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Highlights

- 2D-RPC-MS strategy for main peak purity analysis of pharmaceutical peptides
- Selection of columns / mobile phases with complementary selectivity in ^{1}D and ^{2}D
- 2D-LC allows the determination of isomers that cannot be differentiated by LC-MS
- Results support previously reported peptide RPC column characterisation protocol
- TFA found to reduce selectivity differences for investigated peptides / columns

A Strategy for Assessing Peak Purity of Pharmaceutical Peptides in Reversed-Phase Chromatography Methods using Two-Dimensional Liquid Chromatography Coupled to Mass Spectrometry. Part I: Selection of Columns and Mobile Phases

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Peptides, peak purity, 2D-RPC-MS, mobile phase, stationary phase

Abstract

The current study describes the development of a 2D-LC-MS-based strategy for assessing main peak purity in the analysis of pharmaceutical peptides. The focus is on 2D-LC using reversed-phase (RP) separations in both dimensions, and particularly peptide isomer selectivity, since compounds with the same mass to charge ratio are not readily differentiated by mass spectrometry and therefore must be separated chromatographically. Initially, 30 column / mobile phase combinations were evaluated for both general separation performance (*i.e.*, selectivity and peak shape) and isomer selectivity using forcibly degraded peptide samples and mixtures of synthetic diastereomers. A ranking of more than 300 UV and MS chromatograms suggests that when developing a new method, screening a set of four columns and four volatile mobile phases with differing characteristics should be adequate to both cover the selectivity space, and yield good separation performance. When 2D-LC-MS is to be used to evaluate peak purity for a new method, our results show that a second-dimension separation comprising a C8/C18 column possessing no ionic functionality, and an acetic acid / ammonium acetate mobile phase buffered at pH 5, provides good selectivity at 25°C for peptide isomers with a MW <10 kDa.

Retention data for 29 diverse peptides (1 < MW < 14 kDa, 3.7 < pI < 12.5) measured in this study using a variety of column and mobile phase conditions (*i.e.*, 30 in total) are consistent with the classification of these various chromatographic conditions using the previously reported Peptide

RPC Column Characterisation Protocol. For the investigated peptides trifluoroacetic acid was found to reduce selectivity differences between columns of diverse properties, probably due to its potential to form ion-pairs with peptides. Trifluoroacetic acid often improves peak shape for very large peptides (*i.e.* MW > 10 kDa). In the current dataset which also contain smaller peptides it received the highest ranking for 40% of the column and mobile phase combinations due to better selectivity and/or peak shape.

The reported work here constitutes part 1 of a series of two papers. The second paper focuses on the use of retention modelling for rapid and accurate selection of the shallow gradients (*i.e.*, << 1% ACN/min) required to obtain sufficient peptide isomer retention and separation in the second dimension. The overall results presented in this series of papers provides the guidance needed to develop a 2D-LC-MS method from start to finish for the analysis of main peak purity of therapeutic peptides.

1. Introduction

There is a strong focus within the pharmaceutical industry on biomolecules such as peptides and proteins [1,2]. One important reason for this is that many biological substances have fewer side effects compared with small molecule drugs. However, this trend imposes a marked challenge on analytical chemistry because biopharmaceuticals can be structurally complex, have a high degree of heterogeneity, and diverse degradation pathways.

Reversed-phase chromatography with UV or MS detection is the most important analytical technique for the analysis of intact peptides and their related impurities (*i.e.*, oxidation, deamidation, racemisation, etc.) as well as enzymatic digests of proteins and mAbs. Currently, there is an approach called multiple attribute monitoring (MAM) which intends to replace several traditional analytical methods (*i.e.*, RPC, IEC, icIEF, CE-SDS, HILIC, ELISA) with one RPC-MS method for the characterisation of larger proteins, antibodies and their related impurities at the peptide level [3]. However, the "*!ÛKttŜă heelé* of LC-MS lies in its inability to differentiate compounds which are both difficult to separate chromatographically and have identical mass to charge ratio (*m/z*) such as isomers.

In a series of previous publications, we have characterised 43 RPC columns [4-6] and 51 mobile phases [7] targeting peptide RPC method development. Subsequently, a method development strategy was published based on this characterisation work [8]. Both volatile and non-volatile mobile phase additives were investigated. The study involved not only the characterisation of general peptide selectivity, but also assessed the effect of various mobile phase additives on the corrosion of "*wettable*" LC components and "*memory effects*" after changing LC conditions. References [4, 7] summarises studies of and related to column and mobile phase characterisation.

The work reported here describes a strategy for use of multiple heart-cutting 2D-LC-MS for peak purity analysis of pharmaceutically relevant peptides, where the focus is on isomer selectivity using volatile mobile phases to facilitate MS detection. The objective is to identify RPC column and mobile phase combinations that can be employed in the second dimension to determine isomers co-eluting with the main component in the first dimension. One typical application for 2D-LC-MS peak purity analysis would be during validation of RPC methods used for analysis of the

purity of peptide-based pharmaceuticals, *i.e.* to ensure that there are no isomeric impurities co-eluting with the main peak. An overview of the principles of 2D-LC and the different approaches that can be applied to peak purity analysis can be found in references [9-13].

In addition to the identification of suitable column and mobile phase combinations, another challenge is to define the very shallow gradient required for the second dimension chromatography. The current study also investigated how retention modelling [14] could be used to define such gradients in a quick and rational way.

The study is divided into two papers; Part I is related to the selection of columns and mobile phases [*current publication*], whereas Part II is related to the definition of the second dimension chromatographic gradient conditions and the demonstration of 2D-LC-MS methods developed using this approach [15].

2. Experimental

The following peptides were provided by Sigma-Aldrich (St Louis, MO); bradykinin, rat glucagon-like peptide-2 (1-33) {rat GLP-2 (1-33)}, bovine insulin, bovine ubiquitin and chicken lysozyme. The peptides were dissolved in 20 mM ammonium hydroxide and stressed at 37°C for 40 h to generate 5-30% of related impurities (for insulin 18 h at 37°C, for bradykinin 69 h at 50°C). Post degradation 20 mM of acetic acid was added to reach neutral pH and a peptide concentration of 1 mg/mL.

Thirteen bovine GLP-2 (1-15) and [Gly22, Ile27]-bovine GLP-2 (16-33) related peptides corresponding to a racemisation of different amino acids in the two base sequences (Table 1) were supplied by Apigenex (Prague, Czech Republic). These were all dissolved individually in DMSO/H_2O (80:20 v/v) to a concentration of 0.25 mg/mL. Samples were then combined in the autosampler by on-line mixing 2 uL of the L-form with 1 µL of the corresponding D-form. In this way eight samples were prepared corresponding to a racemisation of a particular amino acid in the sequence. To simplify nomenclature the name of the organism which a peptide originates from has been omitted in subsequent sections of the article.

All data except for those shown in Figure 8 were acquired using a 1290 Infinity II Bio LC coupled to a 6545 Q-TOF MS system (Agilent, Waldbronn, Germany). The LC system incorporated a binary pump with 100 µL Jet Weaver mixer, autosampler, column thermostat equipped with 8-column selector, DAD detector with Max-Light Cartridge Cell LSS (10 mm path length)[1]. The QTOF was equipped with a Dual AJS ESI Source. Agilent MassHunter Workstation LC/MS Data Acquisition (Version 11.0) and MassHunter Qualitative Analysis (Version 10.0) were used for data acquisition and processing.

Injection volumes used for 1D separations were adjusted such that 3 µg of sample was injected on column followed by a five second needle wash with 0.1% v/v FA in ACN/water 50:50 v/v. Columns

[1] High sensitivity detection can cause photo oxidation of bio-molecules. The LSS cell has an aperture at the inlet, which decreases the energy intake and thus can reduce light-induced degradation.

and mobile phases to be evaluated in this study were selected based on previous publications of column [4] and mobile phase characterisation [7]; see Tables 2 and 3. Weak (A) and strong mobile phases (B) were prepared as described in Table 3 and reference [8]. The gradient was standardised to 2.5 – 100 – 100 – 2.5 – 2.5 %B at 0 – 60 – 62 – 65 – 75 min and delivered at 0.3 mL/min. Columns were held at 40°C. The UV detection was at 215 nm and 80 Hz. MS polarity positive, fragmentor: 175 V, skimmer: 65 V, mode: 10 GHz, mass-range: 100 – 3500 m/z, acquisition rate: 5 spectra / s, reference mass: m/z 121.0509 and m/z 922.0098. ESI Source: Gas temp: 320 °C, drying gas: 8 L/min, nebulizer: 35 psi, sheath gas temp: 350 °C, sheath gas flow: 11 L/min, VCap: 3500 V, Nozzle: 500 V.

An Agilent 1290 Infinity II 2D-LC system was used for the collection of the data shown in Figure 8. A generalised illustration of this system for multiple heart-cutting is provided as supplemental information in Part II ([15] Figure S1). The first dimension consisted of an autosampler, a binary pump with a 380 µL Jet Weaver mixer, a thermostated column compartment, and a UV absorbance detector with a 6 mm semi-micro flow cell. The second dimension included a binary pump with a 100 µL Jet Weaver mixer, a thermostated column compartment, and a UV absorbance detector with a 6 mm semi-micro flow cell. A multiple heart-cutting interface (ASM valve + 2 x deck valves) was used to collect fractions of 1D effluent and transfer them to the second dimension. The interface was equipped with 40 µL loops and an 340 mm ASM bypass capillary. A pressure release kit was used between the outlet of the 1D detector and the 2D interface as well as between the outlet of the 2D detector and the MS interface. The mass spectrometer was a 6550 iFunnel QTOF LC/MS. Software and instrumental parameters as described above.

GPMAW version 12.01a, Lighthouse data, Odense, Denmark was used for calculation of MW and pI. OriginPro version 2022, Northampton, MA, USA was used for principal component analysis and generation of graphs.

3. Results and Discussion

The selection of column and mobile phase combinations investigated in this study was designed to yield maximal differences in selectivity while still providing good peak shape. The selection of mobile phases was limited to MS compatible ones to allow generation of EICs that could be used to assess isomer selectivity in both the first and second dimensions. The columns and mobile phases used are described in Tables 2 and 3. It should be stressed, however, that within the biopharmaceutical industry non-volatile salts are frequently used as mobile phase additives due to their complementary selectivity and often better peak shape. The latter is related to a higher ionic-strength that reduces analyte overloading due to mutual repulsion [7,8,16-18]. A previous publication [8] describes a screening strategy for the development of methods to assess the purity of pharmaceutical peptides including both volatile and non-volatile mobile phases.

Five peptides (*i.e.*, bradykinin, GLP-2 (1-33), insulin, ubiquitin and lysozyme, see Table 1) with molecular masses and pIs ranging from 1 to 16 kDa and 3.7 to 12.5, respectively, were subjected to forced alkaline degradation generating 5-30% of related impurities. The molecular weight and *pI* ranges are representative for peptide-based pharmaceuticals as well as peptides found in enzymatic digests of proteins.

UV and MS chromatograms were collected for each sample, column, and mobile phase combination (a total of 150 UV and 150 EIC chromatograms were collected). UV data acquired at 215 nm were used to generate chromatograms reflecting general selectivity and EICs to monitor isomeric selectivity (*i.e.*, selectivity for impurities with the same *m/z* as the main component). In addition, eight synthetic samples consisting of D/L-pairs corresponding to a racemisation of a single amino acid within GLP-2 (1-15) or [Gly22, Ile27] GLP-2 (16-33) were analysed for the purpose of determining isomer selectivity.

3.1. Characterisation of column / mobile phase selectivity

In our previous characterisation of columns for peptide separations [6], nine GLP-2 (1-51) and [Ile27] GLP-2 (16-33) related peptides were used as probes in separations using mobile phases buffered at pH 2.7 and 6.5. In the current work retention data was collected for 29 additional and completely different peptides (*i.e.*, five peptides and their related impurities) using five mobile phases and six columns (i.e., 30 differing column / mobile phase combinations per peptide sample, see Supplementary material S1). Subsequently, principal component analysis (PCA) was used to generate score plots that could be compared with those generated in the previous studies [4-7].

As in the preceding investigation, we observed that columns were grouped differently in the score plot depending on which mobile phase had been used for the collection of retention times. Columns characterised using mobile phases containing formic acid formed different groupings compared to those characterised using mobile phases modified with TFA, which may be rationalized by the difference in ion-pairing properties between formic acid and TFA. It should be noted that these findings may be dependent on the RPC condition and sample used hence extrapolation to other samples and conditions may be problematic. Similar score plots (see Figure 1) where obtained in this study using FA/NH_4FA pH 3.6 and AA/NH_4AA pH 5.1 compared to those in our previous studies [4-7] which used FA (pH 2.7) and NH_4FA (pH 6.5) based mobile phases. The current classification results, based on 29 peptides, support the previous classification results that used only nine peptides, and confirms the validity of the previously published classification protocol [4-7].

The Atlantis Premier BEH C18 AX column was located in quadrant II (*i.e.*, top left quadrant) of the PCA score plot for both sets of data (see Figure 1A and B). The column is designed to carry a significant concentration of positive charge in addition to its RP characteristics (see Table 2). The Acquity CSH Fluoro-phenyl column in quadrants III possesses both a low positive charge and, depending on pH, a significant negative charge attributed to the non-end-capped silica. RP columns possessing a neutral (Acquity BEHC8) or negative character (Ascentis Express Biphenyl and Zorbax 300 SB-C18) were located in quadrant I. Acquity BEH C8 is end-capped and based on a

hybrid silica, which reduces silanol interactions. The Ascentis Express Biphenyl and Zorbax 300 SB-C18 possess bulky ligands, and the latter is not end-capped. Both these properties may result in increased interactions between peptides and exposed silanols. The pH dependent negative charge (*i.e.*, silanol ionisation at intermediate pH) associated with the latter two columns is substantiated by the high Tanaka values (total silanol activity [$\alpha_{B/P}$ at pH 7.6]) of 1.0 to 1.2 for these two columns [19]. In comparison C8 or C18 columns with neutral characteristics such as Acquity BEH C8 typically possess a total silanol activity ($\alpha_{B/P}$ at pH 7.6) value <0.4.

PCA of the retention data obtained for the 29 peptides using low pH combinations (*i.e.*, TFA, MSA and FA/NH_4FA pH 3.6) revealed that the use of TFA reduces the general selectivity, *i.e.* the use of TFA makes columns more similar than when using MSA or FA/NH_4FA pH 3.6. This can be observed in Figure 2 for both diverse columns (Atlantis Premier BEH C18 AX, Acquity CSH Fluoro-phenyl and Acquity BEH C8) and more similar columns (Ascentis Express Biphenyl, Zorbax 300 SB-C18 and Acquity BEH C8). *I.e.* the triangles in Figure 2 formed by the points associated with measurements in TFA are significantly smaller than those described by MSA and FA/NH_4FA pH 3.6. This observation is in line with what was reported in one of our previous studies [4] and was attributed to the ion-pairing properties of TFA which masks the positively charged groups associated with the peptides and/or columns.

3.2. Ranking of chromatographic performance

In addition to determining which column and mobile phase combinations provided large and complementary selectivity differences, a visual ranking was performed to evaluate resolution and peak shape for each of the chromatograms generated with the five peptides and their related impurities (*i.e.*, 30 UV and 30 EIC chromatograms were ranked per column / mobile phase combination, equating to 300 chromatograms ranked in total for the five peptide samples).

The criteria for ranking (one = poorest performance and five = best performance) the various column / mobile phase combinations were based on the number of impurity peaks observed, the resolution around the main peak, and the peak shape. Number of peaks were given the highest importance. Each ranking is an average based on an individual ranking made by two experienced chromatographers. An example of the ranking classification of general selectivity is shown in Figure 3 for UV chromatograms of degraded insulin. Supplementary material S1 highlights an example of the ranking of isomer selectivity for degraded insulin (EIC for the most abundant ion in the main peak).

During the ranking exercise it was observed that for the larger peptides such as ubiquitin and lysozyme (*i.e.*, MW of 9 and 16 kDa respectively), acceptable peak shapes were only observed when TFA or MSA was used in combination with particles with pore sizes of 130 or 300 Å. This is consistent with what generally is observed during the screening of columns and mobile phases at Novo Nordisk A/S, *i.e.*, peptides / small proteins above approximately 10 kDa typically, but not always, require TFA or MSA in combination with large pore size particles to obtain acceptable peak

shapes. Furthermore, lysozyme did not produce enough MS signal with the generic MS settings used to allow evaluation of EIC isomer selectivity.

3.3. Identification of column and mobile phase combinations imparting large differences in general selectivity and good chromatographic performance under first dimension conditions

A PCA score plot (Supplementary material S2) based on the relative retention times of 29 peptides (see Supplementary material S3) were colour coded with the ranking values to provide information on differences in general selectivity (PCA scores) and general chromatographic performance (ranking / colour). A measure of distances in PCA score plots between different column / mobile phase combinations can be used to identify the most dissimilar combinations for method development screening strategies. In this case good chromatographic performance and the largest differences in selectivity was observed between the Acquity BEH C8 with AA/NH_4AA pH 5.1 and the Advance Bio Peptide Plus with TFA, or the Atlantis Premier BEH C18 AX with MSA. However, general method development is typically initiated with an automated screen using a small number of diverse columns and mobile phases. It is therefore, in this case, not necessary to further reduce the number of column / mobile phase combinations. All columns and mobile phases with an average ranking of ≥4 would be suitable to include in a screen for development of a MS compatible first-dimension separation, *i.e.* Acquity BEH C8, Atlantis Premier BEH C18 AX, Advance Bio Peptide Plus, Zorbax 300 SB-C18, MSA, TFA, FA/NH_4FA pH 3.6 and AA/NH_4AA pH 5.1.

Figure 4 provides an alternative overview of all the rankings and indicates individual rankings for the 150 UV chromatograms corresponding to different column, mobile phase and peptide sample combinations in one plot. Figure 4 clearly shows that the Acquity BEH C8 is the column with the best general performance independently of mobile phase but in particular with TFA, MSA, FA/NH_4FA pH 3.6 and AA/NH_4AA pH 5.1. The Advance Bio Peptide Plus, Atlantis Premier BEH C18 AX and Zorbax 300 SB-C18 all provided good performance with TFA and the latter two columns with MSA. AA/NH_4AA pH 7.0 only generated acceptable chromatographic performance for the degraded insulin sample.

The results from the current ranking studies yielded similar high performing column / mobile phase combinations as observed in our previous paper that was based on a smaller retention dataset [8].

There is a common perception that TFA-based mobile phases are needed for the RPC analysis of peptides. However, within the biopharmaceutical industry, mobile phases containing high concentrations of inorganic salts are frequently employed due to their complementary selectivity. In two recent articles we have characterised a number of alternative mobile phases, which in addition to TFA, yielded good peak shapes and large differences in selectivity for peptides [7,8]. In the latter study TFA was found to provide better chromatographic performance in only 21% of the highest ranked column-mobile phase combinations. In the current study, where only volatile mobile phases are evaluated, TFA was given the highest rank for 40% of the best separations. This

difference in ranking may be attributed to the physical / chemical properties of the peptides analysed.

3.4. Comparison of peptide retention observed with different column / mobile phase combinations

Relatively large differences in retention were observed with different column / mobile phase combinations in Figure 5, where the average retention times of bradykinin, GLP-2 (1-33), insulin, ubiquitin and lysozyme relative to the retention observed with the Acquity BEH C8 / TFA combination have been plotted for the different column and mobile phase combinations. The largest difference in retention (*i.e.*, 40%) is observed between Acquity BEH C8 with TFA and Acquity CSH Fluoro-phenyl with MSA. The difference may be explained by the relatively hydrophobic ion-pair formation of the positively charged peptides at low pH with the negatively charged TFA, and the lower hydrophobicity of the Fluoro-phenyl column compared to the C8 (*i.e.*, Tanaka hydrophobicity term k_{PB} = 0.46 and 1.16, respectively [19]). In a column and mobile phase screening strategy the main sample component should ideally elute in the middle of the gradient giving both polar and non-polar impurities a high probability of eluting in the linear part of the gradient on either side of the main component. Depending on the nature of the peptide under investigation and the retentivity of the column and mobile phase combination, it may be necessary to conduct a scouting experiment to determine how much the screening gradient(s) should be extended or shifted.

A similar retention behaviour was observed in a previous study involving another set of peptides and salt based mobile phases in addition to the current volatile mobile phases (see Figure 5 [8]).

For a peptide with basic side chains (*i.e.*, Arg, His, Lys) the Acquity BEH C8 with TFA is likely to be the most retentive combination (see Figure 5). The least retentive combination is likely to be with Acquity CSH Fluoro-phenyl and MSA; the latter forms less hydrophobic ion-pairs than with TFA (see Figure 5).

For a peptide with a larger number of acidic (*i.e.*, Asp, Glu) than basic side chains, the Atlantis Premier BEH C18 AX with AA/NH_4AA at pH 5.1 is likely to be the most retentive combination due to electrostatic attraction between the positively charged column and the peptide's ionised carboxyl groups. For these peptides the least retentive combination is likely to be Acquity CSH Fluoro-phenyl with MSA or AA/NH_4HCO_3 at pH 7 the latter due to electrostatic repulsion of the negatively charged peptide with the negatively charged silanol groups on the silica surface or negative charged MSA adsorbed into the stationary phase layer (see Figure 5 [8]).

3.5. Identification of column / mobile phase combinations giving large differences in isomer selectivity and good chromatographic performance under second dimension conditions

The selection of column / mobile phase combination(s) for the separation of isomers in the second dimension is more complicated since also the selectivity of the ^{1}D column / mobile phase

combination needs to be taken into account. One approach is to make the same type of performance summary as in Figure 4 for the ranking of EICs (see Figure 6). This clearly shows that the BEH C8 column in combination with TFA pH 1.9, FA/NH_4FA pH 3.6 or AA/NH_4AA pH 5.1 is the best compromise for separating the isomers of bradykinin, GLP-2 (1-33) and insulin. Ubiquitin and lysozyme were excluded since they displayed poor performance with these chromatographic conditions. Acceptable chromatography was only observed for the latter two peptides when using particles with large pore sizes in combination with either MSA or TFA, probably due to the peptides large molecular size. The same is typically observed for proteins and could be related to how TFA ion-pairs with the peptide / protein, makes it more hydrophobic and also possibly affecting the peptides / proteins secondary structure thereby altering the size of the solvated peptide / protein.

An alternative approach that involved calculating the average ranking for each column and mobile phase combination gave the same combinations discovered using the first approach (*i.e.*, average ranking ≥4.5 for bradykinin, GLP-2 (1-33) and insulin; see Figure 6).

In an attempt to further narrow down the number of column / mobile phase combinations, an additional evaluation of isomer selectivity was conducted by analysing eight samples, each containing a diastereomeric pair corresponding to a racemisation of one of the amino acids in the GLP- 2 (1-15) or [Gly22, Ile27] GLP-2 (16-33) sequence. The absolute value for the relative retention of the D/L-form -1 was used as a selectivity measure to rank the different column / mobile phase combinations proposed above for ^{1}D and ^{2}D conditions. Table 4 shows again that the BEH C8 in combination with AA/NH_4AA pH 5.1, FA/NH_4FA pH 3.6 and TFA gave rankings of 3.9-4.1. This result and the fact that TFA is frequently used in the purity determination of pharmaceutical peptides suggest that FA/NH_4FA pH 3.6 or AA/NH_4AA pH 5.1 should be employed in the second dimension.

An alternative methodology to select which of the highest ranked (*i.e.*, ≥4) ^{1}D combinations (*i.e.*, general selectivity 215 nm) should be combined with ^{2}D conditions consisting of BEH C8 with AA/NH_4AA pH 5.1, FA/NH_4FA pH 3.6 or TFA is to examine EICs (*m/z* corresponding to the main component) for each of the different peptides (Figure 7 for GLP-2 [1-33] and Supplementary materials S5 for bradykinin, and S6 for insulin). This comparison of EICs demonstrates that AA/NH_4AA pH 5.1 provides a more favourable isomer selectivity than FA/NH_4FA pH 3.6 for GLP-2 (1-33) and insulin. For bradykinin the results are comparable. Lysozyme gave a very poor MS signal and therefore isomer selectivity could not be evaluated.

The data highlights that a BEH C8 column in combination with AA/NH_4AA at pH 5.1 is a good combination to use in the second dimension. Since the Acquity BEH C8 column exhibited good results, it was compared against three other C8 columns (Ascentis Express C8, Poroshell HPH-C8 and Zorbax SB-C8) and the corresponding BEH C18 column and using AA/NH_4AA pH 5.1. The comparison was based on the analysis of the EICs for the degraded samples of bradykinin, GLP-2 (1-33), insulin plus the four D/L stereoisomeric samples of GLP-2 (1-15) and [Gly22, Ile27] GLP-2 (16-33). The result clearly indicated that there were only small differences in performance between the four columns. The non-endcapped Zorbax SB-C8 which possesses bulky di-isopropyl

side ligands exhibited a somewhat different selectivity. The position of these phases in the PCA score plot of the Peptide RPC Column Characterisation Protocol [Figure 1A in [6], except for the Zorbax SB-C8, were located relatively close to each other and can therefore be expected to provide similar selectivity.

In our previous characterisation of mobile phases, it was concluded that the MS response for peptides separated using either FA/NH_4FA pH 3.6 or TFA was approximately 80% lower than that observed when 0.1% v/v formic acid was employed, and 95% lower when AA/NH_4AA pH 5.1 was used [Supplementary material Table S1 Paper IV]. Assuming a typical load of 3 µg of the peptide on column then these responses would still permit 2D-LC-MS determination of the peak purity and isomers at relevant levels below 0.1% of the main component.

From the information above, a logical 2D-LC peak purity strategy would be to combine a ^{1}D separation using low pH mobile phases such as MSA, TFA, FA/NH_4FA pH 3.6 or mobile phase containing inorganic salts as described in reference [7,8]) with a ^{2}D separation consisting of a neutral character C8 column (*i.e.*, BEH C8) and AA/NH_4AA pH 5.1. However, if poor peak shape is obtained with AA/NH_4AA pH 5.1 in the ^{2}D an alternative mobile phase would be either TFA or FA/NH_4FA pH 3.6 depending on what is used in the ^{1}D. In order to obtain acceptable peak shapes for highly basic peptides, TFA would be the mobile phase additive of choice due to its ion-pairing properties. Wang *et al.* [20] have described a 2D-LC-MS system that allows automated screening of ^{2}D columns and mobile phases. A combination of this type of system with the ^{2}D columns and mobile phases which were highest ranked in the current study would provide a very powerful tool for assessment of peak purity.

Based on previous experiences of poor peak shape we decided to not include chiral columns in the current study. It is, however, possible that there are chiral columns that would be suitable for ^{2}D peak purity analysis. Ahmad *et al.* recently published an article where two epimers of a cyclic peptide were separated on a cellulose based column under RPC conditions [21].

The effect of temperature on isomer selectivity for the selected column / mobile phase combination has not been investigated. We have used a temperature of 25°C for the second dimension based on a, somewhat speculative, assumption that high temperature would decrease isomer selectivity as it has been reported to do for chiral separations [22,23].

Figure 8 shows the ^{1}D and ^{2}D chromatograms obtained for [L-Ser5]-GLP-2 (1-15) spiked with 12% of [D-Ser5]-GLP-2(1-15) during evaluation of the suggested peak purity analysis strategy. The ^{2}D gradient was defined using retention modelling as described in Part II of this study together with additional examples [15].

4. Conclusions

The current study describes a strategy for peak purity analysis of pharmaceutical peptides using multiple heart-cutting 2D-LC-MS. The separation of isomers is emphasised, since these compounds with identical *m/z* are not readily differentiated by MS. The study is divided into two parts: part I is related to selection of columns and mobile phases [current publication] whereas part II is

related to the definition of the ^{2}D gradient elution conditions, and additional proof-of-concept 2D-LC-MS separations that illustrate the power of the strategy [15].

The results of PCA classification of six stationary phases based on the retention of 29 diverse peptides (1 < MW < 16 kDA; 3.7 < pI < 12.5; bradykinin, GLP2(1-33), insulin, ubiquitin, lysozyme, and related impurities) have been shown to be consistent with the conclusions of the previously published Peptide RPC Column Characterisation Protocol [4-7]. It must be stressed that these conclusions may not be applicable when differing operational conditions are employed.

Visual ranking of chromatographic performance of the various column / mobile phase combinations with the degraded samples (*i.e.*, total of 300 UV and MS chromatograms) suggests that only four columns (or their equivalent) and four mobile phases are required for screening purposes in peptide RPC-MS method development; Acquity BEH C8, Atlantis Premier BEH C18 AX, Advance Bio Peptide Plus, Zorbax 300 SB-C18, and MSA, TFA, FA/NH_4FA pH 3.6 and AA/NH_4AA pH 5.1. The selected MS compatible column / mobile phase combinations are also consistent with those previously identified in our previous study as promising candidates for RPC-UV method development. In the latter case, the Acquity Fluoro-phenyl and three non-volatile salt-based mobile phases were additionally recommended to be included in screening experiments [8].

TFA was often found to reduce selectivity differences between columns, probably due to its potential to form ion-pairs with cations. TFA may not always be the best additive for peptide analysis but is often necessary to obtain acceptable peak shape for very large peptides (*i.e.*, >10 kDa). MSA is an interesting alternative to TFA that is more UV transparent, less harmful to the environment, and seems to provide larger differences in selectivity between columns. It might, however, have negative long term effects on certain instrument components and this needs to be further investigated [8].

For 2D-LC-MS peak purity analysis, the results indicate that a ^{2}D separation based on the Acquity BEH C8 or an equivalent C8 or C18 column possessing a neutral character and an AA/NH_4AA pH 5.1 mobile phase is likely to provide good selectivity at 25°C for peptide isomers with a molecular weight <10 kDa. However, if the ^{1}D separation is based on the preferred ^{2}D mobile phase conditions (*i.e.*, AA/NH_4AA pH 5.1) or if this mobile phase resulted in poor peak shape in the ^{2}D separation, then TFA (or FA/NH_4FA pH 3.6) is recommended for the second dimension.

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CRediT authorship contribution statement

Stephan Buckenmaier: Conceptualisation, Methodology, Formal analysis, Resources, Review & editing, Funding acquisition

Mel Euerby: Conceptualisation, Methodology, Formal analysis, Review & editing

Patrik Petersson: Conceptualisation, Methodology, Formal analysis, Resources, Writing – review & editing, Funding acquisition

Dwight Stoll: Conceptualisation, Methodology, Review & editing, Funding acquisition

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Figure legends

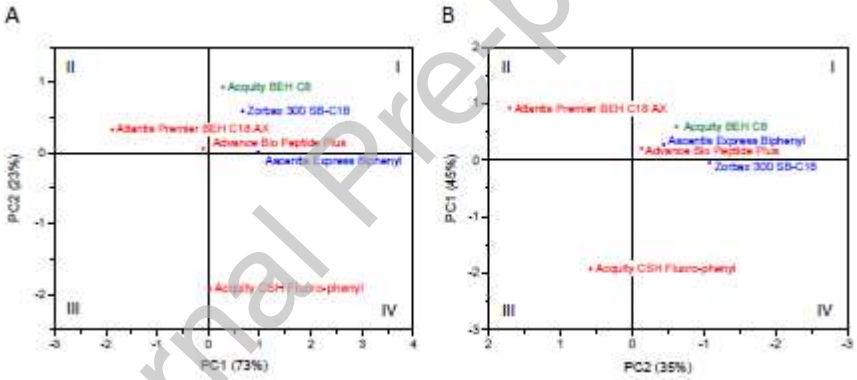

Figure 1. PCA score plots for the same six columns based on retention data obtained for (A) nine GLP-2 (1-15) and [Ile27] GLP-2 (16-33) peptides with FA pH 2.7 and NH_4FA pH 6.5 in previous studies [4-7] *and (B) 29 very different peptides with FA/NH_4FA pH 3.6 and AA/NH_4AA pH 5.1 in the current study. Quadrants II and III contain RPC columns which have a pronounced positive charge.*

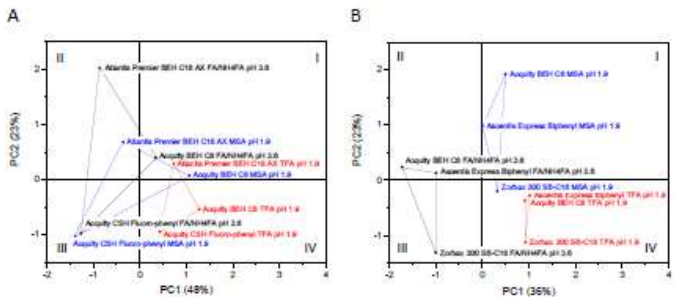

Figure 2. (A) PCA score plot based on retention data obtained for 29 diverse peptides chromatographed using TFA, MSA, FA/NH_4FA pH 3.6 and columns with very different selectivities; Atlantis Premier BEH C18 AX, Acquity CSH Fluoro-phenyl and Acquity BEH C8. (B) Corresponding PCA for more similar columns; Ascentis Express Biphenyl, Zorbax 300 SB-C18 and Acquity BEH C8.

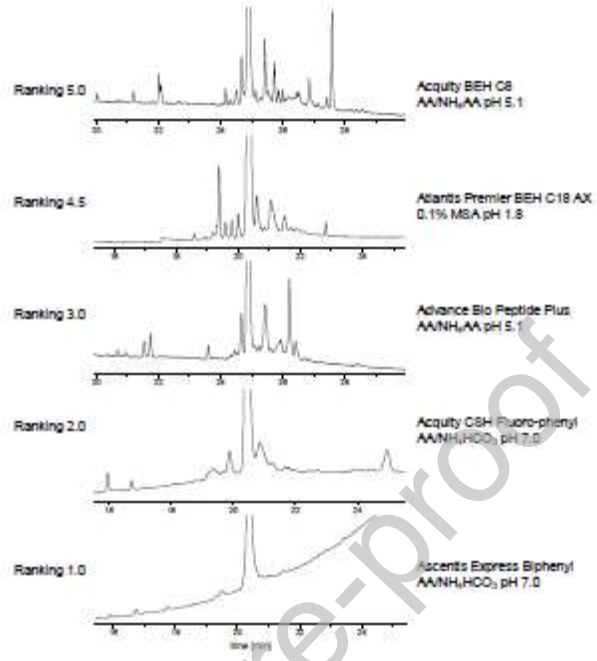

Figure 3. Illustration of general selectivity (215 nm) and ranking of chromatograms from forcibly degraded insulin analysed using different column and mobile phase combinations. Timescales were aligned using the main peak.

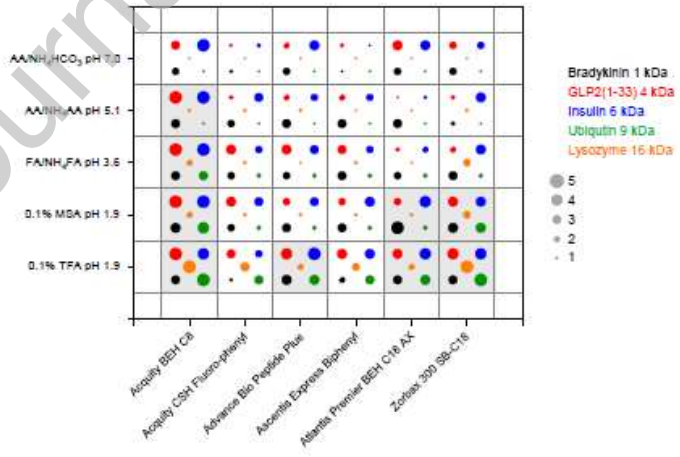

Figure 4. Summary of rankings of general selectivity (UV chromatograms at 215 nm) for 30 column/ mobile phases combinations. The diameters of the circles indicate the ranking for a certain peptide (larger is better). Combinations with an average ranking for bradykinin, GLP- 2 (1-33) and insulin жп ǎă ǎyŘǎǒI·ű ŜŘ ǿǎűK I· ăKI·ŘŜŘ ōI·ǒ[ƎℕƨdzyŘф ¦ōǎIjdzűǎy′I·yŘ fèăƨÎèY′Ŝ LJℕƨŘdzǒŜŘ LJƨƨℕ

peak shape with all column/mobile phase combinations except for particles with large pore sizes used in combination with either MSA or TFA.

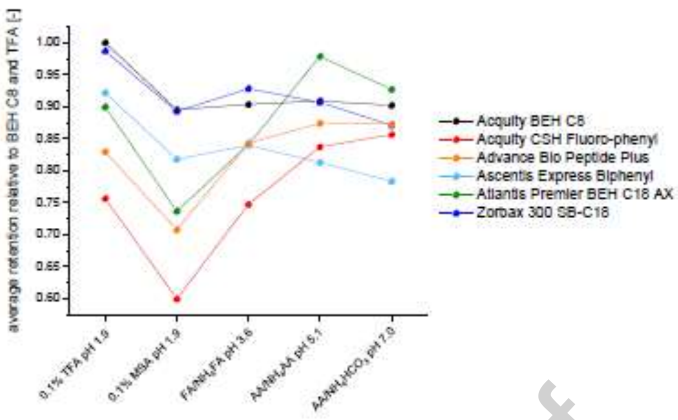

Figure 5. Average retention for peptides with different column / mobile phase combinations relative to retention observed with the BEH C8 - 0.1% TFA combination for bradykinin, GLP-2 (1-33), insulin, ubiquitin and lysozyme.

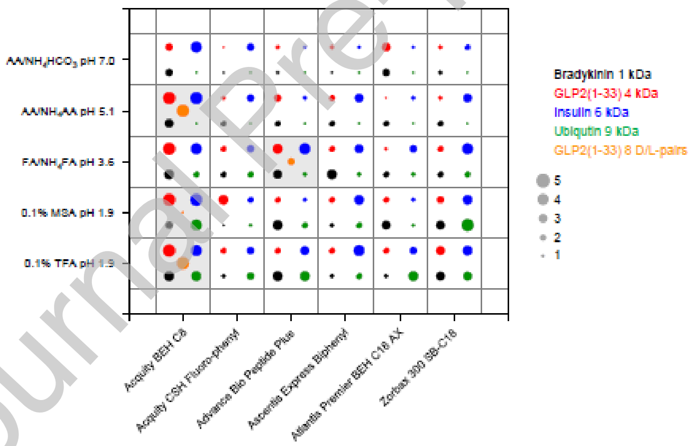

Figure 6. Summary of isomer selectivity (i.e., EIC chromatograms) rankings for 30 column / mobile phase combinations and four peptides with a MW range of 1-9 kDa and a pI range of 3.7-12.5. Circle diameter indicates the ranking level for a particular peptide (larger is better). Combinations with an average ranking for bradykinin, GLP- 2 (1-ooύ I·ўŘ ʎẏădzťʎ ӂпɸp I·ŴŜ ʎẏŘʎŎI·ŰŜŘ ǿʎŰK I· ăKI·ŘŜŘ background. Poor peak shapes were observed for ubiqutin and lysozyme with all column / mobile phase combinations except for particles with large pore sizes in combination with either MSA or TFA. Lysozyme was excluded since a poor MS signal did not allow assessment of isomer selectivity.

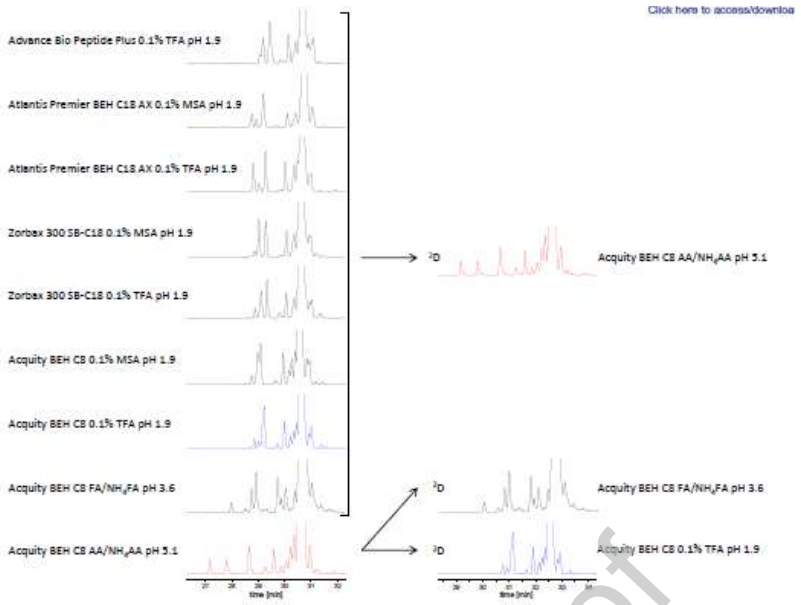

Figure 7. Extracted ion chromatograms for the m/z corresponding to the main component of GLP (1-33) separated using the most promising column / mobile phase combinations identified in the preceding sections. Left ς Column / Y2ōłfŜ LJKI·ăŜ Ŏ2YōλY1·ůł2yă ℕI·y[łyƎ жп ₮2ℕ I·ØŜℕI·ƎŜ ƎŜyŜℕI·t selectivity at 215 nm, and use in the first dimension. Right - Column / mobile phase combinations ranked highest for use in second dimension separations. Time axes are aligned on the main peak in each chromatogram. See also Supplementary materials S5 and S6 for the same type of illustration using bradykinin and insulin.

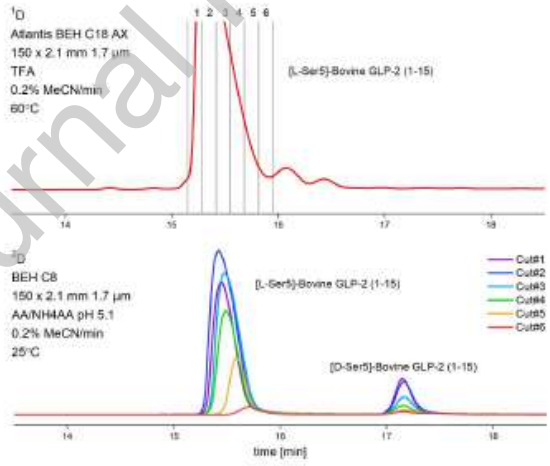

Figure 8. An example of a peak purity analysis used to evaluate the suggested 2D-LC-MS peak purity strategy. [L-Ser5]-GLP-2(1-15) spiked with 12% of [D-Ser5]-GLP-2(1-15). ^{2}D chromatograms have been aligned using the retention of the D-form. Experimental condition 1D: Atlantis Premier BEH C18 AX 150 x 2.1 mm 1.7 µm, 60°C, 0.3 mL/min, gradient 15 ς 19.5 ς 100 ς 100 ς 15 ς 15 %B at 0 ς 30 - 30.1 ς 33 ς 33.1 ς 40 min. Solvent A 0.1% (v/v) TFA in water. Solvent B 0.1% (v/v) TFA in ACN. Experimental conditions 2D: Acquity BEH C8 150 x 2.1 mm 1.7 µm, 25°C, 0.3 mL/min, gradient 4 ς 8 ς 100 ς 100 ς 4 ς 4 %B at 0 ς 20 - 20.1 ς 22 - 22.1 ς 29 min. Solvent A 7.8 mM AA 20

mM NH_4AA pH 5.1. Solvent B 33.1 mM AA 85.5 mM NH_4AA pH 5.1 in water / ACN 2:8 (v/v). The ^2D gradient was calculated based on retention for gradient times of 10, 20 and 30 min for 5 to 40% B (dwell volume 0.19 mL, dead volume 0.36 mL). Predicted retention time 19.9 and 21.8 min actual 15.6 and 16.7 min resulting in prediction error of 23%.

CRediT authorship contribution statement

Stephan Buckenmaier: Conceptualisation, Methodology, Formal analysis, Resources, Review & editing, Funding acquisition

Mel Euerby: Conceptualisation, Methodology, Formal analysis, Review & editing

Patrik Petersson: Conceptualisation, Methodology, Formal analysis, Resources, Writing – review & editing, Funding acquisition

Dwight Stoll: Conceptualisation, Methodology, Review & editing, 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:

Table 1. Peptide amino acid sequence, molecular weight and calculated pI. Uppercase and lowercase letters are used to designate the L- and D-forms of the amino acid residue respectively.

Peptide	**Amino acid sequence**	**MW [kDa]**	**pI [-]**
Bradykinin	RPPGFSPFR	1.1	12.5
Rat GLP-2 (1-33)	HADGSFSDEMNTILDNLATRDFINWLIQTKITD	3.8	3.7
Bovine insulin	GIVEQCCASVCSLYQLENYCN-FVNQHLCGSHLVEALYLVCGERGFFYTPKA	5.7	5.3
Bovine ubiquitin	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEPIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLR	8.5	7.6
Chicken lysozyme	MRSLLILVLCFLPLAALGKVFGRCELAAAMKRHGLDNYRGYSLGNWVCAAKFESNFNTQATNRNTDGSTDYGILQINSRWWCNDGRTPGSRNLCNIPCSALLSSDITASVNCAKKIVSDGNGMNAWVAWRNRCKGTDVQAWIRGCRL	16.2	11.0
Bovine GLP-2 (1-15)	HADGSFSDEMNTVLD	1.6	3.4
[D-His1]-Bovine GLP-2 (1-15)	hADGSFSDEMNTVLD	1.6	3.4
[D-Asp3]-Bovine GLP-2 (1-15)	HAdGSFSDEMNTVLD	1.6	3.4
[D-Ser5]-Bovine GLP-2 (1-15)	HADGsFSDEMNTVLD	1.6	3.4
[D-Ser7]-Bovine GLP-2 (1-15)	HADGSFsDEMNTVLD	1.6	3.4
[isoAsp3]-Bovine GLP-2 (1-15)	HAiDGSFSDEMNTVLD	1.6	3.4
[D-isoAsp3]-Bovine GLP-2 (1-15)	HAidGSFSDEMNTVLD	1.6	3.4
[Asp11]-Bovine GLP-2 (1-15)	HADGSFSDEMDTVLD	1.6	3.3
[D-Asp11]-Bovine GLP-2 (1-15)	HADGSFSDEMdTVLD	1.6	3.3
[Asp21, Gly22,Ile27]-Bovine GLP-2 (16-33)	SLATRDGINWLIQTKITD	2.0	6.6
[D-Asp21,Gly22,Ile27]-Bovine GLP-2 (16-33)	SLATRdGINWLIQTKITD	2.0	6.6
[isoAsp21,Gly22,Ile27]-Bovine GLP-2 (16-33)	SLATRiDGINWLIQTKITD	2.0	6.6
[D-isoAsp21,Gly22,Ile27]-Bovine GLP-2 (16-33)	SLATRidGINWLIQTKITD	2.0	6.6

Table 2. Stationary phases / columns (150 x 2.1 mm I.D.)

Column	Pore size [Å]	Particle size [µm]	Description	Manufacturer
Acquity BEH C8	130	1.7	An end-capped, trifunctional C8 alkyl ligand bonded to ethyl bridged silica hybrid material	Waters
Acquity CSH Fluoro-phenyl	130	1.7	A non end-capped, trifunctional pentafluorophenyl ligand bonded to ethyl bridged silica hybrid material which possesses a low level positive surface charge (pyridyl functionality positively charged below pH 5)	Waters
Advance Bio Peptide Plus	100	2.7	An end-capped superficially porous particle, C18 alkyl ligand with a positive charge on the surface of the particle similar to CSH C18 and Luna Omega PS C18.	Agilent
Ascentis Express Biphenyl	90	2.7	An end-capped, superficially porous particle with a biphenyl ligand	Supelco
Atlantis Premier BEH C18 AX	95	1.7	An end-capped, trifunctional C18 alkyl ligand bonded to ethyl bridged silica hybrid material with a anion exchange functionality (tertiary alkylamine positively charged below pH 8)	Waters
Zorbax 300 SB-C18	300	1.8	A non end-capped, C18 alkyl ligand with diisobutyl sterically protected siloxane bonds on wide pore material	Agilent

Table 3. Weak (A) and strong (B) mobile phases.

Name	Number	Composition	pH	Ionic strength [mM]
0.1% TFA pH 1.9	1A	TFA / water (1:999 v/v)	1.9	13
	1B	TFA / water / ACN (0.9:199.1:800 v/v/v)		
0.1% MSA pH 1.9	2A	MSA / water (1:999 v/v)	1.9	15
	2B	MSA / water / ACN (1:199:800 v/v/v)		
FA/NH_4FA pH 3.6	3A	12.3 mM formic acid and 9.9 mM ammonium formate	3.6	10
	3B	53.1 mM formic acid and 42.9 mM ammonium formate / ACN (2:8 v/v)		
AA/NH_4AA pH 5.1	4A	7.8 mM acetic acid and 20.0 mM ammonium acetate	5.1	20

	4B	33.1 mM acetic acid and 85.5 mM ammonium acetate / ACN (2:8 v/v)		
AA/NH_4HCO_3 pH 7.0	5A	3.2 mM acetic acid and 20.0 mM ammonium hydrogen carbonate	7	20
	6B	16.2 mM acetic acid and 100.0 mM ammonium hydrogen carbonate / ACN (2:8 v/v)		

Table 4. Column and mobile phase combinations with highest average ranking for EIC isomer performance.

Column	Solvent	Bradykinin* 1 kDa	GLP-2(1-33)* 4 kDa	Insulin* 6 kDa	GLP-2(1-15)** 2 kDa average for 5 D/L-pairs	[Gly22,Ile27]-GLP-2(16-33)** 2 kDa average for 2 D/L-pairs	Average
Acquity BEH C8	FA/NH_4FA pH 3.6	4.0	5.0	4.5	3.3	3.5	4.1
Acquity BEH C8	AA/NH_4AA pH 5.1	3.5	5.0	5.0	3.0	3.5	4.0
Acquity BEH C8	0.1% MSA pH 1.9	3.0	5.0	5.0	3.5	4.0	3.9
Acquity BEH C8	0.1% TFA pH 1.9	4.0	5.0	4.5	3.2	3.0	3.9
Advance Bio Peptide Plus	FA/NH_4FA pH 3.6	3.5	4.0	4.5	2.0	3.0	3.6

*Ranking 1-5 based on number of peaks, resolution of main peak and general peak shape (best performance = 5).

**Ranking was based on $|t_{gD}/t_{gL} - 1|$ where t_{gD} is the retention time of the D-form etc.See Supplementary material 3 for ranking of all 8 D/L-pairs.