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Investigation into reversed-phase chromatography peptide separation systems part V: Establishment of a screening strategy for development of methods for assessment of pharmaceutical peptide’s purity

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Keywords
Peptides, RPC, method development screening strategy, mobile and stationary phase selectivity, pH, ion-pair additives

Highlights
- Column/solvent screening strategy for method development of peptides is described
- Use of NH₄PF₆ and CH₃SO₃H as mobile phase additives is described
- TFA was demonstrated not always to be the preferred mobile phase additive

Abstract
The paper describes a simple and rapid reversed-phase UHPLC method development screening strategy for the purity determination of peptide-based pharmaceuticals. The protocol utilises five disparate columns and six volatile or non-volatile mobile phases (i.e., 30 combinations). The method development strategy has been demonstrated to be highly effective in identifying conditions which generate complementary selectivity and good peak shape. Columns with varying degrees of charge (positive and negative), in addition to their differing hydrophobic character, were used in combination with mobile phases within the pH range of 2.3 to 5.1. The novel ion-pair / chaotropic reagent ammonium hexafluorophosphate at pH 2.3 was shown to be an extremely useful mobile phase additive in that it produced excellent complementary separation and good peak shape. Methanesulfonic acid was demonstrated to be a good alternative to the ubiquitously employed trifluoroacetic acid which failed to generate optimum separation for the peptides investigated highlighting the importance of screening disparate mobile phase additives. Both ammonium hexafluorophosphate and methanesulfonic acid were shown not to adversely affect the stability of
C18 columns or demonstrated any irreversible adsorption / memory effects. No pH hysteresis effects were demonstrated with any of the stationary phases on mobile phase pH cycling. No major problems have been observed with the novel mobile phase additives ammonium hexafluorophosphate and methanesulfonic acid, however, it is recommended that they be used with caution until long-term routine use has been established.

1. Introduction

To maximise the probability of separating peptides of interest within a complex mixture, it is necessary to select columns and mobile phases with proven complementary chromatographic selectivities. Papers I and II of this series of articles describe a peptide RPC column characterisation protocol based on the determination of eight selectivity values from nine synthetic peptides (i.e., fragments of [ile27]-bovine GLP-2) chromatographed at pH 2.7 and 6.5 [1,2]. The selectivity values (Δ delta values) facilitate the differentiation between columns based on hydrophobic, electrostatic, hydrogen bonding and aromatic interactions of the peptide probes with the surface of the stationary phase. The protocol was then extended in Paper III to characterise 43 differing RPC columns, via the chemometric tool of Principal Component Analysis (PCA), into various sub-sets based on their chromatographic selectivity differences [3]. Paper IV reported the characterisation of 51 mobile phase compositions (i.e., differing in pH 1.8 - 7.8, salt types, ionic strength, ion-pair reagent and chaotropic / kosmotropic additives) on a new generation C18 stationary phase (i.e., Ascentis Express C18) which was then extended to other RP materials [4].

Based on previous work [3, 4] we selected six column and eight mobile phase compositions (containing both volatile and non-volatile buffers / additives) that provide large differences in selectivity and good peak shape for the development of purity methods for peptide-based pharmaceuticals, i.e., samples consisting of a peptide present at high level and a number of related peptide impurities present at low level (typically 0.05-1% of the peptide drug substance). For this type of sample, it is important to have mobile phases with high ionic strength in order to prevent overloading which generates a very broad / asymmetric main peak resulting in the possible masking of closely eluting peaks impurities [4]. LC-UV is typically employed for the quality control of pharmaceuticals, hence non-volatile salts are included in the scope of this current paper. The study evaluates the inclusion of these disparate column and mobile phase additive combinations as part of an innovative and rapid gradient RPLC method development platform to separate peptide impurities using automated column and mobile phase switching technologies. The success of the selected column and mobile phase additives was assessed using human angiotensin I (1 kDa), bovine
GLP-2 (1-15) (2 kDa), melittin (3 kDa), bovine insulin (6 kDa) and their related impurities as well as a synthetic mixture of bovine GLP-2 (1-15) and four of its diastereomers corresponding to a racemisation of a single amino acid in the base sequence. The chromatographic performance of each of the eight mobile phase additives and the six column combinations for each sample was assessed using a ranking system based on the number of resolved peaks within a set retention window around the main component and the latter’s peak shape. Evaluation of the ranking order facilitated a reduction in the number of column and mobile phase additive compositions that were necessary to maintain a high probability of separating the components of interest.

Additionally, we report the optimisation of mobile phase additive (i.e., formic acid / ammonium formate, acetic acid / ammonium acetate and, NH₄PF₆) concentrations to minimise noise, drift and corrosion while maintaining good chromatographic peak shape. The effect of long-term use of the novel mobile phase additives (i.e., methanesulfonic acid (MSA) [5,6] and NH₄PF₆ at pH 2.3 [7,8]) on C18 column longevity and LC component compatibility (i.e., column frits and metal LC tubing, polytetrafluoroethylene [Teflon] tubing and polyether ether ketone [PEEK] rotor seals) was evaluated using accelerated storage conditions. The use of low pH phosphate buffers containing NaCl was evaluated in place of ammonium sulfate, which previously had rectified the poor peak shape observed in the analysis of certain peptides with sulfate buffers [4]. Ion-pair memory effects with MSA and NH₄PF₆ at pH 2.3 on the six stationary phases was investigated [9]. pH Hysteresis (i.e., cycling between pH 3 → 7 →3) effects were also investigated on the six disparate columns [10]. The use of the universally employed TFA in peptide separations [11-13] was compared to the separations achieved with the mobile phases evaluated in this paper. After careful consideration of the selectivity results obtained and the practical factors of using these novel mobile phase additives, a re-defined gradient RPC method development strategy has been demonstrated.

2. Experimental

2.1 Chemicals and stationary phases

Water, acetonitrile (MeCN) and all mobile phase additives (described in Table 1) used were of LC-MS grade and supplied by Sigma Aldrich (Poole, UK). Dimethylsulfoxide (DMSO) was supplied by Fisher Scientific (Hemel Hempstead, UK). Bovine insulin, human angiotensin I and melittin were supplied by Sigma Aldrich (Poole, UK). The [Ile27]-bovine GLP-2 peptides, which were supplied by Apigenex (Prague, Czech Republic), were all dissolved individually in DMSO / water (8:2 v/v) to a concentration of 0.25 mg/mL. Peptide amino acid sequence, molecular weight and estimated pI can be found in Table 2. Solutions were stored at -20 °C. The base sequence for each peptide used in the Peptide RPC column / mobile phase characterisation protocol is provided in papers I-IV [1-4].
Bovine insulin and bovine GLP-2 (1-15) were degraded (i.e., approximately 24 and 11% area/area increase in impurities levels, respectively) by subjecting them to 20 mM ammonium hydroxide (pH 10.8, 1 mL/mg peptide) for 18 and 41 hours, respectively at 37°C. The solutions were then neutralised by the addition of an equal volume of 20 mM acetic acid resulting in a final concentration of 1 mg/mL. Samples were stored at -20°C when not in use. Angiotensin I and melittin were used as received since both contained a number of related impurities hence forced degradation was not necessary. For simplicity, the name of the organism from which a peptide originates has been omitted in subsequent sections of the article. It should be noted that mobile phases containing fluorinated compounds (i.e., TFA and NH₄PF₆) should be disposed of by an approved waste disposal facility. A brief description of each of the stationary phases can be found in Table 3. A new column was used for each ion-pair reagent to avoid the possibility of column memory effects caused by adsorption of ion-pair reagents.

2.2 Liquid chromatography and experimental settings

Premixed mobile phases were prepared as described in Table 1 for mobile phases A and B. For the non-volatile mobile phase additives, mobile phase B consisted of MeCN/water (8:2 v/v) to prevent any precipitation problems. The gradient was standardised as followed: 5 to 80%B over 60 minutes, with an isocratic hold at the top of the gradient for 3 minutes, before returning to the original conditions in 0.1 minutes and 12 minutes re-equilibration (equivalent to approximately 10 column volumes). The column oven temperature was 45 °C, flow rate was 0.3 mL/min, and detection was at 215 nm (bandwidth 8 nm) referenced at 360 nm (bandwidth 100 nm) and a sampling rate of 12.5 Hz. An injector program was utilised to create online cocktails to minimise consumption of peptides. The peak apex of a water injection was used as the dead time marker [14]. Variable injection volumes were employed to generate a main peak height of approximately 1.5 AU. % area/area values are defined as the area of the impurity peak of interest divided by the sum of the areas of all the impurities plus the main component.

LC separations were performed using a Shimadzu Nexera X3 UHPLC system (Duisburg, Germany) equipped with two binary pumps (LC-40AD) and proportionating valves, degassers (DGU-40S), autosampler with cooling capabilities (SIL-40), column oven (CTO-40C), diode array detector (SPD-M30A), 180 µL mixer and a six-position column switching valve and communication bus module (CBM-40Lite) or a Nexera-i LC-2040 HPLC system. The dwell volumes were 440 and 502 µL, respectively [14].

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2.3 Accelerated column stability studies

Three new Ascentis Express C18 (2.7 µm, 150 x 2.1 mm) columns were exposed to individual conditions of 0.5% v/v TFA in MeCN / water (5:95, v/v), 0.5% v/v MSA in MeCN / water (5:95, v/v) and 100 mM NH₄PF₆ in MeCN / phosphate buffer pH 2.3 (5:95, v/v) at 80 °C and a flow rate of 1 mL/min. The retention time of σ-cresol (1 µL of a 0.1 mg/mL solution in MeOH/water 20:80 v/v) was monitored over the course of 108 injections (15 minute run time x 108 injections equivalent to approximately 5000 column volumes). UV detection at 220 nm (bandwidth 8 nm) with a reference wavelength of 360 nm (bandwidth 100 nm) was employed.

2.4 Corrosion studies

2.4.1 LC frits

Stainless steel 316 (1/4" diameter x 0.03" thickness, 2 µm pore size, VWR International Ltd, Reading, UK) and titanium frits (3/8" diameter x 0.04" thickness, 2 µm pore size, Restek Thames, Buckinghamshire, UK) were washed with water and EtOH before being dried and exposed to the various mobile phase compositions (i.e., corresponding to mobile phase A and B) for seven days at 60 °C (1 frit per 1 mL of mobile phase). The frit was removed, and the solutions evaporated to dryness with the aid of a nitrogen stream. The residue was dissolved in 2% v/v aqueous nitric acid (1 mL) and further diluted before analysis by ICP-MS (Agilent 7500a, Cheadle, Cheshire, UK) for iron, titanium, chromium, and nickel. Corresponding blank controls (i.e., minus frit) were subtracted from the frit results.

2.4.2 LC tubing

Stainless steel 316 (VWR International Ltd) and MP35N® alloy (Waters A/S, Taastrup, Denmark) HPLC tubing (20 mm lengths, 0.025” OD x 0.007” ID, previously washed with water and EtOH) was exposed to the following mobile phase conditions with continuous stirring at 60 °C for 30 days; 0.1% v/v MSA in water, 0.1% v/v MSA in MeCN / water (8:2 v/v), 20 mM NH₄PF₆ in phosphate buffer pH 2.3 103 mM total ionic strength and 20 mM NH₄PF₆ pH 2.3 in 1:1 v/v MeCN/ phosphate buffer. Tubing was washed with water and EtOH and dried at 60°C before being weighed to assess for weight loss or gain and microscopic examination.

2.4.3 Teflon tubing

Tensile strength was assessed before and after 10 litres of either 0.1% v/v MSA in MeCN / water (8:2 v/v), 0.1% v/v MSA in MeCN or 20 mM NH₄PF₆ at pH 2.3 were passed through Teflon tubing (50 cm
lengths, Agilent Technologies, Cheadle, UK,) used to connect the HPLC reservoir sinker to the pump head at a rate of approximately 0.5 mL/min for 14 days. Tensile tests were carried out using an electro-mechanical driven 50kN Instron machine (Instron, High Wycombe, UK).

2.4.4 PEEK rotor seals
PEEK rotor seals (Waters A/S, Taastrup, Denmark) were exposed (30 days at room temperature) to a stirred solution of either 0.1% v/v MSA in MeCN / water (8:2 v/v), 0.1% v/v MSA in MeCN, or MeCN / water (8:2 v/v). Hardness measurements were made using the Duramin A300 hardness tester (Struers Inc, Cleveland, USA) fitted with a Vickers indenter with a load of 1 kgf applied.

2.6 Software and calculations
Shimadzu LabSolution software (Version 5.86, Duisburg, Germany) was used for LC instrument control and data processing. Principal Component Analysis (PCA) was performed using SIMCA (Version 14.1, Sartorius UK Ltd, Surrey, UK) and Origin (Version OriginPro 2017, OriginLab, Northampton, MA, USA). The net charges of the peptide probes were calculated using General Protein / Mass Analysis for Windows (GPMAW) software (Version 9.51, Lighthouse Data, Odense, Denmark). The mobile phase calculations were performed using BufferMaker (Version 1.1.0.0, ChemBuddy, BPP Marcin Borkowski, Poland).

3. Results and discussion
3.1 Rationale for column selection
Six columns which provided large selectivity differences were chosen based on previous results [3]. The stationary phase’s negative / positive charge character, which was dependent on the mobile phase pH, was shown to be the major factor contributing to retention and selectivity of charged peptides while the ligand functionality was observed to be less important. It has been previously demonstrated that the selected columns exhibit the following negative or positive charge / RP characteristic at low pH [3]: Zorbax 300 SB-C18 (non end-capped silica based) low negative charge / intermediate RP character, Ascentis Express Biphenyl (end-capped silica based) low negative charge / intermediate RP character, Acquity BEH C8 (end-capped ethyl bridged silica hybrid technology) [15] no charge / intermediate RP character, Acquity CSH Fluoro-phenyl (non end-capped, low-level positive surface charge, ethyl bridged silica hybrid technology) [15,16] both low positive and negative charge (dependent on mobile phase pH) / low RP character, Luna Omega PS C18 (small amount of positive charge on the silica surface) low positive charge / high RP
character, Atlantis Premier BEH C18 AX (significant amount of positive charged ligand, ethyl bridged silica hybrid technology) [15] high positive charge / high RP character.

3.2 Rationale for mobile phases' selection

20 mM Ionic strength volatile mobile phase compositions covering the pH range of 3.6 to 7.0 were selected as pH was observed to be a major operating parameter controlling retention and selectivity [4]. The ion-pair reagents [17-19] MSA [5,6] and NH₄PF₆ [7,8] (also a chaotropic reagent) at pH 2.3 were included as they had been demonstrated to exhibit large selectivity differences [4]. The non-volatile nature of NH₄PF₆ was confirmed by drying an aqueous solution of NH₄PF₆ in a stream of hot air (300 °C) for 10 minutes where 90% of the theoretical solid was recovered. TFA was included as a control since it is considered to be the default additive for peptide analysis. 100 mM Ionic strength non-volatile phosphate based mobile phases at pH 2.3 and 3.1 were selected as these have been demonstrated to generate good peak shapes and are the mobile phases of choice for low UV wavelength detection.

3.3 Optimisation of mobile phase additive composition

The concentrations of FA/NH₄FA pH 3.6 and AA/NH₄AA pH 5.1 in the mobile phase reservoirs A and B were optimised to generate flat baselines while maintaining a good peak shape to improve the signal to noise ratio and hence the level of quantification (LOQ). The ionic strength of the FA/NH₄FA pH 3.6 had to be reduced from 20 to 10 mM in both mobile phases A and B to reduce noise and thereby achieve acceptable LOQ levels ≤0.1% area/area and peak shape (Table 1). For TFA in 8:2 v/v MeCN / water (mobile phase B) the level was reduced from 0.1% v/v in water (mobile phase A) to 0.09% v/v in 8:2 v/v MeCN / water to obtain a flatter baseline. The quality of acid / base was found to be very important; HPLC gradient grade components and short mobile phase shelf lives are recommended. A composition of 100 mM total ionic strength and 20 mM of the non-volatile NH₄PF₆ gave the lowest tailing factor and narrowest peak width for GLP-2(1-15), see Supplementary Material 1. The low level of NH₄PF₆ was deemed to be advantageous as it should reduce cost, environmental issues, and potential corrosion.

3.4 Initial screening of columns and mobile phases

Initially, degraded samples of GLP-2 (1-15) and insulin were screened on all the six column and eight mobile phase combinations to assess the diversity of the chromatographic selectivity of the column /
mobile phase combinations. In addition, the method development strategy’s ability to separate four very similar diastereomeric impurities of GLP-2 (1-15) was assessed (i.e., in total 6 x 8 = 48 combinations x 3 samples = 144 chromatograms were assessed). The best mobile phase / column combination(s) for each sample were determined by performing a manual ranking exercise on each chromatogram. Each chromatogram for a certain peptide sample was given a ranking of 1 to 5 where 5 denotes the best separations. The criteria for ranking were based on the number of impurity peaks present above a certain level e.g., 0.1% area/area. The high-ranking combinations (i.e., rankings of 4 and 5) were penalised for excessive peak tailing and poor resolution between the main component and adjacent peaks by incurring a subtraction of 1 ranking point. An example of ranking classification and criteria can be seen in Figure 1 for GLP-2 (1-15). Somewhat different ranking criteria were used for the different samples since different peptide samples displayed different degrees of degradation, resolution between main peak and adjacent peak and main peak asymmetry. Detailed ranking criteria for each peptide sample are described in Supplementary material 2.

A reduced number of the most promising column / mobile phase combinations from the initial screening exercise were then further evaluated for the separation of human angiotensin I and melittin and their corresponding synthesis related impurities. The Ascentis Express Biphenyl and Luna Omega PS C18 columns as well as the AA/NH₄HCO₃ pH 7.0 mobile phase were excluded from the additional screening due to the low rankings they produced in the initial screening. The AA/NH₄HCO₃ pH 7.0 mobile phase also produced a low success rate in method development screening within Novo Nordisk. The pH of the AA/NH₄HCO₃ pH 7.0 mobile phase was observed to increase rapidly when stored in a partly capped flask at room temperature due to the loss of carbon dioxide via evaporation (+0.9 pH units per 24 hrs for 48 hrs). Consequently, daily replacement of this mobile phase is necessary. The poor chromatographic performance observed at intermediate pH was due to the negatively charged stationary phase surface at this pH which resulted in unwanted secondary electrostatic interactions with the positively charged analytes. MSA was also excluded despite good performance similar to TFA due to potential corrosion / material incompatibility concerns (see section 3.9). The ranking exercises were then repeated as described above for the additional 48 chromatograms (4 x 6 = 24 combinations x 2 samples).

Ranking for all the different column / mobile phase / peptide combinations has been summarised in Figure 2. Acquity BEH C8 and Atlantis Premier BEH C18 AX in combination with 0.1% TFA were given the highest average ranking (i.e., 3.8) and can be regarded as good generic combinations. However, other combinations were necessary in order to achieve optimal performance and maximal ranking for the angiotensin I, GLP-2 (1-15) and insulin samples.
3.4.1 Degraded GLP-2(1-15)

The ranking exercise demonstrated a high degree of diversity in the chromatographic selectivity provided with the combination of columns and mobile phases selected. The Acquity BEH C8 column in combination with \( H_3PO_4/NH_4H_2PO_4/NH_4PF_6 \) pH 2.3, the Atlantis Premier BEH C18 AX with either \( H_3PO_4/NH_4H_2PO_4/NH_4PF_6 \) pH 2.3 or \( H_3PO_4/NH_4H_2PO_4/NaCl \) pH 2.3 generated the best separations (i.e., rankings of 5, see Figures 2 and 3) based on the criteria employed (see Supplementary material 2). The results show an improved separation for the inclusion of the ion-pair / chaotropic reagent \( NH_4PF_6 \) at pH 2.3 in the mobile phase. The popular ion-pair additive 0.1% TFA pH 1.9 only yielded rankings of between 2 and 3 for all the columns examined and failed to separate a major impurity eluting close to the main peak see Figure 3.

3.4.2 Degraded insulin

Similar to the GLP-2 (1-15) results, the Acquity BEH C8 column in combination with either the \( H_3PO_4/NH_4H_2PO_4/NH_4PF_6 \) pH 2.3 or \( H_3PO_4/NH_4H_2PO_4 \) pH 3.1 generated the best separation of the degraded insulin sample (see Figure 2 and Supplementary material 3). Once again, the popular mobile phase additive TFA failed to separate an abundant impurity eluting close to the main peak. In contrast with the other peptide samples, the AA/NH_4HCO_3 pH 7.0 mobile phase generated a relatively high ranking of 4 with several columns for the insulin sample (see Figure 2).

3.4.3 GLP-2 (1-15) and four diastereomeric racemization products

The degraded samples were best separated on stationary phases possessing neutral to high positive character at low pH with or without an ion-pair reagent. In contrast, the separation of the diastereomeric racemization products of GLP-2 (1-15) (i.e., the \([D\text{-}His \, 1]\), \([D\text{-}Asp \, 3]\), \([D\text{-}Ser \, 5]\) and \([D\text{-}Ser \, 7]\) isomers) (see Table 2) was best achieved using stationary phases possessing mixed mode characteristics (i.e., hydrophobic ligands and positively or negatively charged groups depending on the mobile phase pH). The following column / mobile phase combinations yielded high ranking values, Atlantis Premier BEH C18 AX with 0.1% TFA pH 1.9, Zorbax SB-C18 with AA/NH_4AA pH 5.1 and Acquity CSH Fluoro-phenyl with FA/NH_4FA pH 3.6 or \( H_3PO_4/NH_4H_2PO_4/NaCl \) pH 2.3 or 0.1% MSA pH 1.9 (Figures 2 and 4).

The latter two phases are not end-capped and therefore possess significant negative character (i.e., silanol accessibility). The Acquity CSH Fluoro-phenyl contains a low surface positive character, but this is outweighed by the large silanol activity at higher pH values [20]. The Atlantic Premier BEH C18 AX has a high degree of positive charge in the pH region of 2 – 7. Different stationary and mobile
phase combinations generated varying elution profiles as exemplified in Figure 4. This once again highlights the necessity to screen disparate stationary and mobile phase combinations.

3.4.4 Angiotensin I

The highest ranking (i.e., 5) / best separation was obtained with the Acquity BEH C8 and FA/NH₄FA pH 3.6 combination which compared favourably with the popular mobile phase additive TFA (ranking of 4) (see Figure 2 and Supplementary material 4).

3.4.5 Melittin

As a consequence of the high basicity (total charge +5.3 and +5.0 at pH 2.3 and 5.1, respectively) of this peptide, application of TFA resulted in improved peak shape compared to FA/NH₄FA pH 3.6 and AA/NH₄AA pH 5.1 (see Figure 2 and Supplementary material 5). The highest ranking (i.e., 5) / best separation was obtained with the Acquity BEH C8 (neutral) and Atlantis Premier BEH C18 AX (positive character) columns in combination with the ion-pair reagent TFA. The mobile phase AA/NH₄AA pH 5.1 resulted in poor peak shape for all columns. This is most likely due to secondary electrostatic interactions between the positively charged peptide and partly dissociated silanol groups of the stationary phases.

3.5 Comparison of different column and solvent combinations retentivity

There are relatively large differences in retention between the different column and mobile phase combinations as can be seen in Figure 5 where the average retention of GLP-2 (1-15), insulin, angiotensin I and melittin relative to the Acquity BEH C8 with TFA has been plotted for the different column and mobile phase combinations. Most column and mobile phase combinations have a relative retention within ±20%. The largest differences in retention are observed for AA/NH₄HCO₃ pH 7.0 and, in particular, in combination with the Acquity CSH Fluoro-phenyl and Ascentis Express Biphenyl columns (-56 and -47%). This information is important for the design of column and mobile phase screens. Based on the retention for a scouting gradient employing the Acquity BEH C8 with TFA, it is possible to either extend or shift the screening gradient to achieve sufficient retention with the Acquity CSH Fluoro-phenyl column.

3.6 Rationale for the proposed method development strategy

The results suggest that only five columns in combination with six mobile phases are necessary to provide the analyst with sufficient chromatographic selectivity differences and acceptable peak
shape to generate a high probability of separating all the components of interest. Evaluation of this
design space (i.e., mobile and stationary phase combinations) can be easily achieved on most of the
commercial HPLC and UHPLC instrumentation equipped with column and mobile phase switching
valves.

3.6.1 Columns

The results advocate that a small number of columns which differ in their ratio between
hydrophobic and ionic (i.e., positive / negative ionisable functionality) character should provide
sufficient selectivity differences to maximise the possibility of achieving separation of the impurities
eluting close to the main component. It is recommended that columns of the following classes
should be employed in the method development strategy.

High positive / high RP character

A RP / anion exchange mixed mode phase (i.e., Atlantis Premier BEH C18 AX or equivalent) which
possesses a significant anion exchange character in addition to its RP behaviour.

Low positive / high RP character

A low positively charged surface C18 phase (i.e., Luna Omega PS C18, Agilent Advance Bio Peptide
Plus, Acquity CSH C18 charged surface C18 or equivalent) which possesses a low anion exchange
character in addition to its RP behaviour.

Neutral / intermediate RP character

New generation C8 or C18 column (e.g., Acquity BEH C8 or C18 or equivalent) which possesses
extremely low silanol activity. It has been subsequently shown that there were little selectivity /
retention differences between the C8 and C18 materials from differing manufacturers with the
peptides investigated.

Low positive, low negative / low RP character

A positively and negatively charged surface phase (i.e., typified by the Acquity CSH Fluoro-phenyl)
with RP behaviour. The extent of positive and negative charge on the surface is dictated by the
mobile phase pH and the ionisation of the non end-capped silica surface.

Low negative / intermediate RP character

A phase with a significant silanol activity, typified by the Zorbax 300 Å SB-C18 which is a relatively
wide pore silica bonded with a C18 ligand with di-isobutyl protecting groups, the phase is not end-
capped. The bulky side groups purportedly prevent access of analytes to the ionised silanol groups
on the silica surface, however, in practice this is not always observed. The Tanaka characterisation parameter for the Zorbax 300 Å SB-C18 phase possessed a total silanol activity ($\alpha_{B/P}$ @ pH 7.6) value of 1.00 compared to the mean value of 0.26 (range 0.18 – 0.32) for six new generation C18 phases indicating that the Zorbax 300 Å SB-C18 phase possessed significant silanol activity [20].

3.6.2 Mobile phase additives

Findings suggest that a limited range of mobile phases varying in their pH, ionic strength and ion-pairing characteristics will confer complementary selectivity and generate acceptable peak shape. The following classes of mobile phases additives are suggested to be used in the method development strategy.

Volatile MS compatible mobile phase additives

10 mM ion strength FA/NH$_4$FA pH 3.6 and 20 mM ion strength AA/NH$_4$AA pH 5.1 were selected as they have previously been demonstrated to generate acceptable peak shape and provide differing selectivity [4].

Volatile MS compatible mobile phase ion-pairing reagents

0.1% v/v MSA and TFA, however, initial results may suggest that the former is more corrosive to 316 stainless steel which may necessitate modifications to certain LC components for long-term use. In addition, there is circumstantial evidence that it may also be detrimental to PEEK components within the LC configuration. However, the present study did not observe any effect of MSA on PEEK rotor seals. MSA has been successfully employed in routine use on LC systems in which all PEEK components have been removed [P. Petersson and Mike R. Jackson, Waters R&D, Milford, MA personal communication]. One hypothesis is a removal of plasticizer from the PEEK making it more susceptible to wear from sheer forces in the rotary valve. The authors recommend a cautious approach to the long-term use of MSA until further work is completed as the composition of the wetted parts may differ between LC systems from different manufacturers. Since it exhibited similar results to TFA, it was deemed sensible to use TFA until the validity of using MSA long-term can be established. However, MSA possesses certain advantages over TFA in that it generates reduced UV baseline drift during low wavelength gradient chromatography, lower requirement for large mixers to be needed in gradient chromatography and it possesses better environmental credentials since it not is fluorinated.

Non-volatile UV compatible mobile phase additives
100 mM ion strength \( \text{H}_3\text{PO}_4/\text{NH}_4\text{H}_2\text{PO}_4/\text{NaCl} \) pH 2.3 and \( \text{H}_3\text{PO}_4/\text{NH}_4\text{H}_2\text{PO}_4 \) pH 3.1. Although pH 7 typically resulted in lower ranking than the other mobile phases in the current study, it may provide important differences in selectivity and could therefore be worth including in a mobile phase screening. Due to the limited stability of carbonate buffers a \( \text{NH}_4\text{H}_2\text{PO}_4/(\text{NH}_4)_2\text{HPO}_4 \) buffer would be preferable.

**Non-volatile UV compatible ion-pairing reagents**

The use of \( \text{H}_3\text{PO}_4/\text{NH}_4\text{H}_2\text{PO}_4/\text{NH}_4\text{PF}_6 \) pH 2.3 is recommended in the initial method development screening protocols in order to detect impurities. Despite the fact that we have failed to observe any detrimental effects of \( \text{NH}_4\text{PF}_6 \) at pH 2.3 on LC components and columns, it is recommended that its use should be monitored until sufficient “real time” data has been collected to prove that it is suitable for routine long-term use.

### 3.7 Proposed method development strategy

#### 3.7.1 Screening columns and mobile phase conditions

The proposed screening strategy comprises the evaluation of six MS and non-MS compatible mobile phase additives (mobile phase 2 and 8 were excluded, see Table 1) in combination with five differing columns (the biphenyl column was excluded). The only difference in the LC conditions was that for the non-MS compatible screen, mobile phase B is composed of 8:2 v/v MeCN water to prevent buffer precipitation, whereas for the MS compatible screens the mobile phase A and B are matched with respect to the additive. The best column / mobile phase additive combination is selected based on the number of resolved peaks above a certain % area/area impurity level, the resolution around the main peak and the peak shape. If \( \text{H}_3\text{PO}_4/\text{NH}_4\text{H}_2\text{PO}_4/\text{NaCl} \) pH 2.3 was observed to be the best mobile phase, it may be worthwhile investigating the use of ammonium sulfate in place of NaCl as it could provide a slightly differing selectivity or improved peak shape for certain peptides. The screening of differing organic modifiers such as 2PrOH and MeOH in place of / or in combination with MeCN is an optional activity that could be performed if adequate resolution is not obtained. Due to high viscosity 2PrOH is typically used in combination with MeCN e.g. 20/60/20 2PrOH:MeCN:H\(_2\)O. By examining the screening chromatograms, it is possible to select two complementary LC conditions which can form the basis of a 2D-LC-MS peak purity analysis. This will be reported in a subsequent paper [D.R. Stoll, M.R. Euerby, S.M.C. Buckenmaier, P. Petersson, unpublished results].

#### 3.7.2 Retention time modelling / optimisation and validation and robustness testing
Once a suitable column / mobile phase combination has been selected, the methodology can be further optimised by producing a gradient time versus temperature retention model based on three temperature and three gradient inputs runs (i.e., nine experiments) [21]. A comprehensive model (i.e., all the peaks of interest are modelled [14]) is generated which will facilitate the prediction of optimal conditions. Alternatively, a simple model (i.e., main component plus first and last peaks only are modelled) is generated to identify conditions which provide an acceptable retention. The latter approach has the advantage that it does not require any peak tracking. These conditions can then be subsequently investigated to identify optimal conditions.

The robustness of the optimised methodology can initially be assessed from the retention model. For example, the effect of small permitable changes to the gradient slope and temperature on the separation criteria can be rapidly performed in silico to provide both graphical (calculated chromatograms) and numerical (δtR/δ%MeCN and δtR/δT) guidance for system suitability adjustments. If the methodology appears to be robust, the validation of the method can be confirmed experimentally using a reduced factorial design [22] and ICH guidelines [23].

### 3.8 Column performance / longevity studies using MSA and NH₄PF₆

The longevity of a representative new generation RP column (i.e., Ascentis Express C18 column) when exposed to MSA and NH₄PF₆ (five times the typical concentrations used in chromatography) at an elevated temperature of 80 °C and a linear flow velocity of 8.25 mm/sec was compared to the “gold standard” mobile phases containing TFA. New columns were used for each study. The column stability was assessed by the reduction of the retention factor of o-cresol (i.e., loss of hydrophobic retention as a result of loss of phase C18 / end-capping) as a function of the number of column volumes of the mobile phase passed through the column. 0.5% v/v MSA was observed to be slightly more aggressive to the C18 phase than TFA at the same concentration. 100 mM NH₄PF₆ at pH 2.3 was observed to be less aggressive to the C18 phase than 0.5% v/v TFA. Hence, from a column longevity perspective, both 0.1% v/v MSA and 20 mM NH₄PF₆ should not cause significantly more stationary phase degradation than 0.1% v/v TFA (Figure 6).

### 3.9 Evaluation of the long-term exposure of mobile phase additives to “wettable” LC components

A preliminary investigation into the effect of the selected mobile phase additives on the longevity of the common “wettable” LC components was conducted. It is possible that MSA and NH₄PF₆ might be incompatible with certain LC components as the former has been previously shown to promote
corrosion of stainless steel [24-26] and the latter the formation of fluoride ions [27] under certain conditions.

3.9.1 Potential corrosion of 316 stainless steel and titanium components

The mobile phase additives (see Table 1) were assessed for their propensity to promote corrosion (i.e., extraction of metals from various LC components) on accelerated storage (60°C for 7 days) using Induced Coupled Plasma spectroscopy (ICP). Column frits were selected as a representative LC component as they possessed a large surface area with which the additive could be in contact and hence cause corrosion. Figure 7 highlights that the only potential problem may reside with 0.1% v/v MSA in 80% MeCN / water and stainless steel, where MSA in MeCN or water surprisingly failed to elicit such an effect. These findings have been independently confirmed and will be reported in a subsequent paper [J. Bischof, C Smith, unpublished results]. It has been previously demonstrated that under certain conditions, MSA may promote corrosion of stainless steel [24-26]. All the other additives yielded similar levels of metal extraction to those mobile phase compositions used routinely without concern, e.g., 600 mM NaCl pH 2.4. The major metal extracted in all classes was iron followed by nickel, chromium and then manganese.

The level of titanium extracted from the frits was considerably lower (<1200 ppb) than metals extracted from stainless steel frits (Figure 7). The use of NaCl in place of Na₂SO₄ at pH 2.3 failed to promote increased corrosion (via the formation of HCl) of the stainless steel frits, as evidenced by the extraction of iron, confirming its suitability for use in LC mobile phases. Stainless steel 316 and MP35N® alloy (i.e., nickel-cobalt-chromium-molybdenum alloy) HPLC tubing were exposed to the following mobile phase conditions with continuous stirring for 30 days at 60 °C; 0.1% v/v MSA in water and MeCN / water (8:2 v/v), 20 mM NH₄PF₆ in phosphate buffer pH 2.3 and 1:1 v/v MeCN/ PO₄ buffer. There was no significant weight difference or microscopic change of the tubing observed before and after exposure to the mobile phase additives.

The results indicate that sodium chloride can be successfully used in place of ammonium sulfate, which can promote poor peak shape due to “salting out effects”. The corrosive nature of NaCl has been shown to be comparable to that of the typically employed mobile phases examined and it also possesses the advantage of being more soluble than the sulfate salt.

3.9.2 Compatibility of MSA and NH₄PF₆ on PEEK components

Exposure (i.e., 30 days at room temperature, dynamic conditions) of the PEEK rotor seals to solutions of 0.1% v/v MSA in water or 8:2 v/v MeCN / water yielded no difference in their hardness. Similar results were obtained when exposed to 8:2 v/v MeCN / water in the absence of MSA.
3.9.3 Compatibility of MSA and NH$_4$PF$_6$ on Teflon components

It is known that the mechanical strength of Teflon may be affected by exposure to certain chemicals [28], however, no differences in the tensile strength of the Teflon tubing (used to connect the HPLC reservoir sinker to the pump head) were observed after exposure to the passage of 10 litres of either 0.1% v/v MSA in MeCN / water (8:2 v/v) and in MeCN or 20 mM NH$_4$PF$_6$ at pH 2.3 over 14 days at room temperature.

3.10 Evaluation of the ion-pair memory effects of MSA and NH$_4$PF$_6$, compared to TFA, on differing stationary phase chemistries

Certain ion-pairing reagents may be difficult to remove from RP stationary phase, using typical washing conditions (i.e., 20 column volumes of 50:50 v/v MeCN / water), due to their propensity to adsorb onto the phase [9]. This may result in selectivity changes in the column after exposure to differing mobile phase conditions. Changes in the stationary phase surface chemistry caused by adsorption of the ion-pair reagent were monitored by assessing the Peptide RPC column characterisation selectivity values (i.e., seven $\Delta$ values, see references 1-4, using the mobile phase H$_3$PO$_4$/NH$_4$H$_2$PO$_4$/NaCl pH 2.3) before and after exposure of the column to the mobile phase containing the ion-pair reagent and washing of the column. In all instances no detectable memory / adsorption effects (i.e., $\delta\Delta$ values $\pm$0.003) were observed with MSA, NH$_4$PF$_6$ and TFA on any of the six columns evaluated.

3.11 pH hysteresis effects

The phenomenon of “slow equilibration” of stationary phases after pH cycling between low and intermediate pH is an undesirable characteristic of columns used in screening protocols [10]. To assess if the columns selected for the screening protocol suffered from this phenomenon, we evaluated the selectivity values of the Peptide RPC column characterisation protocol (i.e., seven $\Delta$ values, see references 1-4, using the mobile phase H$_3$PO$_4$/NH$_4$H$_2$PO$_4$/NaCl pH 2.3) before and after pH cycling (pH 2.3 $\rightarrow$ pH 3.6 $\rightarrow$ pH 7.0 $\rightarrow$ pH 2.3). Columns were washed between each pH condition by flushing with 20 column volumes of 1:1 v/v MeCN/water. No evidence of “slow equilibration” was detected with any of the columns (i.e., $\delta\Delta$ values $\pm$0.002), however, there was a very small change in the selectivity values associated with the Luna PS C18 and Fluoro-phenyl phases (i.e., $\delta\Delta$ values 0.004).
4. Conclusions

A high probability of separating peptide impurities can be achieved by screening five columns which possess differing charge / RP character ratios in combination with six differing mobile phase compositions (pH 2.3 to 5.1) in an automated method development screening protocol. Evaluation of the design space (i.e., mobile and stationary phase combinations) can be easily achieved on most commercial HPLC and UHPLC instrumentation equipped with column and mobile phase switching valves. The separation of degradation products of insulin, GLP-2 (1-15) and angiotensin I impurities was best achieved on C8 or C18 columns with neutral to high positive charge at low pH using non-volatile or volatile additives. The use of the ion-pair / chaotropic reagent NH₄PF₆ at pH 2.3 provided excellent separation and peak shape for both degraded samples. The highly basic melittin sample demonstrated the need for an ion-pair reagent such as TFA.

In contrast, it was observed that stationary phases which possessed significant negative character (i.e., silanol accessibility) typified by the SB-C18 and CSH Fluoro-phenyl phases at higher pH values, separated the diastereoisomers of GLP-2 (1-15). The use of novel mobile phase additives such as NH₄PF₆ and MSA have been shown not to affect the stability of C18 columns. All mobile phases were compatible with typical LC instrument materials (i.e., 316 stainless steel and MP35N®, titanium, PEEK and Teflon). The same was true when they were exposed to 316 stainless steel and titanium frits however, 0.1% v/v MSA in 8:2 v/v MeCN / water resulted in exceptionally high levels of iron, nickel and chromium being extracted on accelerated storage. Further investigations are presently underway into this anomalous result. As there is a potential for certain metals to adversely affect the chromatographic performance of a certain analytes [29-31] it is recommended that this be investigated. If this is a cause for concern, then the use of a bioinert LC system coupled with a silica or PEEK lined column and PEEK, titanium or coated stainless steel frits be employed.

No observable pH hysteresis effects were demonstrated with any of the proposed stationary phases on pH cycling between pH 2.3 → pH 7.0 → pH 2.3. The ion-pair additives NH₄PF₆, MSA and TFA failed to demonstrate any memory effects / adsorption onto the RP columns evaluated after washing with 1:1 v/v MeCN / water. While major problems have not been observed with the novel mobile phase additives NH₄PF₆ and MSA, we would recommend caution until their long-term routine use has been established.

The results advocate the use of screening a range of disparate mobile phase additives in conjunction with a small number of stationary phases with differing charge / RP character. The best mobile phase / stationary phase combination will be dependent on the physical / chemical properties of the
peptide under study. The conclusions are based on a limited number of peptides and their corresponding impurities, however, it is believed that the findings will hold true for other peptides as well. The popularly employed ion-pair additive, TFA, failed to generate optimum separation in all the peptides investigated, highlighting the necessity to evaluate other mobile phase additives in method development screening protocols.

**CRediT authorship contribution statement**

Ming Yui Cheung: Methodology, Formal analysis, Investigation.

Jennifer K. Field: Methodology, Formal analysis, Investigation.

Melvin R. Euerby: Conceptualization, Methodology, Formal analysis, Resources, Writing - review & editing, Supervision, Funding acquisition.

James Bruce: Conceptualization, Methodology, Resources, Review & editing, Funding acquisition, Supervision.

Patrik Petersson: Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing, Supervision, Funding acquisition.

**Declaration of interests**

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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References


Figure 1. Examples of typical chromatogram rankings for, in this case, the degraded GLP-2 (1-15) sample. The ranking criteria for the degraded GLP-2 (1-15) was based on the number of impurity peaks present within a retention time window of ±20% of the retention of the main peak and with a signal above 1.0% of the main peak. A ranking value of 1 was given to 1-2 peaks, 2 was given to 3 peaks, 3 was given to 4 peaks, 4 was given to 5 peaks and 5 to 6 or more peaks. The high-ranking combinations (i.e., rankings of 4 and 5) were penalised for excessive peak tailing (i.e., $T_f > 1.5$) or poor resolution (i.e., peak/valley <2) by incurring a subtraction of 1 ranking point. Chromatograms aligned using the main peak.
Figure 2. Summary of screened and ranked column and mobile phase combinations. An initial screen involved all 48 column and mobile phase combinations and three GLP-2 (1-15) and insulin related samples. A second screen involving angiotensin I and melittin related samples was made after removal of combinations marked with a light grey background, i.e., MSA, AA/NaHCO₃ pH 7.0, Ascentis Express Biphenyl and Luna Omega PS C18. These combinations were removed due to low rankings or in the case of MSA potential incompatibility concerns.
Figure 3. Column and mobile phase combinations ranked 5 for the degraded GLP-2 (1-15) sample. Acquity BEH C8 with 0.1% TFA pH 1.9 included for comparison. Chromatograms aligned using the main peak.
Figure 4. Column and mobile phase combinations ranked 5 for GLP-2 (1-15) and four of its diastereomeric racemization products, peak assignment based on the peptide number described in paper 1 of the series [1]: 1 = GLP-2 (1-15), 2 = [D-His1]-GLP-2 (1-15), 3 = [D-Asp3]-GLP-2 (1-15), 6 = [D-Ser5]-GLP-2 (1-15) and 7 = [D-Ser7]-GLP-2 (1-15). Acquity BEH C8 with 0.1% TFA pH 1.9 included for comparison. Chromatograms aligned using isomer 7.
Figure 5. Average retention for 2 or 4 peptides relative to Acquity BEH C8 with TFA for the different column and mobile phase combinations. Closed circles denote GLP2(1-15), insulin, angiotensin I and melittin whereas open circles denotes only GLP2(1-15) and insulin.
Figure 6. Change in the retention factor of \(\sigma\)-cresol using the Ascentis Express C18 column when exposed to the following conditions at 80°C: Filled red circles 100 mM NH\(_4\)PF\(_6\) at pH 2.3, filled blue triangles 0.5% v/v TFA; open black circles 0.5% v/v MSA.
Figure 7. Extraction of metal from 316 stainless steel frits when exposed to the mobile phase conditions (7 days at 60°C). See Table 1 for a description of the mobile phase compositions

a 19.27 mM H$_3$PO$_4$ / 17.38 mM (NH$_4$)$_2$PO$_4$ / 26.37 mM (NH$_4$)$_2$SO$_4$ in water

b 100 mM H$_3$PO$_4$ / NH$_4$H$_2$PO$_4$ pH 2.3 in water

c 10 mM H$_3$PO$_4$ / 20 mM NaH$_2$PO$_4$ H$_2$O / 600 mM NaCl pH 2.4 in water / 2PrOH / MeCN (10:3:7 v/v/v)
Table 1. Weak (A) and strong mobile phase (B) employed in the method development screening strategy.

<table>
<thead>
<tr>
<th>Name</th>
<th>Number</th>
<th>Composition</th>
<th>Volatile</th>
<th>pH</th>
<th>Ion strength [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% (v/v) TFA pH 1.9</td>
<td>1A</td>
<td>TFA / water (1:999 v/v)</td>
<td>Y</td>
<td>1.9</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1B</td>
<td>TFA / water / MeCN (0.9:199:800 v/v/v)</td>
<td>Y</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>0.1% (v/v) MSA pH 1.9</td>
<td>2A</td>
<td>MSA / water (1:999 v/v)</td>
<td>Y</td>
<td>1.9</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>2B</td>
<td>MSA / water / MeCN (1:199:800 v/v/v)</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₃PO₄/NH₄H₂PO₄/NaCl pH 2.3</td>
<td>3A</td>
<td>18.0 mM phosphoric acid, 14.0 mM ammonium dihydrogen phosphate and 80.0 mM sodium chloride</td>
<td>N</td>
<td>2.3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>4A</td>
<td>46.0 mM phosphoric acid, 73.0 mM ammonium dihydrogen phosphate and 20.0 mM ammonium phosphor hexafluoride</td>
<td>N</td>
<td>2.3</td>
<td>100</td>
</tr>
<tr>
<td>H₃PO₄/NH₄H₂PO₄/NH₄PF₆ pH 2.3</td>
<td>5A</td>
<td>9.0 mM phosphoric acid, 99.5 mM ammonium dihydrogen phosphate</td>
<td>N</td>
<td>3.1</td>
<td>100</td>
</tr>
<tr>
<td>80% v/v MeCN</td>
<td>3B, 4B, 5B</td>
<td>MeCN / water (8:2 v/v)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA/NH₄FA pH 3.6</td>
<td>6A</td>
<td>12.3 mM formic acid and 9.9 mM ammonium formate</td>
<td>Y</td>
<td>3.6</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>6B</td>
<td>53.1 mM formic acid and 42.9 mM ammonium formate / MeCN (2:8 v/v)</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7A</td>
<td>7.8 mM acetic acid and 20.0 mM ammonium acetate</td>
<td>Y</td>
<td>5.1</td>
<td>20</td>
</tr>
<tr>
<td>AA/NH₄AA pH 5.1</td>
<td>7B</td>
<td>33.1 mM acetic acid and 85.5 mM ammonium acetate / MeCN (2:8 v/v)</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8A</td>
<td>3.2 mM acetic acid and 20.0 mM ammonium hydrogen carbonate</td>
<td>Y</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>AA/NH₄HCO₃ pH 7.0</td>
<td>8A</td>
<td>16.2 mM acetic acid and 100.0 mM ammonium hydrogen carbonate / MeCN (2:8 v/v)</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 2. Peptide amino acid sequence, molecular weight and estimated pI. Uppercase and lowercase letters are used to designate the L- and D-forms of the amino acid residue respectively.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Molecular weight [kDa]</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine GLP-2 (1-15)</td>
<td>HADGSFSDEMTVLDD</td>
<td>1.6</td>
<td>3.4</td>
</tr>
<tr>
<td>[D-His1]-Bovine GLP-2 (1-15)</td>
<td>hADGSFSDEMTVLDD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[D-Asp3]-Bovine GLP-2 (1-15)</td>
<td>HAdGSFSDEMTVLDD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[D-Ser5]-Bovine GLP-2 (1-15)</td>
<td>HADGSfDEMTVLDD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[D-Ser7]-Bovine GLP-2 (1-15)</td>
<td>HADGSFsDEMTVLDD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine insulin</td>
<td>GIVEQCCASVLQENLYCNG-FVNLHCGLSHELVAYLVCGERGFYTPKA</td>
<td>5.7</td>
<td>5.3</td>
</tr>
<tr>
<td>Human angiotensin I</td>
<td>DRVYIHFHLL</td>
<td>1.3</td>
<td>8</td>
</tr>
<tr>
<td>Melittin</td>
<td>GIGAVLKVLTGLPALISWIKRRKKQ</td>
<td>2.8</td>
<td>12.5</td>
</tr>
</tbody>
</table>

### Table 3. Stationary phases / columns (150 x 2.1 mm I.D.) employed in the method development screening strategy.

<table>
<thead>
<tr>
<th>Column</th>
<th>Pore size [Å]</th>
<th>Particle size [µm]</th>
<th>Description as given by the manufacturer</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acquity BEH C8</td>
<td>130</td>
<td>1.7</td>
<td>An end-capped, trifunctional C8 alkyl ligand bonded to ethyl bridged silica hybrid material</td>
<td>Waters</td>
</tr>
<tr>
<td>Acquity CSH Fluoro-phenyl</td>
<td>130</td>
<td>1.7</td>
<td>A non end-capped, trifunctional pentafluorophenyl ligand bonded to ethyl bridged silica hybrid material which possesses a low level positive surface charge (pyridyl positive charge below pH 5)</td>
<td>Waters</td>
</tr>
<tr>
<td>Atlantis Premier BEH C18 AX</td>
<td>95</td>
<td>1.7</td>
<td>An end-capped, trifunctional C18 alkyl ligand bonded to ethyl bridged silica hybrid material with an anion exchange functionality (tertiary alkylamine positive charge below pH 8)</td>
<td>Waters</td>
</tr>
<tr>
<td>Ascentis Express Biphenyl</td>
<td>90</td>
<td>2.7</td>
<td>An end-capped, superficially porous particle with a biphenyl ligand</td>
<td>Supelco</td>
</tr>
<tr>
<td>Luna Omega PS C18</td>
<td>100</td>
<td>1.6</td>
<td>An end-capped, C18 alkyl ligand with a positive charge on the surface of the particle</td>
<td>Phenomenex</td>
</tr>
<tr>
<td>Zorbax 300 SB-C18</td>
<td>300</td>
<td>1.8</td>
<td>A non end-capped, C18 alkyl ligand with disobutyl sterically protected siloxane bonds on wide pore silica</td>
<td>Agilent</td>
</tr>
</tbody>
</table>
Supplementary material legends

1 Optimisation of the NH$_4$PF$_6$ concentration in the mobile phase.

2 Ranking criteria

3 Column and mobile phase combinations ranked 5 for the degraded insulin sample. Acquity BEH C8 with 0.1% TFA pH 1.9 included for comparison.

4 Column and mobile phase combination ranked 5 for the angiotensin I sample. Acquity BEH C8 with 0.1% TFA pH 1.9 included for comparison.

5 Column and mobile phase combinations ranked 5 for the melittin sample. Zorbax 300 SB-C18 with AA/NH4AA pH 5.1 included to illustrate the need for an ion-pairing reagent to suppress silanol interactions at intermediate pH.