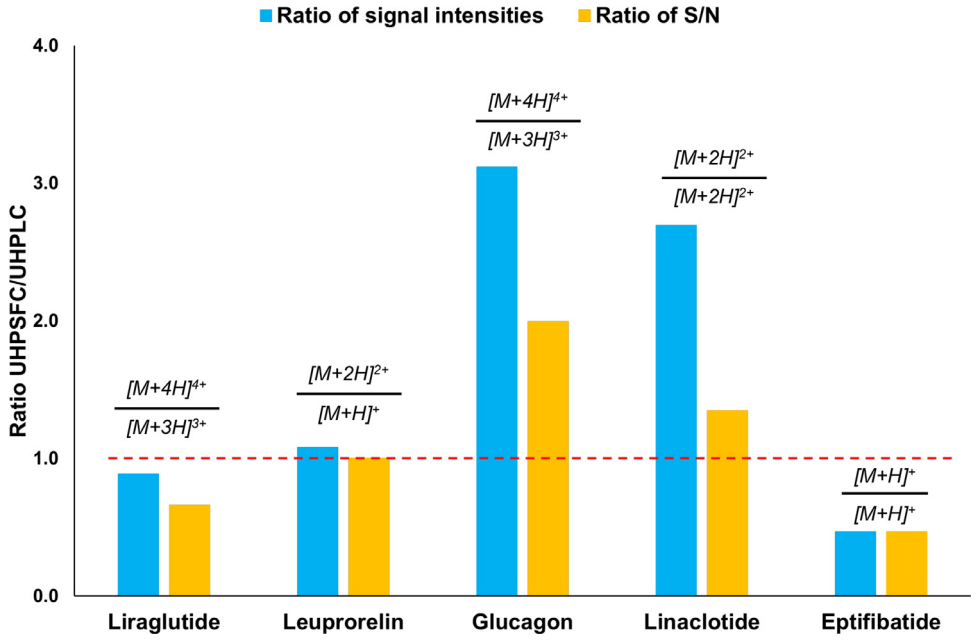

Fig. 9. Table representing the ratio between signal intensities (in blue) and signal-to-noise values (in yellow) obtained in UHPSFC-MS over UHPLC-MS conditions for five commercial peptides. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

as those previously reported for leuprorelin. Under basic conditions, glucagon impurity 1 ($[M + 3H]^{3+} = m/z$ 1319 for UHPLC-MS, $[M + 4H]^{4+} = m/z$ 990 for UHPSFC-MS) and 2 ($[M + 3H]^{3+} = m/z$ 1272 for UHPLC-MS, $[M + 4H]^{4+} = m/z$ 954 for UHPSFC-MS) eluted according to the length of their chain under UHPSFC-MS. The same behavior was also observed for impurities 3 ($[M + 3H]^{3+}$ - $[M + 4H]^{4+}$ of m/z 1179 and 885) and 4 ($[M + 3H]^{3+}$ - $[M + 4H]^{4+}$ of m/z 1168 and 881).

In Fig. S5 of the supplementary material, an example of a cyclic peptide composed of 7 amino acids, eptifibatide, is given. This peptide takes its characteristic cyclic structure after the formation of an intramolecular disulfide bridge between the two cysteine residues present in its chain. Once again, similar results have been observed when this compound was evaluated under UHPLC-MS and UHPSFC-MS conditions as to those previously discussed for linear peptides. While for the control sample, as well as under acidic stress procedure, no major differences were observed, while a larger number of impurities were observed after the exposure to 0.1 M NaOH. Impurities 1, 2 and 3 were better resolved from the main peak in UHPSFC conditions, and a higher number of impurities was visible compared to RP-UHPLC conditions. On the other hand, similarly to leuprorelin and glucagon, impurities produced after an oxidative stress were better resolved under UHPLC conditions.

Overall, this part demonstrated that UHPSFC was able, in almost all examples, to generate comparable performance to UHPLC, and gave complementary information (different elution behavior and selectivity).

3.3.2. Evaluation of MS sensitivity between UHPSFC vs. UHPLC

The use of MSA in the UHPSFC chromatographic method and its compatibility with MS detector was investigated. MSA is, indeed, a highly viscous organic acid with a relatively high boiling point (close to 170 °C, indicating potential issues in its application in chromatographic methods combined to mass spectrometers). Therefore, a systematic study was carried out, focusing on the ratio of the signal intensities, as well as of signal-to-noise values, obtained in UHPSFC and UHPLC for the commercial peptides previously employed (Fig. 9). Although MSA is not highly volatile, it is present at very low concentration in the UHPSFC method. Indeed, its average concentration in the gradient is equal to 4–5 mM (corresponding to 4 – 5 mM in the mobile phase), which is much lower than what is commonly employed in UHPLC (13 mM TFA). Consequently, as shown in Fig. 9, UHPSFC provided comparable signal intensities, as well as signal-to-noise values, to UHPLC. For the remaining two peptides, either the ratio is close to one (in the case of liraglutide) or simply UHPSFC did not provide the same MS sensitivity as UHPLC does (in the example of eptifibatide). Interestingly, the ionic species generated by the two chromatographic techniques were not always similar (Fig. 9). This was also observed in the previous section, as all impurities detected under UHPSFC has a lower m/z ratio than in UHPLC. Indeed, it appeared that UHPSFC was able to better protonate peptides, especially those with a relatively long chain (liraglutide and glucagon) compared to UHPLC, indicating a higher charge state of the ions. This phenomenon was already observed by Wang and Olesik [38], describing how the employment of mobile phases containing liquified CO_2 provided increased charged states and narrower charge state distributions. The authors claimed that the addition of liquified CO_2 mainly improved the desolvation process in the ESI ionization chamber.

3.3.3. Transferability of the UHPSFC method for peptides to preparative scale

We next focused our efforts on a semipreparative purification of a cyclic peptide API. This mixture was subjected to automated SFC column screening [36] on eight different stationary phase columns with gradient elution using MSA-rich modifiers (Fig. 10a). Several columns were found to effectively separate the two components in this reaction showing excellent peak shape and acceptable resolution (2-PIC, DEA and 4-EP). A straightforward optimization to isocratic elution: 35% MeOH/H_2O 95:5 *v/v* + 8 mM MSA/ 65% CO2 on a Waters Torus 2-PIC (30.0 mm x 250 mm, 5 µm) column at a flow rate of 140 mL/min enabled baseline resolution at the semipreparative scale. This procedure facilitated a rapid delivery of 84 mg of peptide (purity > 98%, yield > 95%) by five x 1 mL stacked injections of 20 mg/mL peptide mixture (purity ≈ 69%) in less than 15 min total runtime (Fig. 10b). This serves to illustrate the power of modern SFC technologies and the practical use

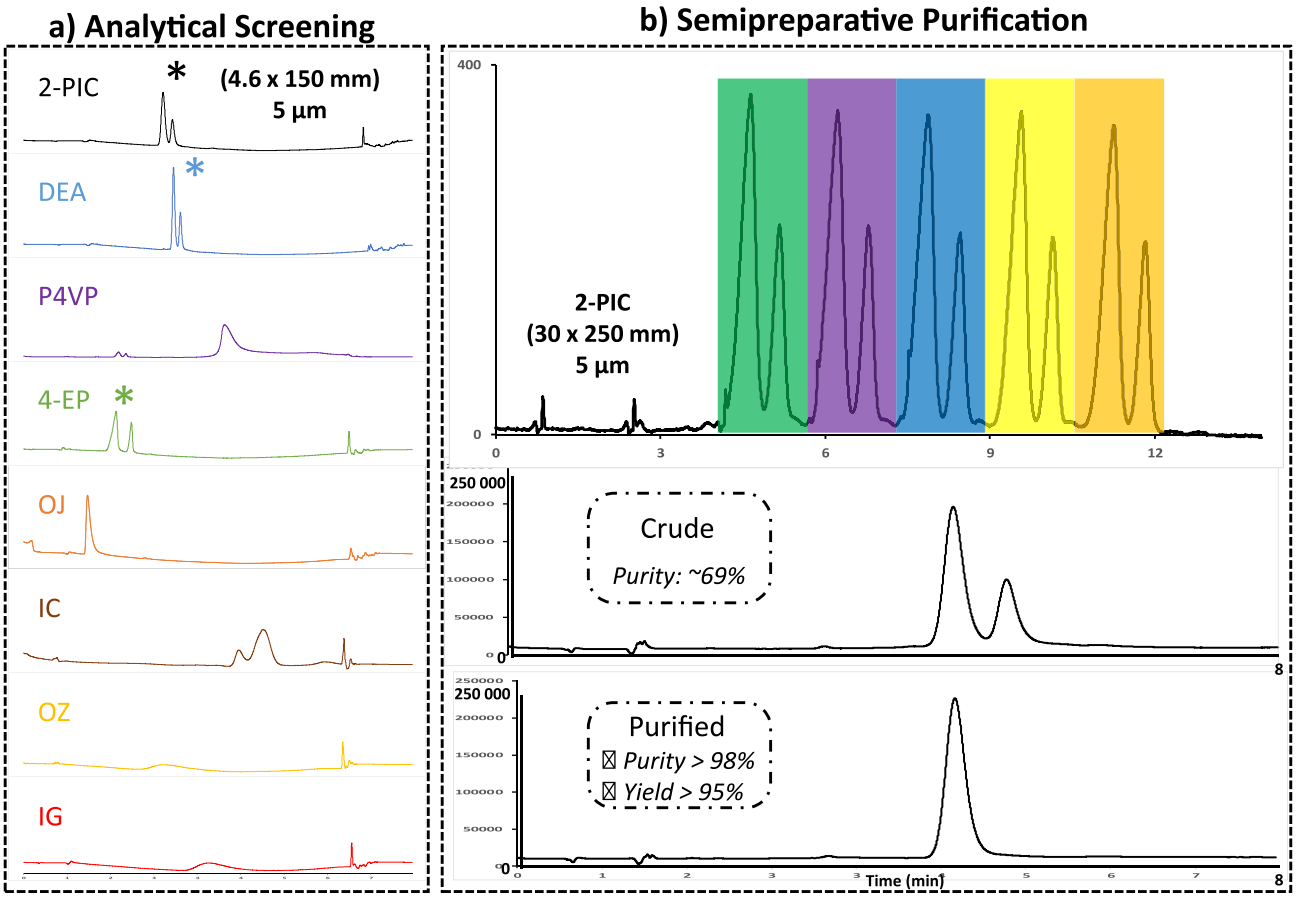

Fig. 10. a) Automated column screening of cyclic peptide using the mobile phase conditions in the Experimental section. b) Semipreparative purification of the cyclic peptide on the 2-PIC column using the conditions listed in the Experimental section (top), the analysis of the sample before purification (middle) and after purification (bottom).

of MSA-rich modifiers in pharmaceutical setting at both analytical and preparative scale.

4. Conclusions

In this work, the possibilities offered by UHPSFC coupled to UV and MS detectors, was evaluated for a series of synthetic and commercially available peptides. A systematic comparison with UHPLC was performed to draw the advantages and limitations of UHPSFC for this kind of analytes. At first, the choice of the stationary phase, as well as an optimization of the mobile phase conditions were achieved for UHPSFC. The combination between a positively charged stationary phase with the addition of an acidic additive in the mobile phase was found to be the one offering the best performance for peptide analysis. Later, the evaluation of a novel additive, methanesulfonic acid (MSA), was carried out and results were compared to the more commonly used trifluoroacetic acid (TFA). MSA demonstrated to provide significantly better chromatographic performance against TFA, at a lower concentration in the mobile phase (8 mM vs 15 mM).

In the second part of this study, the selectivity achieved in UHPSFC was discussed, with a systematic comparison with UHPLC conditions. UHPSFC provided a different separation of peptides according to their isoelectric points vs. UHPLC. Furthermore, UHPSFC allowed a very good discrimination between peptides with different amino acidic sequence lengths, while no such relationship was demonstrated with UHPLC.

In the third part of this work, some applications of UHPSFC in peptide analysis were evaluated and systematically compared to UHPLC, both hyphenated to a MS detector. To this purpose, a set of six commercially available peptides, of which three possessing a linear structure and three with a cyclic one, were employed. Different stressing procedures were employed on each peptide, exposing them to either acidic, basic or oxidative stress. Results showed that UHPSFC gave comparable, if not sometimes even better, performance to those observed with UHPLC. Regarding the MS sensitivities achieved in UHPSFC, it was seen that they were comparable to those observed under UHPLC conditions. Finally, the transferability of the developed analytical method to a semi-preparative level was considered, and the semi-preparative SFC method shows excellent performance in terms of yield and purity for a Merck cyclic peptide.

All these results demonstrated that UHPSFC is a viable alternative for the analysis of highly polar compounds with high molecular weight such as peptides, utilizing a gradient reaching high percentages of co-solvent. Furthermore, UHPSFC has shown once more its orthogonality against UHPLC, fueling even more its utility in analytical laboratories.

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

Gioacchino Luca Losacco: Writing - original draft, Methodology, Investigation. **Jimmy Oliviera DaSilva:** Investigation, Writing - review & editing. **Jinchu Liu:** Investigation, Writing - review &

editing. **Erik L. Regalado:** Supervision, Writing - review & editing, Resources. **Jean-Luc Veuthey:** Supervision, Writing - review & editing. **Davy Guillarme:** Supervision, Writing - review & editing, Project administration.

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