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Investigation into Reversed-phase Chromatography Peptide Separation Systems Part IV: Characterisation of Mobile Phase Selectivity Differences

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Keywords

Peptides, protocol, RPC, characterisation, mobile phase selectivity

Highlights

- 51 RPLC low and mid pH mobile phases were characterised using a peptide based protocol
- Ion pairs, salts, kosmotropic and chaotropic salts
- Larger selectivity differences at low pH than at mid pH
- Most interesting MS compatible mobile phases gave a MS response similar to TFA
- Order of influence: pH and column type > pH > column type > ion-pair reagent

Abstract

The differentiation of mobile phase compositions between sub-classes which exhibit distinct chromatographic selectivity (i.e. termed characterisation) towards a range of peptide probes with diverse functionality and hence the possibility for multi-modal retention mechanisms has been undertaken. Due to the complexity of peptide retention mechanisms in given mobile phase conditions, no attempt has been made to explain these, instead mobile phases have simply been classified into distinct groups with an aim of identifying those yielding differing selectivities for use in strategic method development roadmaps for the analysis of peptide mixtures.

The selectivity differences between nine synthetic peptides (fragments of [Ile27]-Bovine GLP-2) were used to assess how fifty-one RPC mobile phase compositions of differing pH (range 1.8 – 7.8), salt types, ionic strengths, ion-pair reagents and chaotropic / kosmotropic additives affected chromatographic selectivity on a new generation C18 stationary phase (Ascentis Express C18). The mobile phase compositions consisted of commonly used and novel UV or MS compatible additives. The chemometric tool of Principal Component Analysis (PCA) was used to visualise the differences in selectivity generated between the various mobile phases evaluated. The results highlight the importance of screening numerous mobile phases of differing pH, ion-pair reagents and ionic strength in order to maximise the probability of achieving separation of all the peptides of interest within a complex mixture. PCA permitted a ranking of the relative importance of the various mobile phase parameters evaluated. The concept of using this approach was proven in the analysis of a sample of Bovine GLP-2 (1-15) containing synthesis related impurities.

Mobile phases with high ionic strength were demonstrated to be crucial for the generation of symmetrical peaks. The observations made on the C18 phase were compared on three additional stationary phases (i.e. alkyl amide, fluorophenyl and biphenyl), which had previously been shown to possess large selectivity differences towards these peptides, on a limited sub-set of mobile phases. With the exception of the ion-pair reagent, similar trends were obtained for the C18, fluorophenyl and biphenyl phases intimating the applicability of these findings to the vast majority of RPC columns (i.e. neutral or weakly polar in character) which are suitable for the analysis of peptides. The conclusions were not relevant for columns with a more disparate nature (i.e. containing a high degree of positive charge).

1. Introduction

There is a wealth of published information regarding the mechanistic effect ion-pairing reagents [1-6], pH [7-10], temperature [6, 7, 11-13], mobile phase composition [7, 8, 10] and differing stationary phases [6, 7] have on the prediction of peptide retention and selectivity in reversed-phase LC. The groups of Hodges, Hearn, Krokhin and Gilar have devoted extensive research to enhance the current understanding of the retention mechanisms of peptides on RP columns [6, 8, 9, 14-26]. Peptide separations are typically performed on C18 columns using a mobile phase pH of between 2 and 7 with or without an ion-pairing reagent which interacts with protonated amino functionalities within the peptide molecule (i.e. histidine, lysine and arginine residues as well as the N-terminal amino group). The potential value of evaluating ion-pair reagents with differing hydrophobicity on the separation of specific peptides has been reported [1, 5]. Optimization of separation conditions of

complex peptide mixtures are not trivial due to the differing secondary structures that peptides can exhibit in varying organo / aqueous environments and the net charge and charge distribution that the peptide exhibits at various pH values. This can result in a change in the dominance of retention mechanisms as a function of the mobile phase composition.

To date, there has not been a comprehensive study aimed at comparing and characterising (i.e. classifying) various mobile phase compositions composed of differing salts, ion-pair reagents and pH with different stationary phases. Due to the expanding interest in the development of biopharmaceuticals in recent years, there has become a need to identify and, hence, select mobile and stationary phases which provide the chromatographer with the optimal probability of separating relevant peptide components within a complex mixture. Instead of trying to understand the exact retention mechanism in operation with a mobile phase, the primary focus of this paper has been to utilise chemometric tools to identify and characterise 51 novel as well as commonly used mobile phase compositions into differing sub-groupings based on the selectivity profile that they generated.

In order to resolve, identify and quantify small impurities in pharmaceutically relevant peptides it is important that both good selectivity and peak shape are achieved, therefore, in addition to the primary focus on selectivity, the effect of mobile phase additives on peptide peak shape and analyte overloading on selected mobile phases of interest have also evaluated. Literature suggests that both selectivity and peak shape are highly analyte dependent, hence it is important that chromatographers have the option to investigate a range of disparate mobile phases in order to identify conditions that generate optimal resolution for their specific application.

This paper is the fourth in a series which deals with maximising the chromatographic selectivity of peptide separations using reversed-phase chromatography (RPC). Papers one to three of the series focussed on the development of a column characterisation protocol using 26 specifically designed peptide probes [27], the robustness of the optimised protocol [28] and the characterisation of 38 disparate stationary phases [29], respectively. The characterisation protocol, which determines seven retention time differences between nine selected probes based on the 33 amino acid peptide, [Ile27]-Bovine GLP-2 (see Table 1 and references [27-31]), has been demonstrated to successfully discriminate between differing types of RP stationary phases through the peptide probe's hydrophobic, electrostatic, hydrogen bonding and aromatic interactions with the stationary phases, in addition, to the stationary phase's ability to separate diastereoisomeric or isomeric probes [27]. This fourth paper extends the use of the characterisation protocol to 51 novel and commonly used MS compatible and non-compatible mobile phases. In addition to differing pH buffers, the evaluation also included a range of ion-pairing reagents differing in their hydrophobicity and charge

(heptafluorobutyric acid (HFBA), trifluoroacetic acid (TFA), difluoroacetic acid (DFA), sodium butylsulfonate ($BuSO_3Na$) and triethylamine (TEA)), chaotropic sodium perchlorate ($NaClO_4$) and ammonium hexafluorophosphate (NH_4PF_6) or kosmotropic reagents sodium or ammonium sulfate (Na_2SO_4 or $(NH_4)_2SO_4$), the effect of ionic strength (IS) and a range of miscellaneous modifiers (formic acid (FA), phosphoric acid (H_3PO_4), methanesulfonic acid (MSA), ammonium formate (NH_4FA), ammonium acetate (NH_4AA) and ammonium bicarbonate (NH_4HCO_3)) (see Table 2). The MS compatible additives were also evaluated for the signal intensity that they generated in positive mode electrospray ionisation mass spectrometry (ESI-MS). The highly pictorial chemometric tool of Principal Component Analysis (PCA) was employed to provide a simple visualisation of the selectivity differences and similarities between the mobile phase additives and to identify and group mobile phases compositions into different sub-classes dependent on the selectivity profile that they generated towards the peptide probes.

This approach should facilitate the selection of a limited number of mobile phases which can be used to maximise selectivity on a given column. Initially, a large range of mobile phases on a single representative new generation C18 column were investigated. It was then established if these findings could be extrapolated to a range of other disparate RP columns (shown to provide maximal selectivity with two mobile phases [29]) using a limited number of mobile phases (shown to possess large selectivity differences on the C18 phase). It is hoped that the findings can be utilised to identify suitable initial mobile and stationary phase combinations in a RP-LC method development strategy that will provide optimal separation of peptides.

2. Experimental

2.1 Chemicals and Reagents

All water, acetonitrile and mobile phase additives (described in Table 2) used were of LC-MS grade and supplied by Sigma Aldrich (Poole, UK). Dimethylsulfoxide (DMSO) was supplied by Fisher Scientific (Hemel Hempstead, UK). The peptides, which were supplied by Apigenex (Prague, Czech Republic), were all dissolved individually in DMSO/H_2O (80:20 v/v) to a concentration of 0.25 mg/mL. Solutions were stored at -20 °C. The base sequence for each peptide can be located in [27], and further description of the peptide probes described in Table 1.

2.2 Instrumentation

LC separations were performed on a Shimadzu Nexera X2 UHPLC system (Duisburg, Germany) equipped with two binary pumps (LC-30AD) and proportionating valves, degassers (DGU-20A_{SR}), autosampler with cooling capabilities (SIL-30AC), Prominence column oven (CTO-20AC), diode array detector (SPD-M30A) and communication bus module (CBM-20A). The LC configuration had a dwell volume of 342 µL and system retention volume of 14 µL [32]. The MS study was conducted on a Waters Acquity I-Class equipped with PDA and Waters Synapt G2-Si Q-TOF (Wilmslow, UK).

2.3 Stationary Phases

The mobile phases were all assessed on the Ascentis Express C18 (150 x 2.1 mm column dimensions, 2.7 µm particle size, Supelco, Bellefonte, PA, USA). Each ion-pair utilised a dedicated column to avoid memory effects between mobile phases. The Polaris Amide C18 (150 x 2.0 mm, 3 µm, Agilent Technologies, Santa Clara, CA, USA), Acquity CSH Fluoro Phenyl (150 x 2.1 mm, 1.7 µm, Waters, Milford, MA, USA) and Ascentis Express Biphenyl (150 x 2.1 mm, 2.7 µm) were selected as chromatographically diverse stationary phases to ascertain the applicability of the Ascentis Express C18 results on other types of stationary phases [29]. The MS response comparison was performed on an Acquity CSH C18 (150 x 1.0 mm, 1.7 µm) which utilised a translated gradient from the 150 x 2.1 mm column format. A brief description of each of the stationary phases can be located in Table 2 in [29]. The peak apex of a water injection was used as the dead time marker [32].

2.4 Mobile Phase Characterisation Protocol

Premixed mobile phases were prepared as described in Table 2 for the A solvent. The B solvent was prepared using MeCN / H_2O (80:20 v/v). The additives were not matched in the B solvent to prevent wastage of solvent and keep the number of solvents and experiments at an acceptable level. The gradient was standardised as followed: 5.6% to 62.5%B over 40 minutes, with an isocratic hold at the top of the gradient for 2 minutes, before returning to the original conditions in 0.1 minutes and 10 minutes re-equilibration (equivalent to 10 column volumes). The column oven temperature was 40 °C, flow rate was 0.3 mL/min, and detection was 215 nm, bandwidth 8 nm, referenced at 360 nm, bandwidth 100 nm. Where applicable, the Shimadzu 2020 single quadrupole instrument with electrospray ionisation was installed post-PDA to aid peak identification in positive SIM mode. An injector program was utilised to create online cocktails to minimise consumption of peptides.

The MS study which compared the performance of the different buffers was conducted on the Synapt G2-Si MS using positive ESI mode ionisation with a source temperature of 120 °C, capillary

157 voltage of 3.5 kV, desolvation temperature of 250 °C, desolvation gas flow 750 L/hr, nebuliser gas
158 pressure 6.0 bar, cone gas flow of 50 L/hr and scan time of 0.250 s. The mass range was set to 100 –
159 2000 to observe any adduct formation and high-resolution mode was applied.

160

161 ***2.5 Software and Calculations***

162 The Shimadzu LC instrument was controlled, and data processing performed using LabSolutions
163 (Version 5.86). The Waters LC instrument was controlled via MassLynx (Version 4.1). Principal
164 Component Analysis (PCA) was performed using SIMCA (Version 14.1, Umetrics, Umeå, Sweden) and
165 Origin (Version OriginPro 2016, OriginLab, Northampton, MA, USA). The net charges of the peptide
166 probes were calculated at all pH values evaluated in this study using General Protein / Mass Analysis
167 for Windows (GPMAW) software (Version 9.51, Lighthouse Data, Odense, Denmark). The mobile
168 phase calculations were performed using BufferMaker (Version 1.1.0.0, ChemBuddy, BPP Marcin
169 Borkowski, Poland).

170

171 **3. Results and Discussion**

172 ***3.1 Rationale for Mobile Phases' selection***

173 A range of mobile phases composed of differing salts, ionic strengths, anionic / cationic ion-pairing
174 reagents, kosmotropic / chaotropic salts at differing pH values (pH 1.8 - 7.8), were assessed for the
175 selectivity they conferred on a range of peptides of differing physico / chemical properties. Table 2
176 contains the buffers, pH, total ionic strength, mobile phase composition and their MS compatibility.
177 The rationale for the selection of the mobile phase compositions can be found in the Electronic
178 Supplementary Material.

179

180 ***3.2 Effect of pH***

181 The retention of peptides on hydrophobic RP stationary phases is dependent on the mobile phase
182 pH as this influences their net ionisation state (for example the ionisation of the C- and N- terminal
183 of the peptide and or the side chains in aspartic acid, glutamic acid, tyrosine, histidine, lysine and
184 arginine residues), which, in turn, dictates their hydrophilicity and also their propensity to
185 interaction with ion-pairing reagents. The net charge and the number of ionised basic / acidic
186 moieties for each of the peptide probes used is shown in Table 1. In addition, pH will affect the
187 ionisation state of silanol groups on silica based stationary phases and any amino functionality

188 imparted by the manufacturers (i.e. charged surface hybrid phases), hence this will affect the
189 electrostatic attraction / repulsion of the charged peptides and the ionised stationary phase surface.

190 The results were assessed using PCA, the first two principal components described approximately
191 92% of the variability in the dataset. A third principal component did not increase this value
192 substantially but rather added to the complexity of the evaluation; thus, it was not used in the study.
193 The first principal component described approximately 62% of the variability of the data and, as
194 expected, contained the delta values Δ(9,1) and Δ(26,13) (i.e. variables) associated with electrostatic
195 interactions [29], which were diametrically opposite to one another in Fig. 1. In addition to the
196 expected impact of the peptides charge on selectivity, the first principal component was also
197 influenced by the presence of aromatic, phenolic groups and different oxidation state of methionine
198 residues as indicated by the delta values Δ(16,13), Δ(24,13), Δ(8a,1) respectively – these delta values
199 probably describe increased hydrophilicity (i.e. loss of the hydrophobic phenyl group, addition of a
200 phenolic grouping and conversion of a sulfide into a sulfoxide moiety capable of dipole interactions
201 respectively). The second principal component described approximately 30% of the variability,
202 where the results were mainly influenced by steric parameters based on the position of the delta
203 values Δ(14,13) and Δ(15,13) in the biplot (Fig. 1). A PCA contribution plot indicates differences in
204 the selectivity conferred by two mobile phases (i.e. observations). Contribution plots (data not
205 shown) of mobile phases where only pH was varied (MP46, 44, 36, 42, 29, 23 & 4) indicated that the
206 Δ(26,13) value increased with pH, highlighting the increased electrostatic attraction between the
207 positively charge Peptide Number 26 and the negatively charged silica surface. Concomitantly, the
208 Δ(9,1) value decreased as the pH value was increased highlighting the increased electrostatic
209 repulsion between the negatively charge Peptide Number 9 and the negatively charged silica
210 surface. Retention of peptides number 1 and number 9 decreased as the mobile phase pH became
211 more alkaline (switch from a positive to negative net charge) whereas retention increased for
212 Peptide Number 26 which still retained an overall net positive charge even at pH >7. The elution
213 order of hydrophilic peptides was number 1 before number 9 at low pH (both displayed a net
214 positive charge) whereas at pH > 7 there was an elution switch where Peptide Number 9 eluted
215 before number 1 due to Peptide Number 9 now possessing a -5 charge compared to Peptide
216 Number 1 (-4 net charge) hence Peptide Number 9 was more hydrophilic and would also exhibit
217 greater electrostatic repulsion with the ionised silanol groups. Interestingly, the Δ(16,13), Δ(24,13),
218 Δ(8a,1) values all increased as the pH value was increased indicating an enhanced dominance of
219 these hydrophilic terms as the peptides carboxylic acid moieties are progressively deprotonated.
220 The steric parameter Δ(14,13) was shown to be greatest between the pH values of 3.6 and 5.1
221 indicating the importance of evaluating a range of pH values.

Fig. 2A highlights the fact that the greatest selectivity differences between the various additives was observed at low pH. As the mobile phase pH progressively increased in value, the selectivity differences observed between the additives diminished (i.e. a greater spread of mobile phases was seen at pH <2.8 compared to a tighter clustering of observations at pH >6.0). This can be rationalised by the reduced propensity for ion-pair formation at intermediate pH since all but Peptide Number 26 possessed negative or neutral net charges. While hydrophobic interactions typically dominate the RPC of peptides, additional types of interactions such as dipole : dipole and π : π interactions may be important for generating small differences in retention which can give rise to enhanced selectivity. The results suggest that pH should be a major parameter to be explored when optimising the selectivity of peptide separations.

A sample of Bovine GLP-2 (1-15) containing synthetic impurities was compared using the 20 mM IS buffers at pH 2.3, 3.6, 5.1 and 7.5. The chromatographic profiles in Supplementary Material Fig. S1illustrate the large differences in selectivity that can be obtained when different pH mobile phases are screened as part of a method development strategy. The identity of the peaks was not established as the purpose of the exercise was to illustrate selectivity differences; identification would have required 2D-LC-MS due the fact that the mobile phases contained involatile salts.

3.3 Effect of Ion-pair Reagent

It has been previously reported that peptides of differing hydrophobicity require differing ion-pair reagents for optimum separation [1, 5], for example the hydrophobic ion-pair reagents TFA and HFBA and the anionic chaotropic salt ClO_4^- have been reported to yield better separations for hydrophilic peptides *[1, 3, 5].* HFBA is an extremely effective ion-pairing reagent for enhancing the retention of hydrophilic peptides on C18 columns. Whereas the hydrophilic phosphate ion-pair reagent has been shown to be successful for hydrophobic peptides [1, 5]. This highlights the potential value of evaluating various ion-pair reagents / counterions with differing apparent hydrophobicities for the analysis of specific peptide separations. The effect of anionic and cationic ion-pairing reagents was assessed over the pH range 1.8 to 7.8 as in real-life situations there may be a range of peptides with various charged states.

The effect of the anionic (i.e. TFA, HFBA and $BuSO_3Na$) and cationic (i.e. TEA) ion-pair reagents of differing hydrophobicity on peptide selectivity, were compared against the absence of any ion-pairing reagent as a function of mobile phase pH. The chaotropic additives $NaClO_4$ and NH_4PF_6 were included as these also possess ion-pairing properties [34]. From the PCA biplot (Fig. 1) at pH 2.3 in

20 mM ammonium phosphate buffer there appeared to be minimal selectivity differences between the mobile phases (MP5 and 4) containing TFA or not respectively for this specific separation. In comparison, mobile phases containing $BuSO_3Na$ and HFBA (MP2 and 13 respectively) generated differing selectivity compared to TFA highlighting the importance of screening differing ion-pair reagents. The mobile phases containing the hydrophobic ion-pair reagents yielded longer retention times for all the peptides which carried a positive net charge (i.e. +1.1 to +3.4 at pH 2.3) resulting in a more hydrophobic ion-pair which interacts more strongly with the C18 stationary phase. The retention of the peptides was in line with the hydrophobicity of the ion-pair reagents (i.e. none < TFA < $BuSO_3Na$ < HFBA). The selectivity differences between HFBA and the absence of the ion-pair reagent was demonstrated on a sample of Bovine GLP-2 (1-15) containing several synthetic impurities (data not shown).

Comparison of the widely used 0.1% v/v TFA (MP11) mobile phase conditions at low pH, with those containing 0.1% v/v FA (MP1), 0.1% v/v H_3PO_4 (MP3) and 20 mM HFBA (MP14) highlighted significant selectivity differences. Since TFA can yield lower positive mode ESI signals in the MS, there has been a move towards using TFA in combination with FA (MP15) or replacing it with DFA (MP7). It can be observed from the PCA biplot, that there are small to moderate selectivity differences between these alternative approaches compared to TFA.

In general, it has been reported that the retention of positively charged peptides increases with the hydrophobicity of the ion-pair reagent, the number of positive charges on the peptide and the concentration of the ion-pair reagent. However, the magnitude of the increase was dependent on the hydrophobicity of the peptide, for example, more hydrophilic peptides exhibited larger retention time shifts than their corresponding hydrophobic analogues [C.T. Mant and R.S. Hodges, Context-dependent effects on the hydrophilicity/hydrophobicity of side-chains during reversed-phase high-performance liquid chromatography: Implications for prediction of peptide retention behaviour. J Chromatogr A. 2006. **1125**: p. 211-219]. This highlighted the importance of evaluating the hydrophobicity and/or concentration of these hydrophobic anionic ion-pair reagents when fine tuning the optimization of the separation of positively charged peptides.

Fig. 1 highlights that the chaotropic additives $NaClO_4$ (MP16) and NH_4PF_6 (MP18) which can also function as anionic ion-pair regents, yield markedly different selectivity profiles compared to the other ion-pair reagents evaluated. Hodges *et al* have previously observed that the anion effectiveness in forming ion-pairs followed the trend of Cl^- << TFA^- < ClO_4^- [3] and PO_4^- < TFA^- < $HFBA^-$ [5] and that this mirrors the retention of the ion-pairs formed with peptides. The chloride anion is hydrophilic and simply neutralises the positive charge on the peptide thus reducing the peptide's

overall hydrophilicity resulting in an increased retention on RP stationary phases. The results at pH 2.3 in 100 mM phosphate indicate that the retention of the peptides in this study follow the elution order $SO_4^{2-} < PO_4^- \approx Cl^- << ClO_4^- < PF_6^-$ which mirrors the Hofmeister series which is a classification of the ion's ability to influence the structuring of water [35, 36]. Hodges *et al* suggested that ClO_4^- is a more effective ion-pair reagent than TFA in increasing the peptide's hydrophobicity due to the strong chaotropic character (i.e. water structure breaking) of the ClO_4^- anion which competes less effectively for the nearby water molecules than does the bulk water and is, therefore, dehydrated more readily than ions such as Cl^- and TFA^- [3]. It has been suggested that the formation of ion-pairs requires the exclusion of water molecules from the interaction between the positive and negatively charged species (i.e. anions must be dehydrated to form ion-pairs with the protonated amino functionalities of the peptide) [3]. Hodges *et al* stated that the ClO_4^- anion is more readily dehydrated than TFA^- anion and this may partially explain the greater effectiveness of ClO_4^- anion as an ion-pair reagent compared to the TFA^- anion even though the latter is more hydrophobic [3].

Hodges *et al* have suggested that TFA^- anion neutralises the positive charge associated with the peptide and, with its increased hydrophobic nature, augments the hydrophobicity of the peptides whereas the ClO_4^- anion is a more effective ion-pair reagent than TFA even though the latter is more hydrophobic [3].

NH_4PF_6 (MP18) which has recently shown promise in the analysis of small basic analytes [34] generated the largest selectivity differences of the anionic ion-pair reagents evaluated yielding enhanced retention for the +3.4 charged Peptide Number 26 compared to $NaClO_4$. Interestingly, NH_4PF_6 could only be characterised at pH 2.3, due to its very high UV absorbance at pH values $\geq$3.6 (MP48-50) rendering it chromatographically impractical to use except at low pH. As expected, the anionic ion-pair reagents TFA, $NaClO_4$ and $BuSO_3Na$ at pH 7.5 exhibited similar selectivity as shown in the PCA biplot (Fig. 1) since there was less ion-pair formation than at low pH. In acidic environments all the peptide probes possessed a significantly larger amount of positive than negative charge, this facilitated ion-pair formation with the anionic ion-pair reagents, whereas at intermediate pH there is a mixture of positive and negative charges on the peptide. This generated an overall neutral or negatively charged peptide surface which was less likely to interact with the negatively charged ion-pair reagent. In addition, any negative charge on the peptide would presumably repel the anionic ion-pair reagents hence reducing ion-pair formation [37]. This was emphasised with MP51 containing the hydrophobic HFBA anionic ion-pair reagent at pH 6.8 in that the hydrophilic peptides (Peptide Number 1, 8 and 9, net charge of -4 to -5) eluted in the void volume due to their electrostatic repulsion with the HFBA anions adsorbed onto the C18 surface. The location of the charged functionality on the peptide and hence its accessibility and ability to form ion-pairs or

undergo interactions with other ionic species may be as important as the overall net charge of the peptides in controlling retention (See Table 1). HFBA, which has been successfully reported to be a viable ion-pair reagent for the separation of peptides [17, 30, 38-40], yielded different selectivities compared to mobile phases without any ion-pair reagents at pH values between 2.3 and 5.1, as indicated by their position within the score plot (Fig. 2B). This was verified with a sample of Bovine GLP-2 (1-15) which contained synthetic impurities (data not shown). HFBA is not as widely used as TFA since it is known to cause memory effects and noisier UV baselines in LC systems where it has been used, necessitating significant cleaning of LC/MS instrumentation [41]. Memory effects [Ref M.C. García-Alvarez-Coque, G. Ramis-Ramos and M.J. Ruiz-Angel, in Encyclopedia of Analytical Science (Third Edition), p117-126 2015] are due to the very strong affinity of these hydrophobic ion-pairs to hydrophobic stationary phases. In principle, they can be removed by washing with organic solvent, however, in practice the initial properties of the column may have been permanently changed and hence the column cannot be regenerated back to its original state. Typically, this necessitates that once a column has been exposed to an ion-pair reagent it should be dedicated to that specific analysis. There are also potential issues with perfluorinated additives in that LC components (i.e. degasser tubing) that contain Teflon AF (amorphous fluoropolymer) may be affected over time resulting in physical changes to the polymer and contamination the mobile phase [42, 43].

The cationic ion-pair reagent TEA (MP28) generated a different selectivity profile to the anionic ion-pair reagents (MP31, 32 and 33) at pH 5.1. However, the greatest difference in selectivity between the ion-pair reagents evaluated was observed at low pH between the anionic ion-pair reagents NH_4PF_6 and the $BuSO_3Na$ (Fig 2B), where all peptides evaluated possessed a +1.1 to +3.4 overall charge at pH 2.3. The coordinates of each observation (i.e. mobile phase) when plotted in Fig. 2B highlighted that there was a converging trend from low to intermediate pH (Fig. 2B). All the ion-pair reagents behaved in a similar manner, as expected, where there was a greater difference in selectivity at low pH, which narrowed at intermediate pH due to the lack of ion-pair formation as most the peptides exhibited neutrality or a negative net charge.

The use of methanesulfonic acid (MSA) as an additive for small molecules and large biomolecules such as monoclonal antibodies in reversed-phase chromatography has increased due to its promising alternative selectivity [30, 31], UV transparency and MS compatible. McCalley suggested that MSA could offer different selectivity compared to TFA and ammonium salts [30]. It was also expected that MSA would exhibit minimal ion-pairing effects and could be advantageous over TFA. There are, however, potential issues regarding its corrosion of metal components within the LC system [44-46], therefore rinsing is highly recommended after use to prevent component damage

[45]. Fig. 1 highlights that MSA (MP17) generates differing selectivity profiles compared to the commonly used acidic mobile phase additives with the peptides used in the characterisation. Interestingly, MSA resulted in enhanced retention of the peptides, the order of retention was as follows: FA << TFA << MSA < HFBA, suggesting possible ion-pair formation with MSA.

There appeared to be no difference between the asymmetry values obtained for an overloaded sample of Bovine GLP-2 (1-15) over the pH range 1.9 to 7.8 when either cationic or anionic ion-pair reagents were compared. In the absence of ion-pair reagents larger asymmetry values were observed especially at intermediate pH.

3.4 Effect of (Kosmotropic and Chaotropic) Salt

The kosmotropic (i.e. SO_4^{2-} ion) and chaotropic (i.e. PF_6^- and ClO_4^-) salts are water structure making and breaking respectively, where they are known to affect the solvation shell of peptides and proteins. This affects the way in which the peptides interact with the stationary phase. Both kosmotropic and chaotropic salts follow the Hofmeister series [11, 12], which describes the minimum concentration required to salt out proteins [47-52]. As shown in section 3.3, both the chaotropic salts PF_6^- and ClO_4^-, which are also anionic ion-pair reagents yielded interesting selectivities at low pH. The position of the chaotropic agents (ClO_4^- and PF_6^- (i.e. MP16 and 18 respectively)), which are also anionic ion-pair reagents, within the score plot (Fig. 1 and Fig. 2C) suggests a much greater degree of selectivity differences compared to the kosmotropic SO_4^{2-} and Cl^- salts (i.e. MP9 and 10). The two kosmotropic salts assessed, $(NH_4)_2SO_4$ and Na_2SO_4 (MP8 and 9 respectively), demonstrated very little selectivity differences between them, which suggested that the cation had minimal effect on selectivity. In general, good peak shapes were obtained using SO_4^{2-} additives, however, significant tailing was observed (see Fig. 3B) for three of the nine peptides using a mobile phase containing SO_4^{2-}. In comparison, excellent peak shape was observed when no additional salt or NaCl was added (Fig. 3A and C respectively, 100 mM total IS). The poor shape observed for the three Peptide Numbers 16, 24 and 26 could be attributed to “salting out effects” which are not related to the overall net charge of the peptide.

The ClO_4^- chaotropic salt offered marked selectivity differences, although, the use of ClO_4^- is not recommended due to health and safety and environmental considerations. Nevertheless, it could be useful as an alternative mobile phase additive if critical species are problematic to resolve. The PF_6^- additive also offered an even larger difference in selectivity and does not exhibit explosive properties. PF_6^- clearly is an interesting additive, however, there is currently limited experience of its

long term use, there is also the potential risk of PF_6^- generating HF in aqueous solutions, which may cause ligand cleavage of the stationary phase [53].

3.5 Effect of ionic strength on peak shape

Addition of salt to the mobile phase was intended to suppress the electrostatic interactions between the negatively charged silanol groups on the silica surface and the positively charged peptides, hence improving the peak shape. The effect of salts was evaluated using a buffer at 20 mM IS with a total IS of 100 mM provided by the addition of salt. This was compared against 100 mM IS buffers without salt. IS has been shown to be crucial for chromatographic performance [15, 54-57]. Mass overloading of analytes on columns generates asymmetric / tailing peaks due to a mixture of more than one retention mechanism. For charged analytes like peptides it is often due to a mutual repulsion which increases as the concentration of the peptide increases in the stationary phase. By increasing the IS the mutual repulsion is reduced, as described by McCalley *et al* [57].

The peptides employed in the characterisation protocol [29] were chromatographed in their non-overloaded state, thus a comparison of peak capacity and asymmetry has little impact. Therefore, a comparison of an overloaded sample of Bovine GLP-2 (1-15) was used to highlight the advantages of using an increased IS on peak shape. The asymmetry values for the overloaded Bovine GLP-2 (1-15) sample using the 100 mM IS kosmotropic and chaotropic salts ranged between 0.90 and 2.69 (average 1.40) which indicates an improvement compared to the 20 mM IS mobile phases (0.99 to 4.21, average 2.82). The mobile phase additives specified in Table 2 were only prepared in A solvent, thus the actual IS experienced in the column outlet at the point of elution was lower (i.e. 30%). The commonly used additives 0.1% v/v FA, 0.1% v/v TFA and 20 mM IS TFA were also evaluated (Fig. 4). Within the pharmaceutical industry, phosphate buffers are frequently used instead of TFA, as the peak shape is often superior, improved UV baselines with concomitant lower quantification limits being observed. The overloaded Bovine GLP-2 (1-15) sample exhibited substantial tailing with ionic strength mobile phases below 10 mM, in particular 0.1% v/v FA. The asymmetry improved with 0.1% v/v TFA and 20 mM TFA, however, the peak possessed tailing of greater than 2.0. Mobile phases with greater than 20 mM IS during elution of the peak generally produced near Gaussian peaks, illustrating the need for increased IS for the chromatographic analysis of this peptide. The type of salt did not appear to have much influence on the symmetry of the peak. This corroborates the findings reported by Hodges' *et al*. who observed improved peak shape with mobile phases containing 50 mM NaCl and $NaClO_4$ salts [15]. The improved peak shape was illustrated in Fig. 5

which compared the effect of pH 2.5 0.1% v/v FA and pH 3.6 20 mM NH_4FA / FA mobile phases on the peak shape of a sample of Bovine GLP-2 (1-15) containing synthetic impurities.

In comparison to the frequently used ammonium phosphate, sodium or ammonium sulfate (Fig. 3B), with their potential problems of salt precipitation at elevated organic levels and / or precipitation of the peptides, it has been demonstrated that NaCl (Fig. 6C) could be a viable alternative to the use of sulfate or phosphate as it produced improved peak shapes at 100 mM IS compared to the sulfate salts and better solubility than phosphate. Caution must however be exercised in the use NaCl at low pH as it may be converted to HCl which could potentially be corrosive to the LC system and limit its applicability.

3.6 Effect of Stationary Phases on Mobile Phase Characterisation

Six diverse mobile phases which exhibited moderate to good selectivity differences in the PCA biplot (Fig. 1) with the Ascentis Express C18 column were evaluated on three additional chromatographically dissimilar stationary phases, the Ascentis Express Biphenyl, Polaris Amide C18 and the Acquity CSH Fluoro Phenyl, which were shown to exhibit large selectivity differences in the Peptide RPC Column Characterisation Protocol [29]. The six differing mobile phases varied between pH 1.9 and 7.5, different ionic strengths and the absence or presence of ion-pair reagents. 0.1% v/v FA pH 2.5 (MP1) and 0.1% v/v TFA, pH 1.9 (MP11) were selected as these are probably the most commonly employed low pH mobile phase additives for peptide, the latter being an ion-pair reagent suitable for enhancing retention. The MS compatible and non-compatible mobile phases, 20 mM NH_4FA pH 6.5 (MP42) and $NH_4H_2PO_4$ / $(NH_4)_2HPO_4$ pH 7.5 (MP44) respectively, were chosen as they represent common intermediate pH additives. A mobile phase containing the ion-pairing reagent 20 mM AA / NH_4AA / $BuSO_3Na$ pH 5.1 (MP 32) was included as it exhibited selectivity differences. The low pH mobile phase 100 mM H_3PO_4 / $NH_4H_2PO_4$ / $(NH_4)_2SO_4$ pH 2.3 (MP8) was additionally included as experience at Novo Nordisk has shown that it often yields better peak shape and selectivity than the universally used TFA.

The selectivities of the differing mobile / stationary phase combinations were compared against the previously determined Ascentis Express C18 to ascertain their similarities in the score plot (i.e. can the results and conclusions for the Ascentis Express C18 be applied to a greater range of stationary phases). The Ascentis Express Biphenyl (see Supplementary material Fig. S2B) and Acquity CSH Fluoro Phenyl (see Supplementary material Fig. S2C) presented a similar pattern to the Ascentis Express C18 (see Supplementary material Fig. S2A) for the mobile phases evaluated. This suggested

that neutral, negative / polar and weakly positive stationary phases respond similarly as they generated a similar pattern within the score plots. MP32 (pH 5.1 20 mM AA / NH_4AA / $BuSO_3Na$) did not behave similarly, such a deviation could be due to how the ion-pair reagent interacts with either the C18, biphenyl or propyl pentafluorophenyl ligands attached to the surface of the stationary phase [58-60]. This may alter how the peptides would interact with the ion-pair reagent and stationary phase, thereby potentially offering different selectivity profiles. Delta values for MP44 in Supplementary material Fig. S2C (i.e. Acquity CSH Fluoro Phenyl phase at pH 7.5) could not be obtained due to the fact that the hydrophilic peptides (Peptide Numbers 1, 8 and 9, net charge of -4 to -5) eluted in the void volume as a result of their electrostatic repulsion with the excess of negatively charged silanol groups on this low retentive stationary phase's surface. Despite the $BuSO_3Na$ mobile phase result, it is an encouraging observation that neutral, negative / polar and weakly positive stationary phases behave in a similar manner which indicates that these results should be transferable to a wider array of commercially available columns of these classifications.

The pattern in the score plot (see Supplementary material Fig. S2D) for the Polaris Amide C18, which possessed a high positive character, indicated that there was no correlation between this type of phase and the neutral Ascentis Express C18. The Polaris Amide C18 phase was additionally observed to generate large selectivity differences using the Peptide RPC Column Characterisation Protocol [29], thus, it was reasonable to expect that this stationary phase would behave differently with the range of mobile phases compared to the C18 column.

Contribution plots (data not shown) of MP42 (pH 6.5) *versus* MP1 (pH 2.5) as a function of the four stationary phases highlighted the similarity between the response of the C18, biphenyl and fluoro phenyl phases. The later three exhibited greater hydrophilic (*Δ*(8a,1), *Δ*(16,13), *Δ*(24,13)]) positive electrostatic interaction (*Δ*(26,13)) and a lower negative electro repulsive descriptors ((*Δ*9,1)) at pH 6.5 compared to pH 2.5 due to the increase ionisation of the silica surfaces. In comparison the positively charged Polaris Amide stationary phase exhibited lower electrostatic interaction (*Δ*(26,13)) and hydrophilic (*Δ*(8a,1), *Δ*(16,13), *Δ*(24,13)) descriptors due to the positive charge on the phase offsetting the increased negative charge of the ionised silanol groups at pH 6.5.

This limited evaluation of stationary phases indicated that the results from the Ascentis Express C18 could be applied to the group of columns consisting of neutral, negative / polar and weakly positive stationary phases, i.e. majority of commercially available columns. However, where the stationary phase offered vast selectivity differences, the mobile phase characterisation results are less applicable.

3.7 MS Response

The intensity of the MS signals for Peptide Number 1 (Bovine-GLP-2 (1-15)) in the positive mode electrospray ionisation (ESI) was assessed as a function of a range of volatile mobile phase additives shown in the Supplementary material Table S1. The intensity of the MS signal ranged between 6E+02 to 2E+05, highlighting a large disparity in MS signal intensity between the differing mobile phase additives. Mobile phases corresponding to pH 2.5 0.1% v/v FA (MP1) and pH 7.8 20 mM FA / NH_4HCO_3 / TEA (MP37) gave the maximal and minimal MS signal intensities respectively. A significant number of the volatile mobile phases gave %MS responses (compared to FA (MP1)) of between 0 - 10%, which included some of the typically employed MS additives such as pH 6.5 20 mM NH_4FA (MP42), pH 7.0 20 mM NH_4AA (MP36) and pH 7.8 20 mM NH_4HCO_3 (MP46). The ubiquitously employed pH 1.9 0.1% v/v TFA (MP11) yielded only a 20% response compared to FA, this is in line with previous reports [39]. Replacing 50% of the TFA with FA in the mobile phases (MP15) partially rectified this reduced positive ESI sensitivity [39, 61]. DFA has been historically avoided as an additive in LC/MS due to poor purity and significant metal content [62], however, due to enhanced production processes, the quality of DFA (MP7) has recently improved, making it a viable alternative to TFA (MP11) [63]. Under the test conditions pH 1.9 13 mM 0.1% v/v DFA (MP7) yielded a 50% response (2.5 times higher than TFA). Interestingly, the infrequently used pH 1.9 0.1% v/v MSA (MP17) yielded a reasonable MS response of 15% compared to FA. The adduct formation was considered negligible for all additives.

The volatile ion-pairing reagents TEA (MP21, 28, 31 and 37) and HFBA (MP14, 26 and 33) containing mobile phases produced poor MS signal responses with this peptide, which could prohibit their use for low impurity measurements. HFBA also caused memory effects and necessitated significant cleaning of the MS instrument [41].

Caution must be taken when extrapolating these findings to other peptides as the MS response is highly analyte and MS operating conditions dependent. Formic acid is typically stated as the "*gold standard*" for the generation of high sensitivity positive mode ESI-MS but unfortunately, exhibits poor peak shape, in comparison to TFA or phosphate buffers [57]. A significant reduction in the MS response was observed when TFA was employed suggesting that DFA should be considered instead of TFA as the reduction in MS signal is not so pronounced. The results indicate that the volatile mobile phase additives possessing differing selectivity properties over the pH range should generate acceptable MS responses comparable to the commonly used 0.1% TFA.

4. Conclusions

The chemometric tool of PCA has been employed to visualise the large differences in selectivity that can be generated between various mobile phases. The results highlight the importance of screening several mobile phases of differing pH and ion-pair reagents in order to maximise the probability of achieving separation of all the peptides of interest within a complex mixture. PCA demonstrated that for this specific range of peptides, the use of ion-pair reagents generated large selectivity differences when they were employed at a suitable pH which facilitated ion-pair formation. The PCA score plot (Fig. 2) and Euclidian distances (data not shown) highlighted that the anionic ion-pair reagents HFBA, ClO_4^- and PF_6^- had the largest impact of the ion-pair reagents on selectivity at low pH. The PCA score plot and Euclidian distances for the four differing columns and six differing mobile phases (Fig. S2) intimates the relative importance of mobile phase parameters on selectivity to be: pH and column type > pH > column type > ion-pair reagent (however, if more diverse ion-pair reagents such as HFBA, ClO_4^- and PF_6^- had been included then a greater importance to the ion-pair reagent would have been observed).

The exploitation of mobile phases with differing selectivity profiles was proven in the analysis of a sample of Bovine GLP-2 (1-15) containing synthesis related impurities. Mobile phases with high ionic strength were demonstrated to be crucial for the generation of symmetrical peaks. The observations made on the C18 phase were compared on three additional stationary phases (i.e. alkyl amide, fluorophenyl and biphenyl), which had previously been shown to possess large selectivity differences towards these peptides, on a limited sub-set of mobile phases. With the exception of the ion-pair reagent $BuSO_3Na$, similar trends were obtained for the C18, fluorophenyl and biphenyl phases suggesting that these findings are applicable to the vast majority of RPC columns (i.e. neutral or weakly polar in character) which are suitable for the analysis of peptides. The conclusions were not relevant for columns with a more disparate nature (i.e. containing a high degree of positive charge).

The findings from this work, in combination with the stationary phase characterisation study, [29], will hopefully assist in the definition of method development strategies for RPC peptide separations. The manuscript is intended to provide the analyst with a quick and simple visualisation of the similarity / dissimilarity of mobile phases for method development selection purposes and it is not the intention to state which is the best mobile phase composition as this will be unique to a specific peptide application and it is, of course, the responsibility of the analyst to verify the best mobile phase additives for their specific peptide separation.

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CRediT authorship contribution statement

Jennifer K. Field: Methodology, Formal analysis, Investigation, Writing - original draft. Melvin R. Euerby: Conceptualization, Methodology, Writing - review & editing, Supervision. Kim F. Haselmann: Formal analysis, Investigation. Patrik Petersson: Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing, Supervision, Funding acquisition.

Figure legends

Fig. 1 Biplot of the mobile phase additives. The observations were colour coded based on pH and observed a trend of low pH to intermediate pH along the first principal component, in line with the electrostatic variables $\Delta(9,1)$ and $\Delta(26,13)$. The second principal component was attributed to steric interactions $\Delta(14,13)$ and $\Delta(15,13)$. MP48-50 could only be characterised at pH 2.3, due to its very high UV absorbance at pH values ≥3.6 (see Sec. 3.3).

Fig. 2 Comparison of (A) pH (B) ion-pair and (C) kosmotropic and chaotropic salts. Simplified versions of the biplot shown in Fig. 1. The green region denotes the pH <2.5, purple region denotes pH 3.6, orange area denotes pH 5.1 and the blue region denotes pH >6.0.

Fig. 3 Comparison of the characterisation probes using (A) MP25 100 mM H_3PO_4 / $NH_4H_2PO_4$, (B) MP9 100 mM H_3PO_4 / $NH_4H_2PO_4$ / Na_2SO_4, and (C) MP10 100 mM H_3PO_4 / $NH_4H_2PO_4$ / NaCl.

Fig. 4 Comparison of the asymmetry of an overloaded Bovine GLP-2 (1-15) sample *versus* ionic strength at column outlet at the point of elution for different mobile phase additives. The plot highlights the poor symmetry of the peptide using the commonly used additives 0.1% v/v FA or TFA, and that with increased ionic strength the asymmetry significantly improves. Results at 70 mM (i.e. NH_4HPO_4, $(NH_4)_2SO_4$, Na_2SO_4 and NaCl) indicate that the type of salt is less important than IS.

Fig. 5 Chromatograms of a sample of Bovine GLP-2 (1-15) containing synthetic impurities using either pH 2.5 0.1% v/v FA (blue trace, MP1.) or pH 3.6 20 mM NH_4FA / FA (red trace, MP23.) to illustrate the effect of increased ionic strength on peak shape. There are significant improvements in chromatographic performance using the higher ionic strength conditions.

Supplementary material figure legends

Fig. S1 Comparison of the UV chromatographic profile of Bovine GLP-2 (1-15) and synthetic impurities sample using the 20 mM IS base buffers on the Ascentis Express C18. (A) MP4 pH 2.3 20 mM H_3PO_4 / $NH_4H_2PO_4$, (B) MP23 pH 3.6 20 mM FA / NH_4FA, (C) MP29 pH 5.1 20 mM AA / NH_4AA and (D) MP44 pH 7.5 20 mM $NH_4H_2PO_4$ / $(NH_4)_2HPO_4$.

Fig. S2 Score plot comparing the effect of stationary phase on six chromatographically diverse mobile phases. Mobile phase MP1 pH 2.5 0.1% v/v FA, MP8 pH 2.3 100 mM H_3PO_4 / $NH_4H_2PO_4$ / $(NH_4)_2SO_4$, MP11 pH 1.9 0.1% v/v TFA, MP32 pH 5.1 20 mM AA / NH_4AA / $BuSO_3Na$, MP42 pH 6.5 20 mM NH_4FA, MP44 pH 7.5 20 mM $NH_4H_2PO_4$ / $(NH_4)_2HPO_4$. NB. Delta values for MP44 could not be obtained for the Acquity CSH Fluoro Phenyl phase due to the hydrophilic peptides eluting in the void volume.

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Electronic Supplementary Material

Rationale for Mobile Phases' selection

Typically, the buffering capacity was greater than 5 mM / pH in order to provide sufficient capacity to resist significant changes to the mobile phase pH. pH 1.8 – 7.8 was investigated as this range is typically employed in many laboratories. While alkaline pH conditions may appear attractive, they may result in numerous problems such as the deamidation of asparagine, disulfide shuffling (i.e. the breaking of the disulfide bond in cystine which can then undergo reformation of new disulfide bridges [33]) and the decreased longevity of many RP stationary phases.

The protocol that had been reported for the characterisation of 38 commercially available stationary phases [29] was applied to 51 differing mobile phase conditions. Selectivity (referred to as delta, Δt_g^*) was determined by the normalised retention time differences between two probes of interest [27, 28]. The selectivity was measured for changes in negative charge (Δ(9,1)), changes in positive charge (Δ(26,13)), oxidation (Δ(8a,1)), racemisation (Δ(14,13)), steric (Δ(15,13)), aromatic (Δ (16,13)) and the phenolic character (Δ(24,13)) of the peptide probes.

The characterisation was performed on columns from a single batch of a representative new generation “low acidity” C18 material to remove any variation caused by the stationary phase. Columns were dedicated to specific ion-pair reagents to avoid any potential memory effects (i.e. irreversible binding of the ion-pair reagent to the stationary phase) compromising the validity of the results.

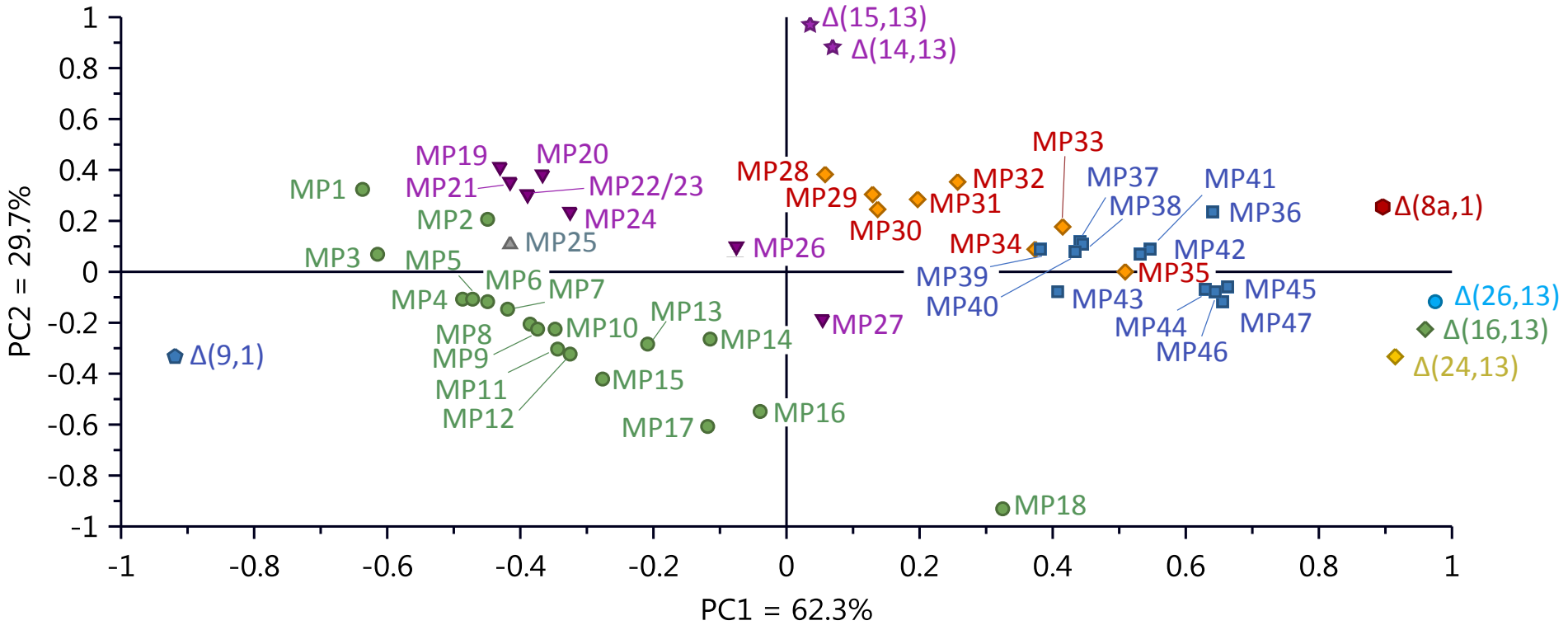
Ideally, the concentration of additive should be the same in both the aqueous (A) and MeCN / H_2O (80:20 v/v) (B) mobile phase reservoirs to avoid any pH / ionic strength changes throughout the gradient and to obtain a more horizontal UV baseline. However, due to practical limitations such as solubility and the number of parameters / mobile phases required for the study, additives were omitted from the B solvent. The gradient employed was the same as in the original Peptide RPC Column Characterisation Protocol [29] with respect to the rate of change of %MeCN/min. MeCN is the primary organic solvent used for peptide separations due to its physicochemical properties such as low UV cut off (ideal for peptides monitored using 215 nm), low viscosity and low pressure drop.

The mobile phases were characterised using the peptide probes described in reference [28, 29], in conjunction with an evaluation of the peak shape and chromatographic performance for the peptide probes as well as an overloaded sample of Bovine GLP-2 (1-15) containing synthetic impurities for mobile phases of interest.

807 The mobile phases were standardised at pH values of 1.8-2.3, 5.1 and 7.5-7.8 with a contribution
808 from the buffer components to the total ionic strength (IS) of 20 mM in the aqueous solvent which
809 should maintain the ionisable functionalities in predominantly the protonated or deprotonated state
810 (i.e. >90%, the exception was the C-terminal amino acid). Lower pH values were not used as most
811 commercially available stationary phases did not recommend these conditions due to the potential
812 of ligand hydrolysis. Ammonium phosphate and formate pH 3.1 and 3.6 respectively were also
813 included as experience has shown them to provide alternative / beneficial selectivities [Petersson,
814 Novo Nordisk personal communication].

815

Fig 1

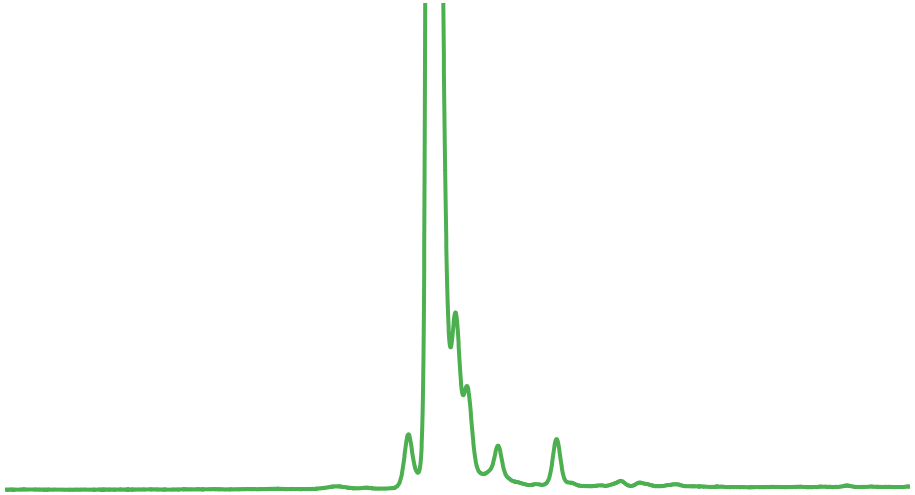


MP1 pH 2.5 0.1% v/v FA
MP2 pH 2.3 20 mM H_3PO_4/$NH_4H_2PO_4$/$BuSO_3Na$
MP3 pH 2.2 0.1% v/v H_3PO_4
MP4 pH 2.3 20 mM H_3PO_4/$NH_4H_2PO_4$
MP5 pH 2.3 20 mM H_3PO_4/$NH_4H_2PO_4$/TFA
MP6 pH 2.3 100 mM H_3PO_4/$NH_4H_2PO_4$
MP7 pH 1.9 0.1% v/v DFA
MP8 pH 2.3 100 mM H_3PO_4/$NH_4H_2PO_4$/$(NH_4)_2SO_4$
MP9 pH 2.3 100 mM H_3PO_4/$NH_4H_2PO_4$/Na_2SO_4
MP10 pH 2.3 100 mM H_3PO_4/$NH_4H_2PO_4$/NaCl
MP11 pH 1.9 0.1% v/v TFA
MP12 pH 1.8 20 mM TFA
MP13 pH 2.3 H_3PO_4/$NH_4H_2PO_4$/HFBA
MP14 pH 1.8 20 mM HFBA
MP15 pH 2.2 0.05% TFA/0.05% FA
MP16 pH 2.3 100 mM H_3PO_4/$NH_4H_2PO_4$/$NaClO_4$
MP17 pH 1.9 0.1% v/v MSA
MP18 pH 2.3 100 mM H_3PO_4/$NH_4H_2PO_4$/NH_4PF_6
MP19 pH 3.6 100 mM FA/NH_4FA/$(NH_4)_2SO_4$
MP20 pH 3.6 20 mM FA/NH_4FA/$BuSO_3Na$
MP21 pH 3.6 20 mM FA/NH_4FA/TEA
MP22 pH 3.6 20 mM FA/NH_4FA/TFA
MP23 pH 3.6 20 mM FA/NH_4FA
MP24 pH 3.6 100 mM FA/NH_4FA/NaCl
MP25 pH 3.1 100 mM H_3PO_4/$(NH_4)_2HPO_4$
MP26 pH 3.6 20 mM FA/NH_4FA/HFBA
MP27 pH 3.6 100 mM FA/NH_4FA/$NaClO_4$
MP28 pH 5.1 20 mM AA/NH_4AA/TEA
MP29 pH 5.1 20 mM AA/NH_4AA
MP30 pH 5.1 100 mM AA/NH_4AA /$(NH_4)_2SO_4$
MP31 pH 5.1 20 mM AA/NH_4AA/TFA
MP32 pH 5.1 20 mM AA/NH_4AA/$BuSO_3Na$
MP33 pH 5.1 20 mM AA/NH_4AA/HFBA
MP34 pH 5.1 100 mM AA/NH_4AA/$NaClO_4$
MP35 pH 5.1 100 mM AA/NH_4AA/NaCl
MP36 pH 7.0 20 mM NH_4AA
MP37 pH 7.8 20 mM FA/NH_4HCO_3/TEA
MP38 pH 7.5 20 mM $NH_4H_2PO_4$/$(NH_4)_2HPO_4$/TEA
MP39 pH 7.5 100 mM $NH_4H_2PO_4$/$(NH_4)_2HPO_4$/NaCl
MP40 pH 7.5 100 mM $NH_4H_2PO_4$/$(NH_4)_2HPO_4$
MP41 pH 7.5 20 mM $NH_4H_2PO_4$/$(NH_4)_2HPO_4$/TFA
MP42 pH 6.5 20 mM NH_4FA
MP43 pH 7.5 100 mM $NH_4H_2PO_4$/$(NH_4)_2HPO_4$/$(NH_4)_2SO_4$
MP44 pH 7.5 20 mM $NH_4H_2PO_4$/$(NH_4)_2HPO_4$
MP45 pH 7.5 20 mM $NH_4H_2PO_4$/$(NH_4)_2HPO_4$/$BuSO_3Na$
MP46 pH 7.8 20 mM NH_4HCO_3
MP47 pH 7.5 100 mM $NH_4H_2PO_4$/$(NH_4)_2HPO_4$/$NaClO_4$

Fig. 1S

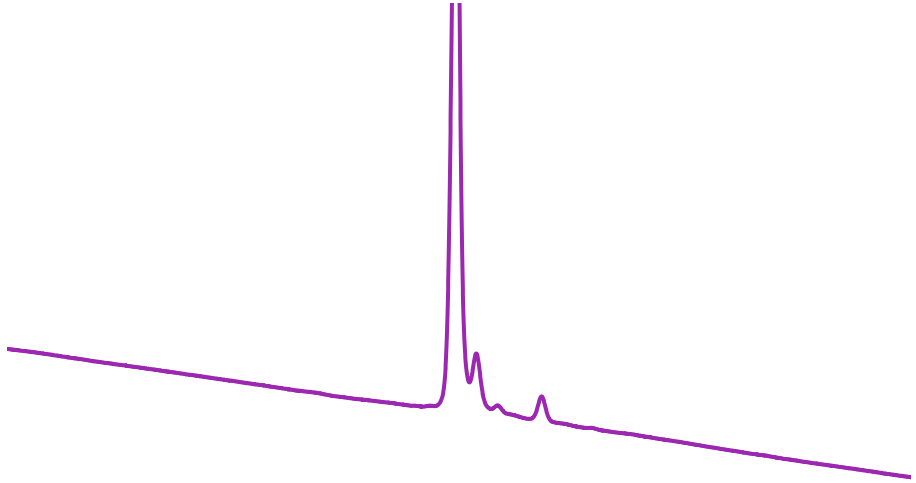
A

MP4 pH 2.3 20 mM $H_3PO_4/NH_4H_2PO_4$



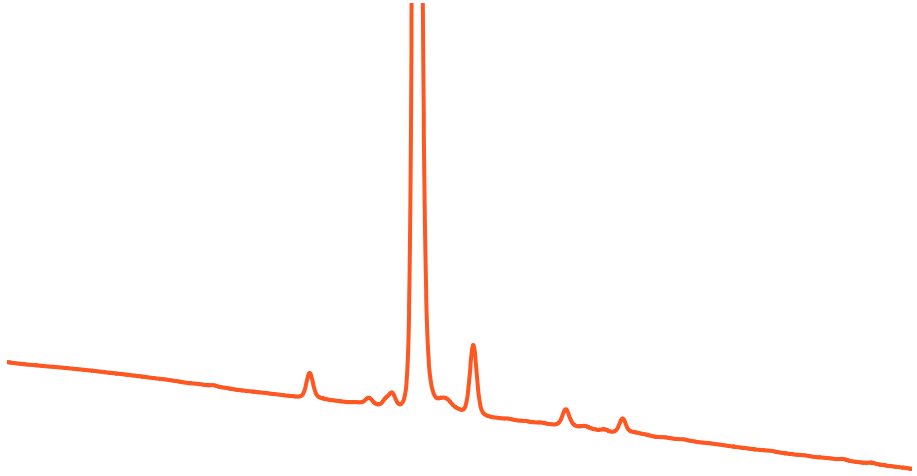
B

MP23 pH 3.6 20 mM FA/NH_4FA



C

MP29 pH 5.1 20 mM AA/NH_4AA



D

MP44 pH 7.5 20 mM $NH_4H_2PO_4/(NH_4)_2HPO_4$

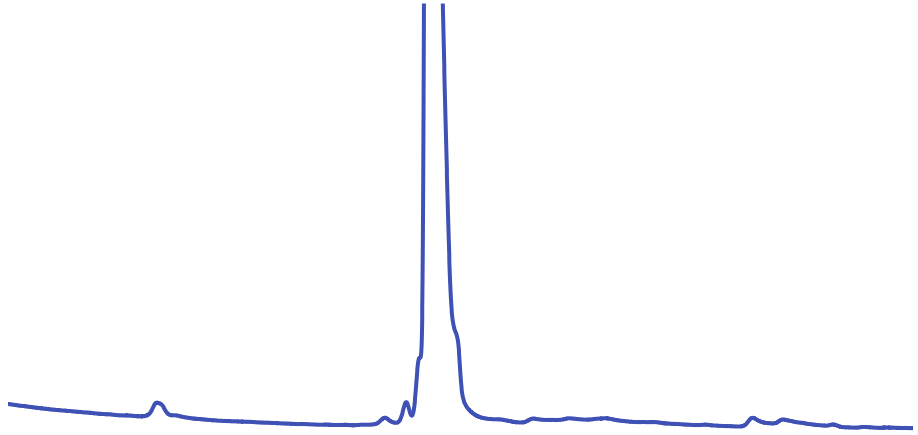
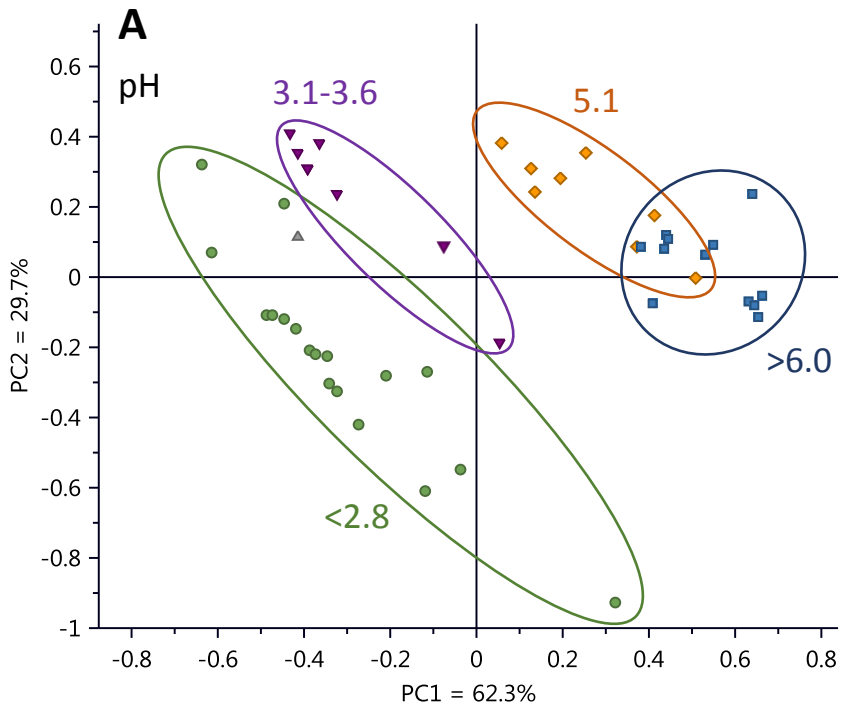
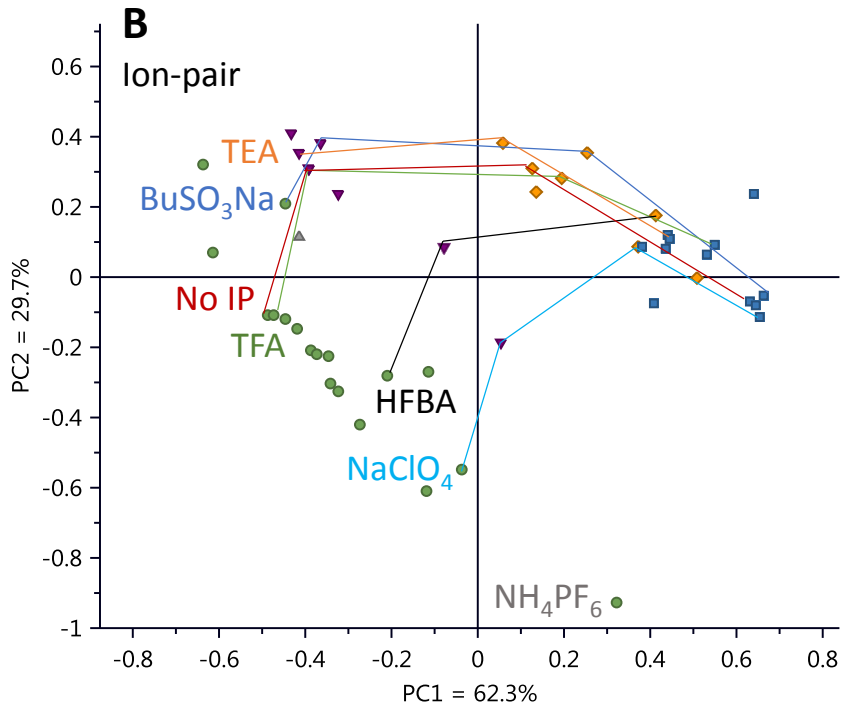
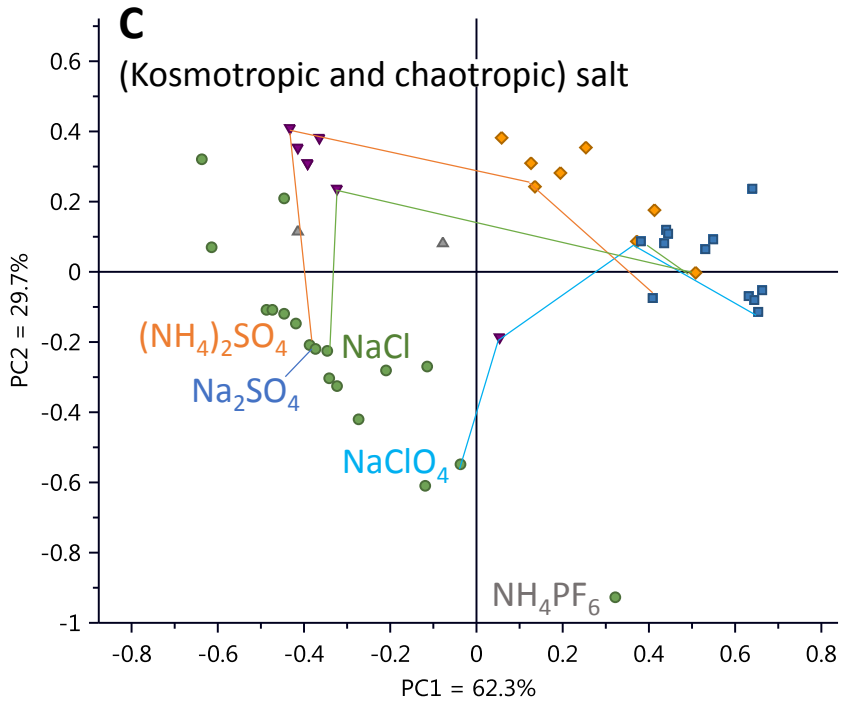


Fig.2


A
pH
3.1-3.6
5.1
>6.0
<2.8
PC2 = 29.7%
PC1 = 62.3%


B
Ion-pair
TEA
BuSO3Na
No IP
TFA
HFBA
NaClO4
NH4PF6
PC2 = 29.7%
PC1 = 62.3%


C
(Kosmotropic and chaotropic) salt
(NH4)2SO4
NaCl
Na2SO4
NaClO4
NH4PF6
PC2 = 29.7%
PC1 = 62.3%

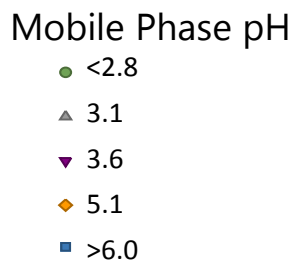
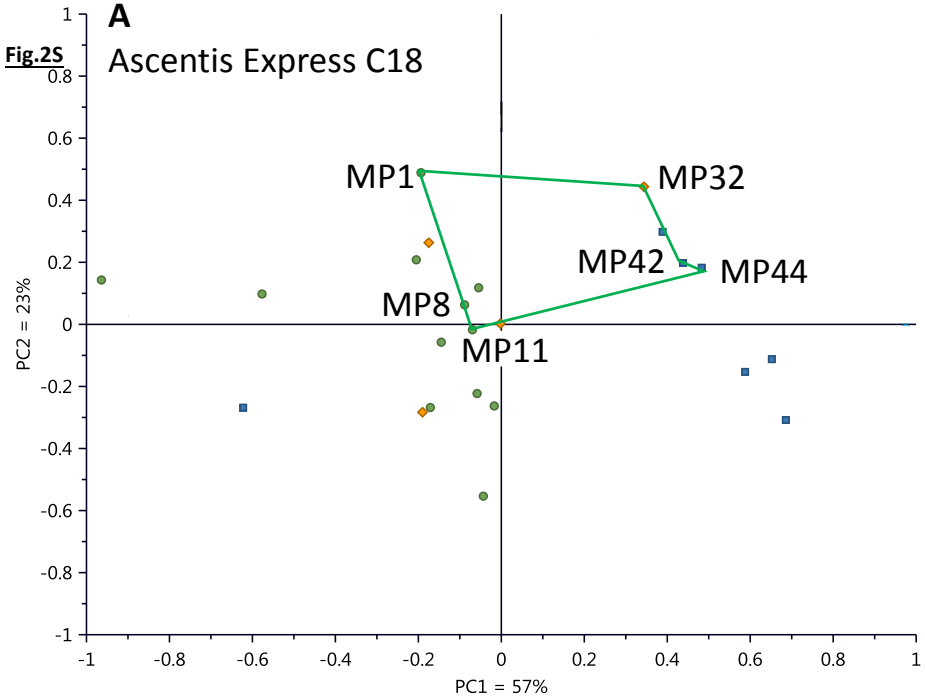
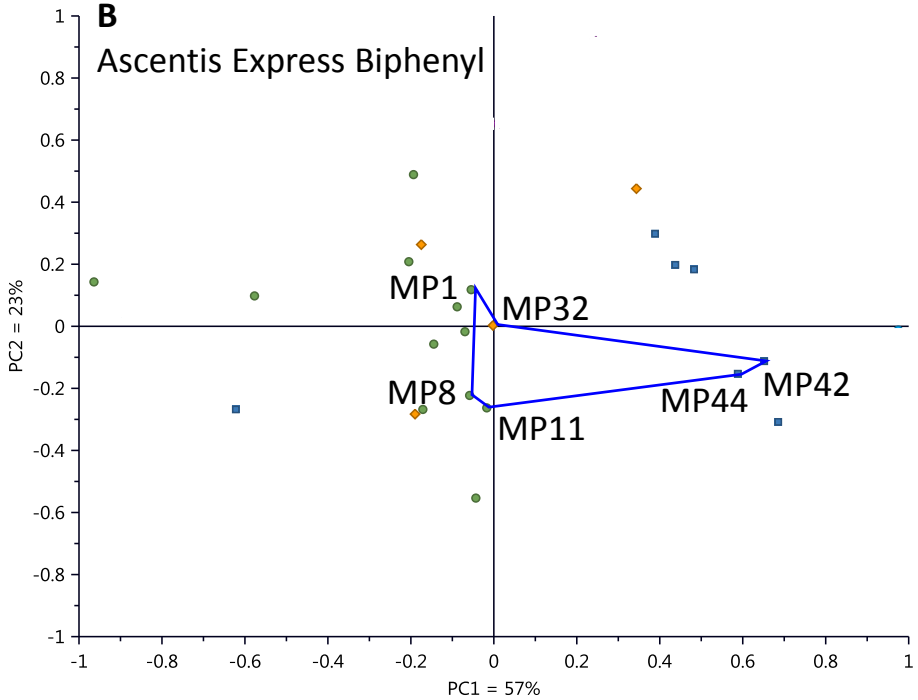
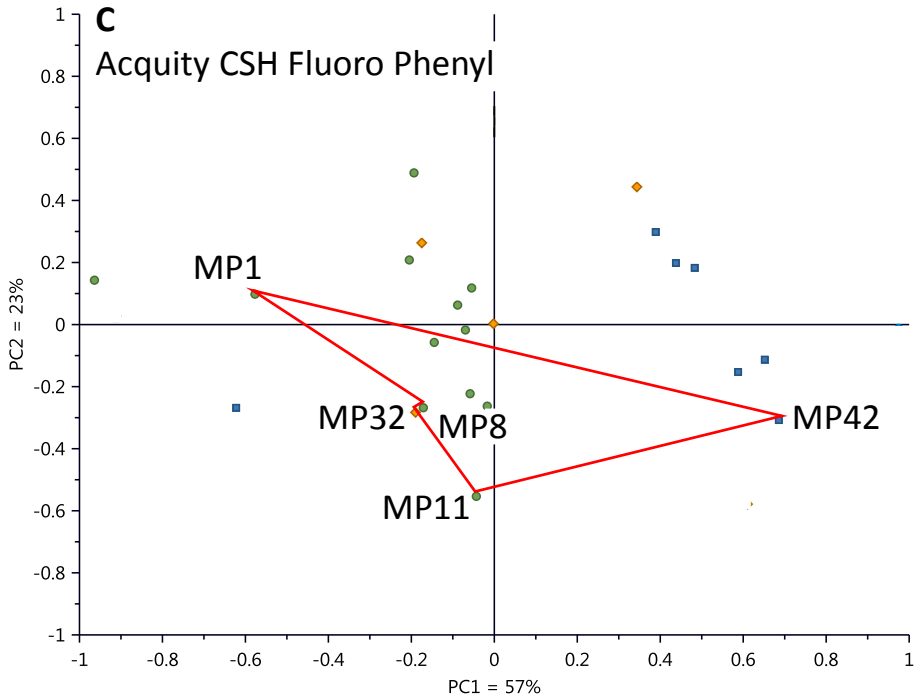
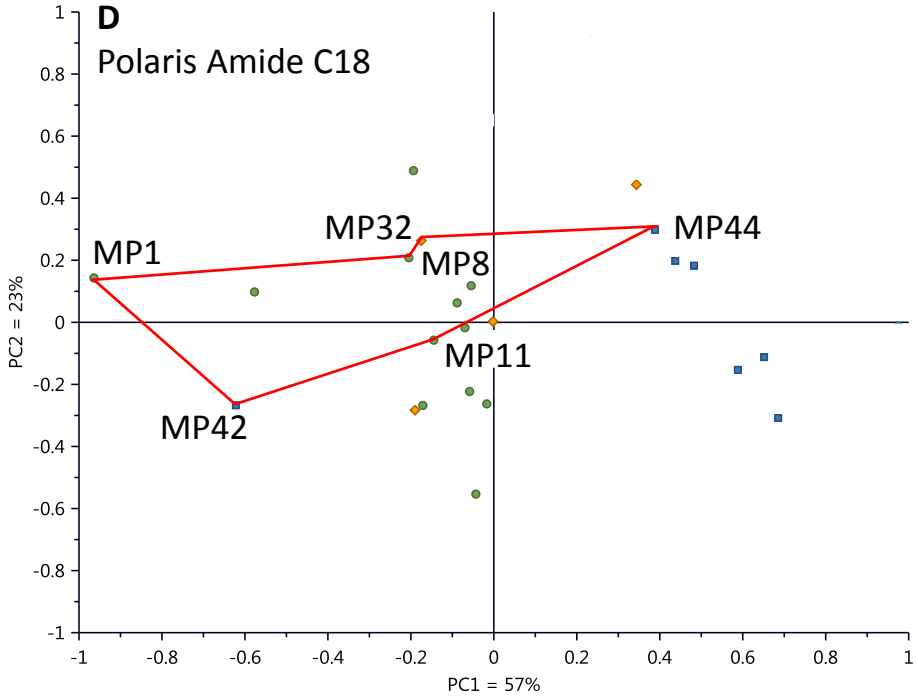

Mobile Phase pH
<2.8
3.1
3.6
5.1
>6.0

Fig.2S









Mobile Phase Name

MP1 pH 2.5 0.1% FA
MP8 pH 2.3 100 mM $H_3PO_4/NH_4H_2PO_4/(NH_4)_2SO_4$
MP11 pH 1.9 0.1% TFA
MP32 pH 5.1 20 mM $AA/NH_4AA/BuSO_3Na$
MP42 pH 6.5 20 mM NH_4FA
MP44 pH 7.5 20 mM $NH_4H_2PO_4/(NH_4)_2HPO_4$

Mobile Phase pH

- <2.8
- 3.1
- 3.6
- 5.1
- >6.0

Fig. 3

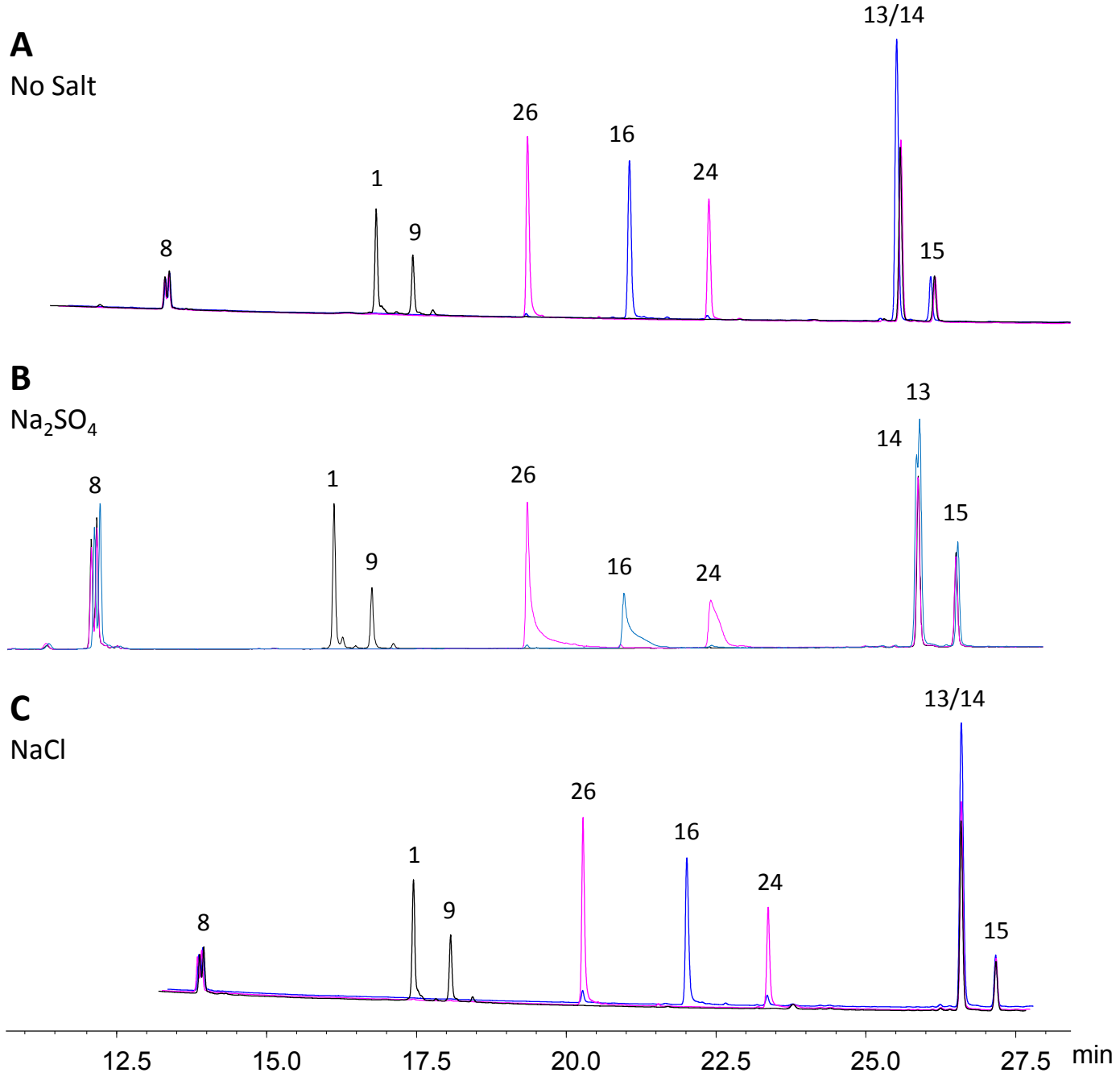

A
No Salt
8
1
9
26
16
24
13/14
15
B
Na_2SO_4
8
1
9
26
16
24
14
13
15
C
NaCl
8
1
9
26
16
24
13/14
15
12.5
15.0
17.5
20.0
22.5
25.0
27.5
min

Fig. 4

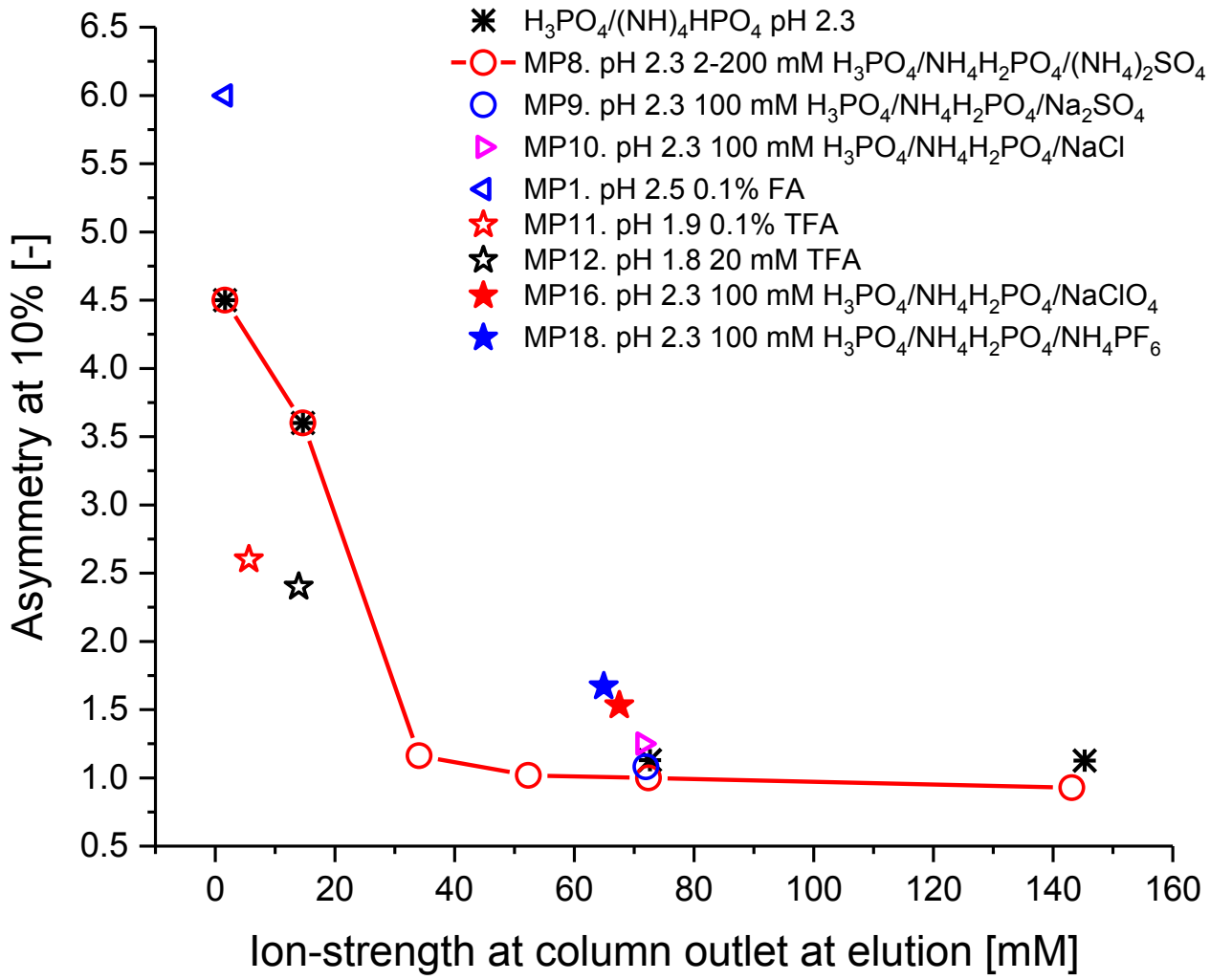

Asymmetry at 10% [-]
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
20
40
60
80
100
120
140
160
Ion-strength at column outlet at elution [mM]
H3PO4/(NH)4HPO4 pH 2.3
MP8. pH 2.3 2-200 mM H3PO4/NH4H2PO4/(NH4)2SO4
MP9. pH 2.3 100 mM H3PO4/NH4H2PO4/Na2SO4
MP10. pH 2.3 100 mM H3PO4/NH4H2PO4/NaCl
MP1. pH 2.5 0.1% FA
MP11. pH 1.9 0.1% TFA
MP12. pH 1.8 20 mM TFA
MP16. pH 2.3 100 mM H3PO4/NH4H2PO4/NaClO4
MP18. pH 2.3 100 mM H3PO4/NH4H2PO4/NH4PF6

Fig. 5

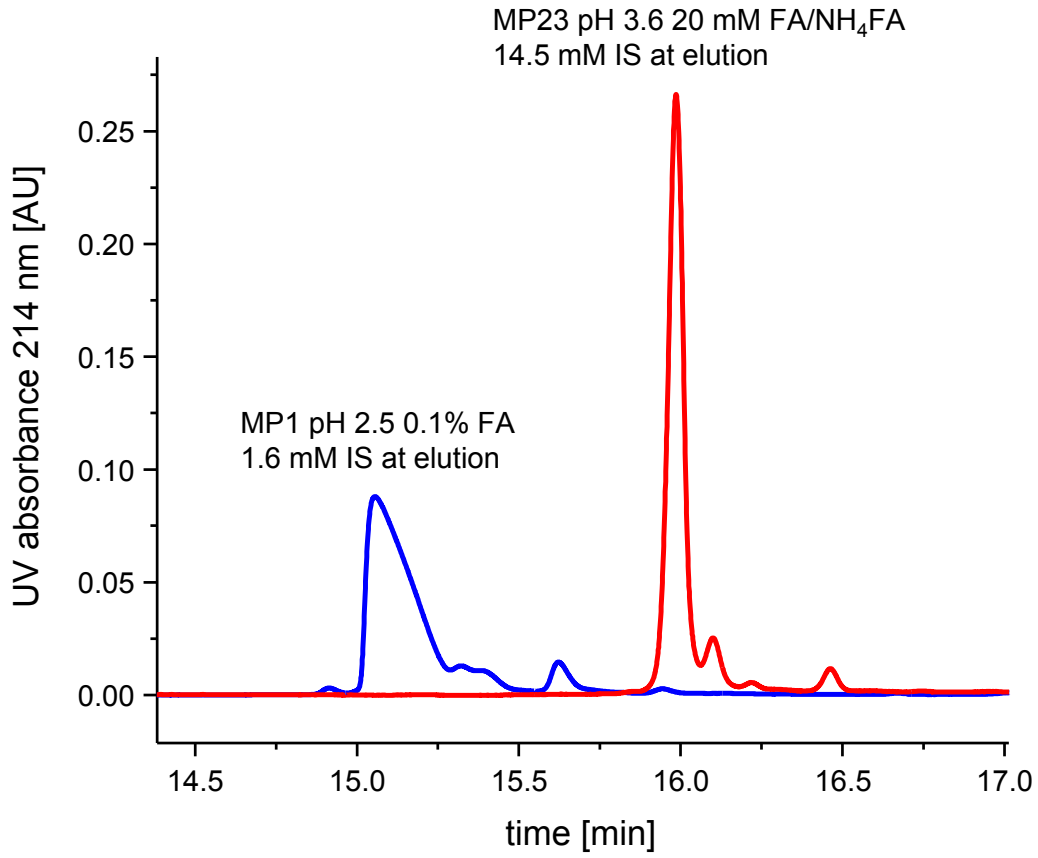

MP23 pH 3.6 20 mM FA/NH4FA
14.5 mM IS at elution
MP1 pH 2.5 0.1% FA
1.6 mM IS at elution
UV absorbance 214 nm [AU]
0.25
0.20
0.15
0.10
0.05
0.00
14.5
15.0
15.5
16.0
16.5
17.0
time [min]

Table 1

Charge of the characterisation peptide probes at different pH values

Peptide Name	Peptide Number	pI	Total Net Charge at pH *x* / # positive groups / # negative groups						
			1.8	**2.3**	**3.6**	**5.1**	**6.5**	**7.5**	**7.8**
Bovine GLP-2 (1-15)	1	3.5	1.5/2.0/0.5	1.1/2.0/0.9	-0.6/2.0/2.6	-2.9/1.9/4.8	-3.8/1.2/5.0	-4.0/1.0/5.0	-4.0/1.0/5.0
[Met(O)10]-Bovine GLP-2 (1-15)	8	3.5	1.5/2.0/0.5	1.1/2.0/0.9	-0.6/2.0/2.6	-2.9/1.9/4.8	-3.8/1.2/5.0	-4.0/1.0/5.0	-4.0/1.0/5.0
[L-Asp11]-Bovine GLP-2 (1-15)	9	3.4	1.5/2.0/0.5	1.1/2.0/0.9	-1.1/2.0/3.0	-3.9/1.9/5.7	-4.8/1.2/6.0	-5.0/1.0/6.0	-5.0/1.0/6.0
[Ile27]-Bovine GLP-2 (16-33)	13	6.7	2.7/3.0/0.3	2.4/3.0/0.6	1.1/3.0/1.9	0.1/3.0/2.9	0.0/3.0/3.0	0.0/3.0/3.0	0.0/3.0/3.0
[D-Ser16,Ile27]-Bovine GLP-2 (16-33)	14	6.7	2.7/3.0/0.3	2.4/3.0/0.6	1.1/3.0/1.9	0.1/3.0/2.9	0.0/3.0/3.0	0.0/3.0/3.0	0.0/3.0/3.0
[Ile26,Leu27]-Bovine GLP-2 (16-33)	15	6.7	2.7/3.0/0.3	2.4/3.0/0.6	1.1/3.0/1.9	0.1/3.0/2.9	0.0/3.0/3.0	0.0/3.0/3.0	0.0/3.0/3.0
[Gly22,Ile27]-Bovine GLP-2 (16-33)	16	6.7	2.7/3.0/0.3	2.4/3.0/0.6	1.1/3.0/1.9	0.1/3.0/2.9	0.0/3.0/3.0	0.0/3.0/3.0	0.0/3.0/3.0
[Tyr26,Ile27]-Bovine GLP-2 (16-33)	24	6.7	2.7/3.0/0.3	2.4/3.0/0.6	1.1/3.0/1.9	0.1/3.0/2.9	0.0/3.0/3.0	0.0/3.0/3.0	0.0/3.0/3.0
[Lys26,Ile27]-Bovine GLP-2 (16-33)	26	9.7	3.7/4.0/0.3	3.4/4.0/0.6	2.1/4.0/1.9	1.1/4.0/2.9	1.0/4.0/3.0	1.0/4.0/3.0	1.0/4.0/3.0

Footnote

The sequence for the peptide probes followed either the base sequence for Bovine GLP-2 (1-15) HADGSFSDEMNTVLD or [lle27]-Bovine GLP-2 (16-33) SLATRDFINWLIQTKITD

Table 2

Mobile Phase Preparation

	Mobile Phase	Mobile Phase Number	pH	Total Ionic Strength (mM)^	MS Compatible	Concentration Reagent 1* (mM)	Concentration Reagent 2* (mM)	Concentration Reagent 3* (mM)
Base Buffers	$H_3PO_4/NH_4H_2PO_4$	4	2.3	20		19.61	17.05	-
	FA/NH_4FA	23	3.6	20	✓	24.55	19.85	-
	AA/NH_4AA	29	5.1	20	✓	7.75	20.00	-
	$NH_4H_2PO_4/(NH_4)_2HPO_4$	44	7.5	20		1.73	6.23	-
	$H_3PO_4/NH_4H_2PO_4$	6	2.3	100		58.24	94.44	-
	$H_3PO_4/(NH_4)_2HPO_4$	25	3.1	100		59.02	49.98	-
	$NH_4H_2PO_4/(NH_4)_2HPO_4$	40	7.5	100		5.81	31.97	-
Salt (NaCl additive)	$H_3PO_4/NH_4H_2PO_4/NaCl$	10	2.3	100		19.39	17.61	78.92
	$FA/NH_4FA/NaCl$	24	3.6	100		22.55	21.85	77.80
	$AA/NH_4AA/NaCl$	35	5.1	100		7.41	23.40	76.60
	$NH_4H_2PO_4/(NH_4)_2HPO_4/NaCl$	39	7.5	100		1.23	6.73	78.95
Kosmotropic Salt (Na_2SO_4 or $(NH_4)_2SO_4$ additive)	$H_3PO_4/NH_4H_2PO_4/Na_2SO_4$	9	2.3	100		19.39	17.61	26.31
	$H_3PO_4/NH_4H_2PO_4/(NH_4)_2SO_4$	8	2.3	100		19.39	17.61	26.31
	$FA/NH_4FA/(NH_4)_2SO_4$	19	3.6	100		22.55	21.95	25.94
	$AA/NH_4AA/(NH_4)_2SO_4$	30	5.1	100		6.74	21.06	26.33
	$NH_4H_2PO_4/(NH_4)_2HPO_4/(NH_4)_2SO_4$	43	7.5	100		1.23	6.77	26.27
Chaotropic Salt ($NaClO_4$ additive)	$H_3PO_4/NH_4H_2PO_4/NaClO_4$	16	2.3	100		19.11	22.55	75.40
	$FA/NH_4FA/NaClO_4$	27	3.6	100		22.55	21.85	77.80
	$AA/NH_4AA/NaClO_4$	34	5.1	100		6.75	21.00	79.00
	$NH_4H_2PO_4/(NH_4)_2HPO_4/NaClO_4$	47	7.5	100		1.23	6.73	78.95
Chaotropic Salt (NH_4PF_6 additive)	$H_3PO_4/NH_4H_2PO_4/NH_4PF_6$	18	2.3	100		19.73	17.27	78.92
	$FA/NH_4FA/NH_4PF_6$	48	3.6	100		22.80	22.20	77.59
	$AA/NH_4AA/NH_4PF_6$	49	5.1	100		6.79	21.21	78.83
	$NH_4H_2PO_4/(NH_4)_2HPO_4/NH_4PF_6$	50	7.5	100		1.23	6.77	78.82
TEA (Ion-Pair)	$FA/NH_4FA/TEA$	21	3.6	20	✓	29.55	14.85	5.00

	AA/NH_4AA/TEA	28	5.1	20	✓	12.75	15.00	5.00
	$NH_4H_2PO_4$/$(NH_4)_2HPO_4$/TEA	38	7.5	20		6.80	1.16	5.00
	FA/NH_4HCO_3/TEA	37	7.8	20	✓	5.00	15.40	5.00
TFA (Ion-Pair)	H_3PO_4/$NH_4H_2PO_4$/TFA	5	2.3	20		9.88	14.12	5.00
	FA/NH_4FA/TFA	22	3.6	20	✓	13.71	20.06	5.00
	AA/NH_4AA/TFA	31	5.1	20	✓	0.90	20.04	5.00
	$NH_4H_2PO_4$/$(NH_4)_2HPO_4$/TFA	41	6.8	20		0.00	8.50	5.00
HFBA (Ion-Pair)	HFBA	14	1.8	20	✓	20.00	-	-
	H_3PO_4/$NH_4H_2PO_4$/HFBA	13	2.3	20		9.88	14.12	5.00
	FA/NH_4FA/HFBA	26	3.6	20	✓	13.71	20.06	5.00
	AA/NH_4AA/HFBA	33	5.1	20	✓	0.90	20.04	5.00
	$NH_4H_2PO_4$/$(NH_4)_2HPO_4$/HFBA	51	6.8	20		0.00	8.50	5.00
$BuSO_3Na$ (Ion-Pair)	H_3PO_4/$NH_4H_2PO_4$/$BuSO_3Na$	2	2.3	20		15.48	10.09	5.00
	FA/NH_4FA/$BuSO_3Na$	20	3.6	20		18.80	14.97	5.00
	AA/NH_4AA/$BuSO_3Na$	32	5.1	20		5.90	15.04	5.00
	$NH_4H_2PO_4$/$(NH_4)_2HPO_4$/$BuSO_3Na$	45	7.5	20		1.32	4.70	5.00
Miscellaneous	0.1% v/v H_3PO_4 (85% w/w)	3	2.2	8			-	-
	0.1% v/v FA	1	2.5	2	✓		-	-
	0.1% v/v TFA	11	1.9	13	✓		-	-
	TFA	12	1.8	20	✓	20.00	-	-
	0.1% v/v DFA	7	1.9	16	✓		-	-
	0.05% v/v FA / 0.05% v/v TFA	15	2.2	7	✓		-	-
	0.1% v/v MSA	17	1.9	15	✓		-	-
	NH_4FA	42	6.5	20	✓	20.00	-	-
	NH_4AA	36	7.0	20	✓	20.00	-	-
	NH_4HCO_3	46	7.8	20	✓	20.00	-	-

^ Ion-pair reagents were added at 5 mM concentration, whilst the 100 mM IS mobile phases composed of salt and buffers were made by keeping the buffer contribution at 20 mM IS and the salt was added to contribute with 80 mM IS.

** Reagent 1 = Acid, Reagent 2 = Base, Reagent 3 = Additive*

Table S3

The MS signal and average charge for each MS compatible mobile phase using the Bovine GLP-2 (1-15) peptide probe #1 (load 0.25 µg)

Mobile Phase	Mobile Phase Number	MS Signal	Average Charge	%MS Response of FA
pH 7.8 20 mM FA / NH_4HCO_3 / TEA	37	6E+02	1.5	0%
pH 1.8 20 mM HFBA	14	9E+02	1.9	0%
pH 5.1 20 mM AA / NH_4AA / HFBA	33	1E+03	2	1%
pH 3.6 20 mM FA / NH_4FA / HFBA	26	2E+03	2	1%
pH 5.1 20 mM AA / NH_4AA / TEA	28	2E+03	1.6	1%
pH 3.6 20 mM FA / NH_4FA / TEA	21	2E+03	1.6	1%
pH 6.5 20 mM NH_4FA	42	6E+03	2	3%
pH 5.1 20 mM AA / NH_4AA / TFA	31	9E+03	2	5%
pH 5.1 20 mM AA / NH_4AA	29	1E+04	2	5%
pH 7.0 20 mM NH_4AA	36	1E+04	2	5%
pH 7.8 20 mM NH_4HCO_3	46	1E+04	2	5%
pH 3.6 20 mM FA / NH_4FA / TFA	22	2E+04	2	10%
pH 1.9 3 mM 0.1% MSA	17	3E+04	2	15%
pH 3.6 20 mM FA / NH_4FA	23	4E+04	2	20%
pH 1.9 8 mM 0.1% TFA	11	4E+04	2	20%
pH 2.2 7 mM 0.05% FA / 0.05% TFA	15	5E+04	2	25%
pH 1.9 16 mM 0.1% DFA	7	1E+05	2	50%
pH 2.5 2 mM 0.1% FA	1	2E+05	2	100%