This is a peer-reviewed, accepted author manuscript of the following article: Field, J. K., Euerby, M. R., & Petersson, P. (2020). Investigation into reversed phase chromatography peptide separation systems part III: establishing a column characterisation database. *Journal of Chromatography A*, *1622*, [461093]. https://doi.org/10.1016/j.chroma.2020.461093

Investigation into Reversed Phase Chromatography Peptide Separation Systems Part III: Establishing a Column Characterisation Database

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Abstract

The Peptide RPC Column Characterisation Protocol was applied to 38 stationary phases, varying in ligand chemistry, base silica, end capping and pore size, which are suitable for the analysis of peptides. The protocol at low and intermediate pH is based on measuring retention time differences between peptides of different functionality to calculate selectivity delta values. The characterisation was designed to explore increases / decreases in positive or negative charge (deamidation), steric effect (i.e. racemisation / switch in amino acid order), oxidation and addition / removal of aromatic moieties. The necessity of developing a characterisation protocol specifically for peptide analysis was highlighted by the fact that the small molecule databases (Snyder's Hydrophobic Subtraction Model and the extended Tanaka protocol) failed to correlate with the Peptide RPC Column Characterisation Protocol. Principal Component Analysis was used to demonstrate that the protocol could be used to identify columns with similar or dissimilar chromatographic selectivity for the purpose of selectivity back-up or method development columns respectively. This was validated using peptide fragments derived from the tryptic digest of bovine insulin and carbonic anhydrase. It was also demonstrated that the presence of positively charged functional groups on the stationary phase was advantageous as it yielded very different chromatographic selectivity and improved peak shape.

Keywords

Database; Reversed Phase Chromatography; Characterisation; Peptides; Stationary Phase

Highlights

- 38 RPC columns were characterised at low and mid pH using a peptide based protocol
- Probes for hydrophobic, electrostatic, polar, steric and aromatic interactions
- No correlation with two protocols based on small molecules could be observed
- Prediction of similarities and differences were validated using protein digests
- Addition of positively charged functionality alters separation selectivity and improves resolution

1 Introduction

The number of stationary phases with different ligand functionalities has increased substantially over the past few decades. This greater range of chemistries has vastly increased the number of column-analyte interactions with the possibility of different chromatographic selectivities available for the chromatographer. However, the plethora of choice can make column selection a daunting task. The selection of the stationary phase is one of the most critical parameters which affects selectivity, and hence can impact on the success of developing chromatographic methods. By choosing stationary phases with different chromatographic properties, the likelihood of maximising selectivity differences increases, which improves the efficiency of the method development process. Alternatively, selecting stationary phases which possess similar chromatographic selectivity is also an important activity for the identification of a back-up column for the methodology. One effective means of doing both activities is to compare column selectivity from a database where the stationary phases have been characterised. Well-defined column characterisation protocols have been described in the literature which utilise small molecules as probes to establish the interactions of a stationary phase. Two of the largest databases are available free on the internet, which use either Snyder's Hydrophobic Subtraction Model or extended Tanaka protocol by Euerby *et al.* [1-7].

However, the relevance of small molecule column characterisation protocols for peptides was found to be minimal, with no correlation and it is believed that stationary phases should be characterised using relevant probes i.e. peptide probes to assess columns used for peptide separations [8]. With the recent development of the Peptide RPC Column Characterisation Protocol, it is now feasible to characterise stationary phases which highlight interactions, relevant for peptide separation methods, which was not feasible before [9, 10]. This protocol characterises the stationary phase at both low and intermediate pH using gradient chromatography, where the solvents used were rationalised in reference [9]. Twenty-six probes were designed to interrogate the phases, before being iteratively assessed to reduce the number of peptides required but still adequately describe the column with

minimal loss of information [9]. The robustness of the protocol was critically assessed using a reduced factorial design to establish which variables must be carefully controlled to ensure the integrity of the protocol [10]. The definitive protocol and the mitigation necessary to ensure robustness can be found elsewhere [10]. The mitigation included a further reduction of the number of peptides and delta values used in the definitive protocol applied in the current study [10].

The newly developed peptide-based protocol was performed on 43 columns provided by six different stationary phase manufacturers, the database of these results is available upon request from the corresponding author. The results were collated and evaluated using principal component analysis (PCA). To validate the protocol's ability to predict similarities and differences in selectivity a range of stationary phases was selected from the PCA to highlight the similarity between phases for back-up column purposes and phases which demonstrate selectivity differences for method development strategies. Both approaches were validated using tryptic digests of carbonic anhydrase and bovine insulin which produced multiple peptide fragments. The protocol was also compared against characterisation approaches based on small molecules to ascertain its necessity for selecting appropriate stationary phases for peptide separations. Finally, the protocol highlighted potential areas for new column chemistries which could be exploited to achieve further improvements in separation selectivity.

2 Experimental

2.1 Chemicals and Reagents

All water, acetonitrile and mobile phase additives (ammonium formate (AF) and formic acid (FA)) 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. Carbonic anhydrase, bovine insulin, trypsin, TRIS base, TRIS HCl and hydrochloric acid which were used to digest the proteins to demonstrate the applicability of the Peptide RPC Column Characterisation database, were supplied by Sigma Aldrich. The sequences for the peptide probes followed either the base sequence for Bovine GLP-2 (1-15) "HADGSFSDEMNTVLD" or Bovine GLP-2 (16-33) "SLATRDFINWLIQTKITD". A further description of the peptide probes can be found in references [9, 10].

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). A Shimadzu single quadrupole Mass Spectrometer (LCMS 2020) was used as a secondary detector with positive electrospray ionisation. The LC configuration had a dwell volume of 342 µL and system retention volume of 14 µL [11].

2.3 Stationary Phases

All columns were new as supplied by the manufacturer and used a standardised column format of 150 x 2.1 mm. The Peptide RPC Column Characterisation database was expanded using 43 stationary phases from several column manufacturers, which differed by ligand chemistry, pore size and base silica. The database is available from the corresponding author upon request. A brief description of each of the phases assessed can be located in Table 1. The peak apex of a water injection was used as the dead time marker [11].

2.4 Column Characterisation Protocols

The description of the procedure for the Peptide RPC Column Characterisation protocol and mitigation to ensure the integrity of the protocol are all described within reference [10] in Appendix 1. The Peptide RPC Column Characterisation Protocol was compared against the extended Tanaka protocols and Snyder's Hydrophobic Subtraction Model which are well described within literature [1-4, 6, 7, 12-15].

2.5 Protein Digests

Trypsin was added to either carbonic anhydrase or bovine insulin (all 0.4 mg/mL) in a ratio of 1:20 respectively, and dissolved in 50 mM TRIS buffer pH 7.5 (7.4 mM TRIS base and 42.6 mM TRIS HCl). The solutions were stored at 37 °C for 24 hours. The digestion was stopped by the addition of 37% HCl to reduce the pH to 2.5 (3 mL HCl was added to 20 mL of sample). The digest was chromatographed using either low or intermediate pH. The low pH conditions used 0.1% formic acid in water for the aqueous component and 0.1% v/v formic acid in acetonitrile for the organic component. The intermediate pH conditions used 20 mM ammonium formate in water for the

aqueous and pure acetonitrile for the organic component. The gradient for both mobile phases was as follows: 5-60 %B over 60 minutes, where it was held at 60%B for 2 minutes, before dropping to initial conditions in 0.1 minutes, and re-equilibrated for 12 minutes (equivalent to 10 column volumes). The gradients were performed on a range of columns selected to give very similar or different selectivity based on PCA (see Section 3.4 below). Analyses were performed at 40 °C and 0.3 mL/min flow rates.

2.6 Software and Calculations

The LC instrument was controlled and data processing performed using LabSolutions (Version 5.86, Shimadzu, Duisburg, Germany). Principal Component Analysis (PCA) was performed using SIMCA (Version 14.1, Umetrics, Umeå, Sweden) and Origin (Version OriginPro 2016, OriginLab, Northampton, MA, USA). The net charges of the peptide probes were calculated at both pH 2.5 and 6.45 using General Protein / Mass Analysis for Windows (GPMAW) software (Version 9.51, Lighthouse Data, Odense, Denmark).

3 Results / Discussion

3.1 Principal Component Analysis

Each stationary phase was assessed using nine peptide probes; three from the 1-15 portion of Bovine GLP-2, and six from the 16-33 portion of Bovine GLP-2, all nine probes differed by just one amino acid residue in order to evaluate subtle selectivity differences of the stationary phases towards these probes. The full 33 amino acid sequence had the propensity to form higher order structures, thus the sequence was divided into two, where the first base sequence contained amino acids 1-15 and the second contained amino acids 16-33 (Bovine-GLP-2 (1-15) and Bovine-GLP-2 (16-33)). Selectivity (referred to as delta, Δt_g^*) was determined by the normalised retention difference ([9, 10]) between certain modified peptides and its corresponding original base sequence (either Peptide Numbers 1 or 13, Table 2). The delta values enabled evaluation of the effect of oxidation, changes in negative charge, steric interactions such as racemisation and switches in amino acid sequence, aromatic and phenolic interactions and changes in positive charge. Initial studies using circular dichroism deduced there was a lack of secondary structure of the peptides within the mobile phase. However, it was later found that the selectivity differences observed could only be explained by the presence of a second order structure of the peptides within the hydrophobic acetonitrile / ligand layer of the stationary phase [9, 16-18].

Delta values for each of the stationary phases were collated and evaluated using PCA. Approximately 82% of the variability was described using two principal components. The addition of a third principal component contributed with an additional 10% but complicates visual comparisons. It was therefore decided to limit the number of principal components to two. Each stationary phase was assigned a classification based on prior knowledge of the phase chemistry and location in the PCA score plot. The score plot in Fig. 1A demonstrates that there are regions of high electrostatic interactions (positive and negative) and a region of low electrostatic / polar interactions. The negative / polar stationary phase character, predominantly in the upper right quadrant of the score plot, also aligns itself with the increase in positive charge probe, Δ(26,13) AF, the changes in aromatic / phenolic character probe, Δ(24,13) AF and oxidation probe, Δ(8a,1) FA (Fig. 1B). It is possible that the Δ(26,13) AF probe is more important in identifying stationary phases with negative character in comparison to Δ(26,13) FA due to the presence of negatively charged silanol groups at intermediate pH. The aromatic / phenolic character probe could potentially distinguish phases which can form hydrogen bonds with the hydroxyl group of the tyrosine substituent, form *pi-pi* interactions with aromatic ligand moieties, or potentially could differentiate phases based on steric interactions, where the larger tyrosine amino acid might alter the secondary structure of the peptide within the hydrophobic, acetonitrile layer in the stationary phase [9, 16, 17]. The oxidised probe is also capable of forming hydrogen bonds with accessible silanol groups at low pH, thus its position within the loading plot can also be rationalised. The region of low electrostatic interactions in the lower right quadrant can be characterised by high Δ(15,13) AF and Δ(16,13) FA, which mostly assess steric interactions. Δ(26,13) FA is also located in the lower right quadrant, where its position is less obvious to interpret. The stationary phases possessing positively charged functional groups are in the upper and lower left quadrants of the score plot which aligns with the increase in negative charge probe, Δ(9,1) FA, in the loading plot (Fig. 1B).

The neutral grouping in the lower right quadrant consists of a plethora of phases with alkyl moieties with high ligand density and end capping. These phases (green circles) include the C18 alkyl ligand phases, but also the Acquity BEH C8, Poroshell HPH C8 and Kinetex C8. One exception to the cluster of neutral phases is the location of the Kinetex C18, which was positioned within the negative / polar phases, close to the Poroshell SB-AQ. This position was affirmed by evaluation of a brand-new column. Comparison of the Kinetex C18 against other C18 alkyl phases using PCA, based on the extended Tanaka protocol or the Hydrophobic Subtraction Model data, did not indicate any potential rational for its divergence in the current study. Hence, it is unclear why the Kinetex C18 displays more polar properties in the peptide characterisation compared to other C18 columns. It is interesting to note that during peptide analysis within Novo Nordisk, the Kinetex C18 column has

been found to display different selectivities compared to other C18 columns on numerous occasions (unpublished results).

The blue square markers in the upper right quadrant denote the negative / polar phases. These consist of phases which either possess a negative moiety in the ligand chemistry and / or contain significant silanophilic interactions by means of low ligand density or no end capping, or a ligand architecture which can promote access to the silica surface. The group contains phenyl phases such as phenyl hexyl, diphenyl and biphenyl phases, where the ligand architecture is such that the peptide probe can potentially gain accessibility to the silica surface and its silanol groups [19, 20]. There are also two pentafluorophenyl phases which, in addition, have the ability to form electrostatic *pi-pi* interactions. This category also contains the Acquity HSS C18-SB, Acquity BEH C4, Poroshell SB-AQ, Zorbax SB-C8, Zorbax SB-C18 and Zorbax SB-C18 300 which are all non end capped phases and possess low ligand density, properties which promote silanol interactions. Although the Acquity BEH C4 has reduced hydrophobic interactions due to the shorter alkyl chain and is non end capped, it is actually located within the neutral alkyl phases. This is perhaps contrary to expectations but the phase is based on hybrid silica with fewer silanol groups to interact with the solutes. In addition, smaller ligands normally yield a higher surface coverage compared to C18 type columns. The PLRP-S is a hydrophobic polymeric phase with no silanol groups. However, its position within the score plot would suggest there are either polar or negative retention mechanisms which this phase can undertake. This could be attributed to either the presence of negatively charged cation-exchange sites on the phase as suggested for the Hamilton PRP-1 [21] or a cation-π aromatic interaction between the benzene functionality of the stationary phase and the positively charged peptide [22]. The Acclaim WCX is a mixed mode stationary phase with a carboxylic acid moiety. The Acclaim WCX was not included in the PCA due to repulsion of the hydrophilic peptides which possessed a negative net charge from the deprotonated carboxylic acid ligand at intermediate pH conditions. However, the negatively charge carboxylic acid using ammonium formate at native pH would suggest this type of phase would be located within the negative / polar character group. At low pH the Acclaim WCX behaves like a C-alkyl phase because of the protonated carboxylic acid terminal ligand.

The stationary phases with positively charged functionalities were scattered within the upper and lower left quadrants of the score plot and consisted of the Acquity CSH range, the Luna Omega PS C18, the Poroshell 120 Bonus-RP and the Polaris Amide C18. These phases were characterised using the extended Tanaka protocols where positive character can be measured using the selectivity between benzene sulfonic acid and toluene ($\alpha_{(BSA/T)}$). The negatively charged benzene sulfonic acid was retained on all the phases, which suggests positive character on the phase. These results would imply that the Polaris Amide C18 and the Poroshell Bonus-RP were both synthesised in a two-step

process where unreacted / non-acylated amino groups are positively charged, thus capable of electrostatic interactions [13]. The CSH range and the Luna Omega PS C18 have all been designed to possess a low amount of positive surface charge in order to improve peak shape of positively charged compounds at low pH and low ion strength [20, 23].

It should be noted the Ascentis Express F5, Bioshell CN, Bioshell C18 and AdvanceBio Peptide Map were not included in the PCA. The Ascentis Express F5 retained the multiple positively charged peptides in ammonium formate which was initially hypothesised due to the dense electronegative charge associated with the fluorinated aromatic ring. However, this was not an issue for the Kinetex F5 or Poroshell PFP which possess similar characteristics for aromatic and dipole character to the Ascentis Express F5 based on the Extended Tanaka protocol. There is greater silanophilic activity on the Ascentis Express F5, as highlighted from the Extended Tanaka protocol results for $\alpha_{(B/P\ pH\ 7.6)}$ and $\alpha_{(B/P\ pH\ 2.7)}$, which could possibly help elude to the added retention of the positively net charged peptides in ammonium formate. The Bioshell CN could be characterised using the formic acid conditions, which exhibited typical peak shapes. Whilst the hydrophobic peptides could be satisfactorily retained under the ammonium formate conditions, the hydrophilic peptides eluted in the void. This prevented the determination of t_g^* and consequently Δt_g^*. The diisopropylcyanopropyl phase has limited hydrophobicity due to the short alkyl linker, thus there is insufficient hydrophobicity to retain the hydrophilic peptides. The silanophilic interaction under intermediate pH conditions ($\alpha_{(Benzylamine/Phenol\ @\ pH\ 7.6)}$) measured in the Extended Tanaka protocol also suggests there is some negative character to the phase, which could also explain the lack of retention of the negatively net charged hydrophilic peptides. Conversely, the peptides were eluted from the Bioshell C18 and AdvanceBio Peptide Map within the given gradient time in ammonium formate. However, under formic acid conditions, the hydrophobic peptides could not be eluted from the stationary phase. The gradient was extended to 100% MeCN with the corresponding %B/min change used on the original gradient without success. This would suggest the phases possess significant hydrophobicity, making them potentially more suitable for less hydrophobic peptides, whilst the Bioshell CN is better suited to more hydrophobic compounds. In comparison, all peptides eluted on the Bioshell C18 and AdvanceBio Peptide Map when TFA was employed as the additive. This is difficult to rationalise as TFA is a more hydrophobic ion pair than formic acid. The AdvanceBio Peptide Map yielded excellent peak shapes for all the peptides with TFA. Consequently, 38 stationary phases were characterised and not the original 43.

The use of PCA enables the user to visualise the difference or similarities between stationary phases. For example, phases which are located within close proximity are presumed to possess similar chromatographic selectivity, whilst those which are separated can be viewed as chromatographically

diverse. This can help to direct the design of new silanes for stationary phases. It is clear that although hydrophobic interactions dominate the retention mechanisms in reversed phase chromatography, there are also other interactions which are important, namely polar and electrostatic interactions.

Phases which could incorporate some form of charge or polar functionality could be of particular interest for peptide chromatography specialists. Some of the most diverse selectivity was achieved on phases which can incorporate these types of interactions, such as the Polaris Amide C18, Acquity CSH Fluoro Phenyl and the Acclaim WCX. These phases are capable of electrostatic and / or polar interactions with the peptides, with different modes of interactions possible depending on pH. These columns were compared against the neutral Ascentis Express C18 phase, using stick plots of the characterisation data in both formic acid or ammonium formate (Fig. 2). The Ascentis Express C18 was selected to represent a standard C18 type stationary phase. The comparison demonstrates the different retentivity of charged peptides on phases with positive or negative character. The Ascentis Express C18 (Fig. 2D) is a neutral phase with little acidic silanol activity, thus the mode of interaction between the stationary phase and peptide is mainly hydrophobic. The Acclaim WCX possessed a similar elution profile under formic acid conditions to the neutral C18 phase which suggests retention is primarily related to the alkyl proportion of the WCX ligand functionality. [Tyr26]-Bovine GLP-2 (Peptide Number 24) possessed greater relative retentivity on the Acclaim WCX compared to the Ascentis Express C18 which is possibly indicative of polar interactions between the polar carboxylic acid moiety and the tyrosine substituted peptide such as hydrogen bonding. The Acquity CSH Fluoro Phenyl and the Polaris Amide C18 (Fig. 2B and C respectively) possessed similar retentivity for the majority of peptides, where the more positively charged [Gly22]-, [Tyr26]- and [Lys26]-Bovine GLP-2 (16-33) (Peptide Numbers 16, 24 and 26, respectively) were repulsed from the positive character stationary phases in comparison to Bovine GLP-2 (1-15) and [L-Asp11]-Bovine GLP-2 (Peptide Numbers 1 and 9, respectively). The change in relative retention for the peptide [Lys26]-Bovine GLP-2 (16-33) (Peptide Number 26) was to a greater degree than the other peptides due to the greater positive net charge (Table 2). The more positively charged hydrophobic peptides experience a greater repulsion on the Polaris Amide C18 which has a higher degree of positive charge on the ligand, thus having a narrower elution window. This is also evident from the extended Tanaka protocol, where the negatively charged benzene sulfonic acid was greatly retained on the Polaris Amide C18 [13].

Under intermediate pH conditions, the Acclaim WCX was negatively charged, which caused the negatively charged hydrophilic peptides to elute on the void. [Lys26]-Bovine GLP-2 (16-33) (Peptide Number 26) possessed significant increases in retentivity on the Acclaim WCX in comparison to the remaining peptides due to the positive net charge of the peptide and the fully charged WCX ligand or

accessibility into the stationary phase, and selectivity was notably different to other stationary phases evaluated. The Polaris Amide C18 and the Acquity CSH Fluoro Phenyl, whilst chromatographically similar at low pH, demonstrated significant selectivity differences under intermediate pH conditions. The positive character on the Polaris Amide C18 was still present, as demonstrated by the low relative retention of [Lys26]-Bovine GLP-2 (16-33) (Peptide Number 26), whilst the negative charged Bovine GLP-2 (1-15) and [L-Asp11]-Bovine GLP-2 (1-15) (Peptide Number 1 and 9) had greater retentivity in comparison to the other phases under the same conditions. The selectivity on the Acquity CSH Fluoro Phenyl, on the other hand, seemed to resemble the Acclaim WCX which suggests a greater degree of negative character on the stationary phase. This could be due to the non end capped silica which results in a higher number of negatively charged silanol groups at intermediate pH causing repulsion of the negatively charged peptides, and greater retention of the positive net charge of [Lys26]-Bovine GLP-2 (16-33) (Peptide Number 26).

The peptide [Lys26]-Bovine GLP-2 (16-33) (Peptide Number 26) was compared in both formic acid and ammonium formate on the four phases in Fig 2. The peak shape on the C18 phase under ammonium formate conditions was significantly improved compared to the low pH conditions, (peak width decreased from 0.107 to 0.052), as expected with the low ionic strength of the formic acid and the multiply charged peptide. The amide phase gave good peak shape under both mobile phase conditions with peak widths of 0.054 and 0.074, which demonstrates the usefulness of the phase with positive character with low ionic strength additives like formic acid. The fluoro phenyl phase also produced similar peak widths under both mobile phase conditions (0.100 and 0.093), but exhibited broader peaks compared to the amide. The WCX phase produced peak widths of 0.154 and 0.161, under low and intermediate pH, respectively. Note type of particle as well as particle sizes differ between these phases as described in Fig. 2.

The score plot in Fig. 1A suggests an empty space in the upper left quadrant which could be occupied by a mixed mode phase with positive character to give a selectivity which is completely different to a standard C18 column whilst maintaining an excellent peak shape (See Section 3.2 for a discussion on the peak capacity). Despite the selectivity potential for mixed mode phases, the successful implementation within the laboratory depends on the batch to batch reproducibility, which has been poor for many traditional SCX, WCX and mixed alkyl / SCX or WCX phases. Newer mixed mode phases would need to be evaluated for batch to batch reproducibility, but they are likely to offer an interesting alternative selectivity for stationary phase development as shown in the current study.

3.2 Peak Capacity

The sample peak capacity (PC^{**}) was used to measure the performance of the separation in both formic acid and ammonium formate, which calculates the number of peaks that can be separated between the first and last eluting analyte in a sample (Eq. 1) [24]. This measurement was selected to represent the performance for a particular stationary phase in the retention window.

$$PC^{**} = \frac{t_{g\,max} - t_{g\,min}}{w_{ave}} + 1 \qquad \text{Eq. 1}$$

Where t_{gmin} and t_{gmax} are the retention times of the first and last eluting peptide and w_{ave} is the average width at base for all the peaks that are separated except for the diastereoisomeric oxidised methionine probes (Peptide Number 8) which are typically poorly separated on most of the stationary phases. The average peak width and the retention time for the first and last eluting peptide are recorded in the supplementary material.

The PC^{**} values for the stationary phases in formic acid ranged between 22-155 (mean 91) whilst the ammonium formate conditions provided a range of 72-272 (mean 189), which illustrates the increased performance which can be generated when using ammonium formate in comparison to formic acid. The Acclaim WCX, PLRP-S and Polaris Amide C18 stationary phases generated PC^{**} of 50, 64 and 83 using the formic acid conditions, respectively, whilst the ammonium formate conditions generated a value of 72 on the Polaris Amide C18. It should be noted that these phases had a greater particle size compared to most other phases assessed (totally porous particle (TPP) 3 µm *versus* TPP 1.8 µm or 2.7 superficially porous particle (SPP)). It is therefore expected that peak capacity should increase by approx. 1.4x when reducing the particle size.

The ammonium formate in the aqueous portion of the mobile phase was 20 mM which is sufficient to improve peak shape by reducing mutual repulsion effects. McCalley *et al*. suggested that the poor peak shape often observed for basic species, such as the positively charged peptides used in this study, is related to overloading caused by mutual repulsion between adsorbed ions of the same charge [21, 25, 26]. Mobile phases such as formic acid exacerbate this issue because there is insufficient ionic strength, where a 0.1% formic acid v/v solution has approximately 2 mM ionic strength in comparison to 20 mM ionic strength for a 20 mM ammonium formate solution. Formic acid mobile phases can, however, produce acceptable levels of peak capacity for phases which possess some positive charge, such as the Polaris Amide C18, the CSH range of stationary phases and the Luna Omega PS C18 [23]. These phases can produce improved peak shapes for basic species, where the peak width at 50% and asymmetry factor at 10% decreased on average for the positive character stationary phases by 43 and 24%, respectively between formic acid and ammonium formate (data not shown). This contrasted with the neutral and negative character stationary phases,

which increased in peak width by 37% on average between formic acid and ammonium formate, and tailing increased by 30% on average between the two mobile phases.

The PC^{**} value observed on the Polaris Amide C18 was lower than expected despite narrow peak widths. This was attributed to a narrower elution window compared to the other phases, where the elution window was 27 and 32% smaller on average in formic acid and ammonium formate, respectively. This is a potential limitation of the PC^{**} measure, which can present a false impression of the results if the elution window is narrower than comparative columns. However, this can be addressed by applying the conventional peak capacity (*PC* – Equation 2) equation to all the stationary phases, where the two different measurements complement each other to provide an overview of the chromatographic performance. The *PC* values in formic acid ranged between 71-561 (mean 297) whilst ammonium formate ranged between 122-470 (mean 341). The Polaris Amide C18's PC values increased to 421 in formic acid and 173 in ammonium formate. There are limitations in both PC^{**} and *PC* measurements, where the former can underestimate the value due to a narrow elution window, whereas the latter can overestimate the separation power, as peaks do not elute across the entire gradient time. However, when used in conjunction, they can accurately represent the chromatographic performance of the stationary phase.

$$PC = \frac{t_G}{w_{ave}} + 1 \qquad \text{Eq. 2}$$

Where t_G is the gradient time for the linear gradient.

3.3 Comparison of the Peptide RPC Column Characterisation Protocol against protocols based on small molecules

The Peptide RPC Column Characterisation Protocol was devised to bridge the deficit in column characterisation approaches for stationary phases used for peptide separations, where it is believed that probes which represent the analytes of interest are best suited for characterising the stationary phase. However, it is important to compare and contrast results produced by the peptide protocol against small molecule column characterisation protocols in order to evaluate its necessity. The small molecule databases are vast with nearly 400 columns characterised by the extended Tanaka protocol and over 600 columns characterised by the Hydrophobic Subtraction Model. Therefore, logically the larger databases would be used to compare columns if there was a correlation. Small molecule characterisation data was obtained from Snyder's Hydrophobic Subtraction Model (HSM) via the

PQRI database [5] with the expanded version on the web [7], the Tanaka protocol in the ACD database, and experimental results for the extended Tanaka protocol [3, 5, 7, 13]. Although 38 columns were successfully characterised using the peptide approach, the comparison was limited to 30 stationary phases which were common between all three databases (see Table 1). The characterisation data for these 30 columns on the three different protocols were combined into a single loading plot, where the locations of the different terms were observed (Fig. 3). The closely located terms highlight a greater correlation compared to terms with larger spacing.

The results from the peptide protocol were compared against the two small molecule approaches which indicated that there were some weak correlations, particularly for electrostatic types of interactions (i.e. Δ(26,13) FA and C(2.8) or $\alpha_{(B/P)\ pH\ 2.7}$ and Δ(9,1)FA and $\alpha_{BSA/T}$, Fig. 3). The correlation between probes were also compared pairwise using traditional linear regression analysis (Table 3). The resulting R^2 values varied between 0.15 – 0.75 and 0.32 – 0.73 between the delta values and the Tanaka terms, and the delta values and HSM terms, respectively (Table 3). The Polaris Amide C18 possessed extreme properties in all three characterisation protocols, which caused the phase to be distinct from other phases. With the Polaris Amide C18 excluded in the comparison, the regression coefficients were reduced with R^2 values between 0.10 – 0.45 and 0.20 – 0.41, respectively. This is due to the extreme properties attributed to the positively charged functionality on the ligand which skewed the relationship analysis.

The results essentially show that there is no correlation between the Peptide RPC Column Characterisation Protocol and the Hydrophobic Subtraction Model or the extended Tanaka protocol. Thereby it proves that there is a need for a RPC column characterisation protocol based on peptide probes.

3.4 Validation of the Protocol

Tryptic digests of carbonic anhydrase and bovine insulin were made in order to generate peptide fragments which could be tracked for selectivity differences. The aim was to establish if the Peptide RPC Column Characterisation database could be used to select stationary phases with diverse selectivity thus producing a different chromatographic profile. It was previously demonstrated that the protocol can be used to find columns with different and similar selectivity for different purposes. However, the same peptides were used to characterise the stationary phases as well as illustrate differences and similarities with respect to selectivity [9]. In the current study based on the tryptic

digests of bovine insulin and carbonic anhydrase, completely different peptides were used for the validation which would be more appropriate.

The Polaris Amide C18, Zorbax 300 SB-C18, Acquity BEH C8, Ascentis Express C18, Kinetex Biphenyl, Acquity CSH C18 and Acquity CSH Fluoro Phenyl were all identified as being chromatographically dissimilar via the score plot in Fig. 1A. Eleven carbonic anhydrase fragments were monitored using mass spectrometry with ions selected via extracted ion chromatograms (EIC) on the six columns using the formic acid additive conditions described in Section 2.5 (Fig. 4). There are clear selectivity differences between the six phases, with the elution order differing between all six phases as well as the degree of resolution between peaks. The Ascentis Express C18, Zorbax 300 C18-SB and Acquity BEH C8 were all categorised as neutral phases, but all demonstrated different elution profiles, which illustrates that phases within the same classification can bestow alternative selectivity. The differences in intensity observed for certain fragments can be explained by signal suppression caused by co-eluting fragments which are not displayed. In addition to the different selectivity, the Acquity CSH C18 and Polaris Amide C18 both reveal superb peak shape using formic acid conditions.

Selectivity can also be measured between two chromatographic conditions (i.e. two different columns or mobile phases) by plotting the retention time of a set of compounds to derive the regression coefficient (R^2) [27, 28]. The R^2 value can be inserted into Eq. 3 to determine the selectivity correlation, where a S value of 0 signifies identical selectivity, whilst a value of 100 identifies orthogonal selectivity.

$$S = 100\ \sqrt{1 - R^2} \qquad \text{Eq. 3}$$

This approach was applied to the retention times of digest fragments, where each column in Fig. 4 was compared directly to the Ascentis Express C18, as a typical C18 phase. Results greater than 10 would typically suggest there are selectivity differences between the two parameters [28]. The S values (Fig. 4) were all greater than 11, with the largest differences achieved between the Polaris Amide C18 and the Ascentis Express C18 (S = 33), which is quite significant.

As important as choosing stationary phases with different selectivity, it is often crucial to identify phases with similar selectivity. The following phases were identified as potentially providing similar selectivity profiles from the score plot (Fig. 1A); Ascentis Express C18 and Poroshell HPH-C18, the Acquity CSH C18 and Luna Omega C18 PS, and finally Kinetex Biphenyl and Ascentis Express Biphenyl. A tryptic digest of carbonic anhydrase was chromatographed using the formic acid gradient conditions described for the peptide characterisation protocol (Fig. 5). The Poroshell HPH-C18 and

Ascentis Express C18 were superimposed in the score plot (Fig. 1A), and clearly demonstrate a very similar eluting profile for the peptide fragments. The order of elution as well as the resolution between peaks are extremely similar. The Ascentis Express Biphenyl and Kinetex Biphenyl were within close proximity to one another in the score plot (Fig. 1A) and also contain a similar ligand moiety. In general, the elution profile is very similar, with some subtle differences between the peaks (see peaks 9-11). The final pair, the Luna Omega PS C18 and Acquity CSH C18, have an alkyl moiety with some degree of positive charge on the surface of the phase. Their location within the score plot (Fig. 1A) suggest that some similarity could be expected but also that there are some differences as their positions are not overlaid. The profiles in Fig. 5 confirms that this is the case, where in general, the elution profile is similar but there also are selectivity differences between some of the critical pairs. The selectivity correlation was applied to the set of columns, where the Poroshell HPH-C18 was compared to the Ascentis Express C18, the Ascentis Express Biphenyl was correlated against the Kinetex Biphenyl and the Luna Omega PS C18 was evaluated against the Acquity CSH C18. The S-values ranged between 2 and 8, which all demonstrate a close correlation between the predicted pair of similar columns.

Similar selectivity differences were observed for the digest of carbonic anhydrase chromatographed at mid pH as well as for the tryptic digest of bovine insulin. Overall, the score plot was successfully used to select stationary phases which could be chromatographically dissimilar with a diverse elution profile or can be used to select phases with similar profiles.

4 Conclusions

A novel column characterisation protocol which employed peptides as probes was applied successfully to 38 out of 43 stationary phases. The results were placed in a database and critically assessed using principal component analysis. Each phase was classified into one of three groups; neutral, negative / polar or positive character, where the column classification was justified based on its position within the score plot and prior knowledge of the phase. This approach allows easy identification of phases which could be chromatographically similar or dissimilar, depending on its location relative to another column.

This protocol facilitates the identification of potential novel stationary phases which could provide alternative selectivity for method development strategies. The study suggests that phases which offer different selectivity and good peak shape for peptides also often possess electrostatic interactions. In particular, mixed mode phases possessing some form of positive moiety show great potential, provided good batch to batch reproducibility also can be obtained.

The results of the Peptide RPC Column Characterisation Protocol were compared against two protocols based on small molecule which highlighted a lack of correlation between the test parameters. Consequently, there is a need for a peptide-based protocol to select appropriate stationary phases for peptide separations.

Finally, the use of stationary phase peptide database to select columns of similar and dissimilar chromatographic selectivities at low and mid pH was proven using tryptic digests. Further work is currently underway to expand the protocol to also cover mobile phases.

5 References

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6 Figure Captions

Fig. 1 A) Score and B) loading plot for 38 stationary phases characterised using the Peptide RPC Column Characterisation Protocol. The different stationary phases have been grouped and colour coded based on prior knowledge of stationary phase properties.

Fig. 2 Comparison of normalised retention of the three test mixtures used to characterise the stationary phase in formic acid and ammonium formate on the (A) Acclaim WCX, (B) Acquity CSH Fluoro Phenyl, (C) Polaris Amide C18, and (D) Ascentis Express C18. Peptide Number 1: Bovine GLP-2 (1-15), 8a/b: [Met(O)10]- diastereoisomers, 9: [L-Asp11]-Bovine GLP-2 (1-15), 13: Bovine GLP-2 (16-33), 14: [D-Ser14]-, 15: [Ile26,Leu27]-, 16: [Gly22]-, 24: [Tyr26]-, 26: [Lys26]-Bovine GLP-2 (16-33). On the right hand side are overlaid chromatograms of [Lys26]-Bovine GLP-2 (16-33) (Peptide Number 26) in formic acid (pink trace) and ammonium formate (black trace).

Fig. 3 Loading plot containing the parameters from the Peptide RPC Column Characterisation Protocol, Hydrophobic Subtraction Model and extended Tanaka protocol. Parameters which are close together could show potential correlation whilst parameters which are at a distance have a limited relationship.

Fig. 4 Extracted ion chromatogram of a tryptic digest of carbonic anhydrase analysed on six stationary phases selected to demonstrate different chromatographic profiles. Chromatographic conditions were as follows: A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile, 5-60%B over 60 minute gradient, 40 °C, 0.3 mL/min, 4 µL injection. *m/z* 1: 330.40, 2: 605.10, 3: 639.10, 4:509.75, 5: 577.75, 6: 487.75, 7: 549.10, 8: 839.10, 9: 759.10, 10: 802.40, 11: 787.10

Fig. 5 Extracted ion chromatograms of a tryptic digest of carbonic anhydrase analysed on three pairs of stationary phases to demonstrate chromatographic similarities. Chromatographic conditions were as follows: A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile, 5-60%B over 60 minute gradient, 40 °C, 0.3 mL/min, 4 µL injection. *m/z* 1: 330.40, 2: 605.10, 3: 639.10, 4:509.75, 5: 577.75, 6: 487.75, 7: 549.10, 8: 839.10, 9: 759.10, 10: 802.40, 11: 787.10

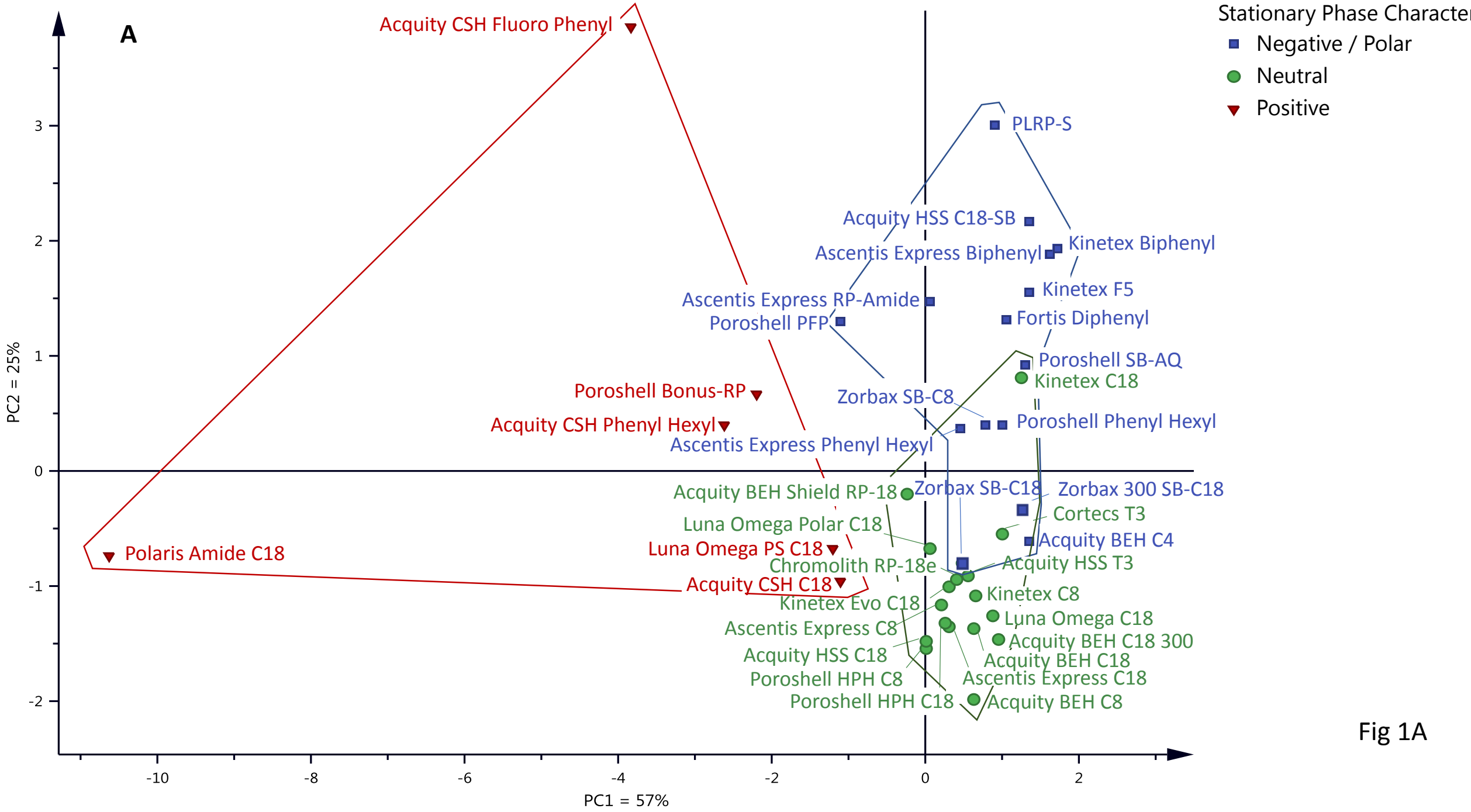

A
Stationary Phase Character
Negative / Polar
Neutral
Positive
Acquity CSH Fluoro Phenyl
PLRP-S
Acquity HSS C18-SB
Kinetex Biphenyl
Ascentis Express Biphenyl
Kinetex F5
Ascentis Express RP-Amide
Fortis Diphenyl
Poroshell PFP
Poroshell SB-AQ
Kinetex C18
Poroshell Bonus-RP
Zorbax SB-C8
Acquity CSH Phenyl Hexyl
Poroshell Phenyl Hexyl
Ascentis Express Phenyl Hexyl
Acquity BEH Shield RP-18
Zorbax SB-C18
Zorbax 300 SB-C18
Cortecs T3
Luna Omega Polar C18
Acquity BEH C4
Luna Omega PS C18
Polaris Amide C18
Chromolith RP-18e
Acquity HSS T3
Acquity CSH C18
Kinetex C8
Kinetex Evo C18
Luna Omega C18
Ascentis Express C8
Acquity BEH C18 300
Acquity HSS C18
Acquity BEH C18
Poroshell HPH C8
Ascentis Express C18
Poroshell HPH C18
Acquity BEH C8
PC2 = 25%
PC1 = 57%
3
2
1
0
-1
-2
-10
-8
-6
-4
-2
0
2

Fig 1A

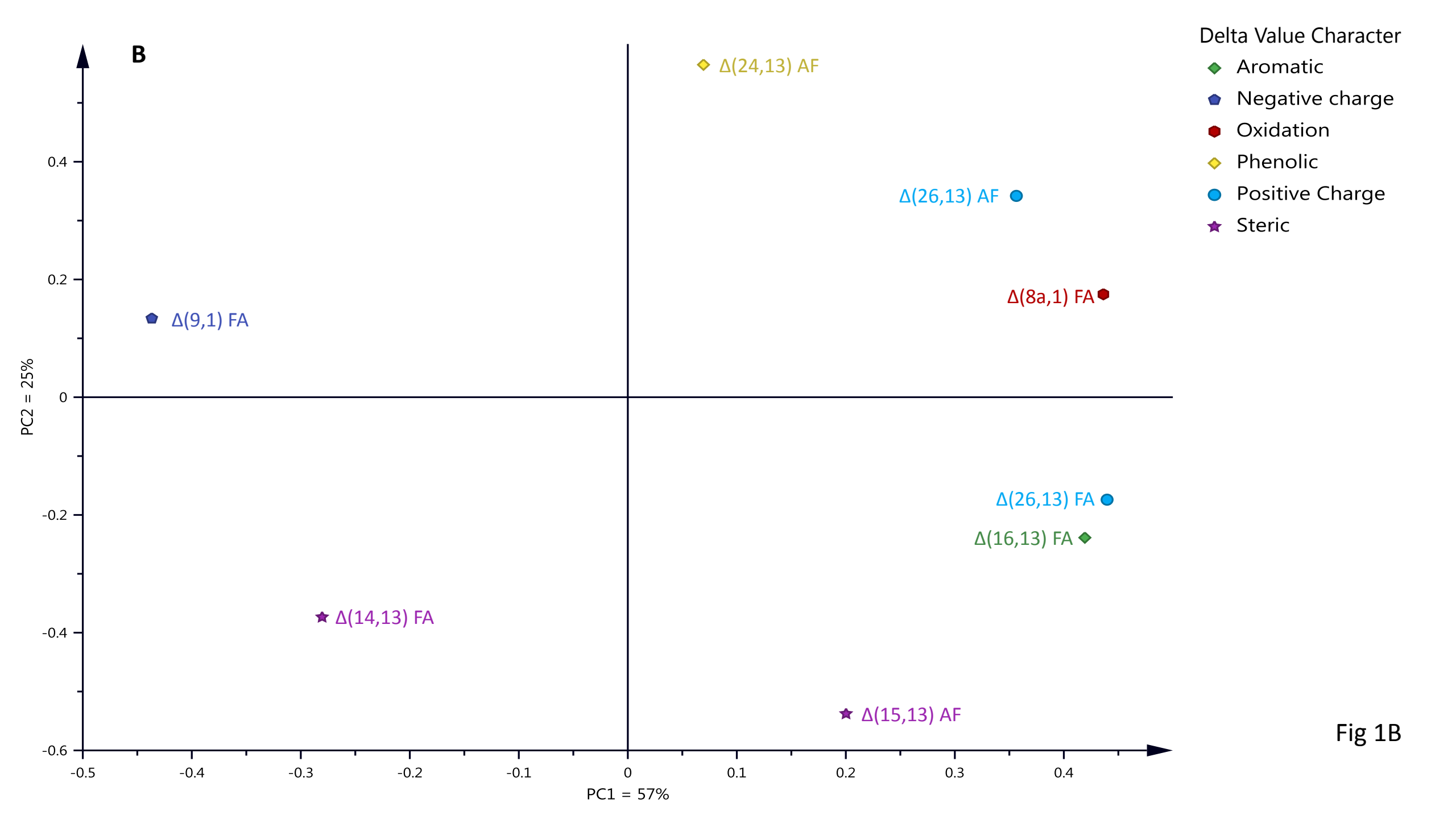

B
Δ(24,13) AF
Δ(26,13) AF
Δ(8a,1) FA
Δ(9,1) FA
Δ(26,13) FA
Δ(16,13) FA
Δ(14,13) FA
Δ(15,13) AF
Delta Value Character
Aromatic
Negative charge
Oxidation
Phenolic
Positive Charge
Steric
PC2 = 25%
PC1 = 57%
0.4
0.2
0
-0.2
-0.4
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4

Fig 1B

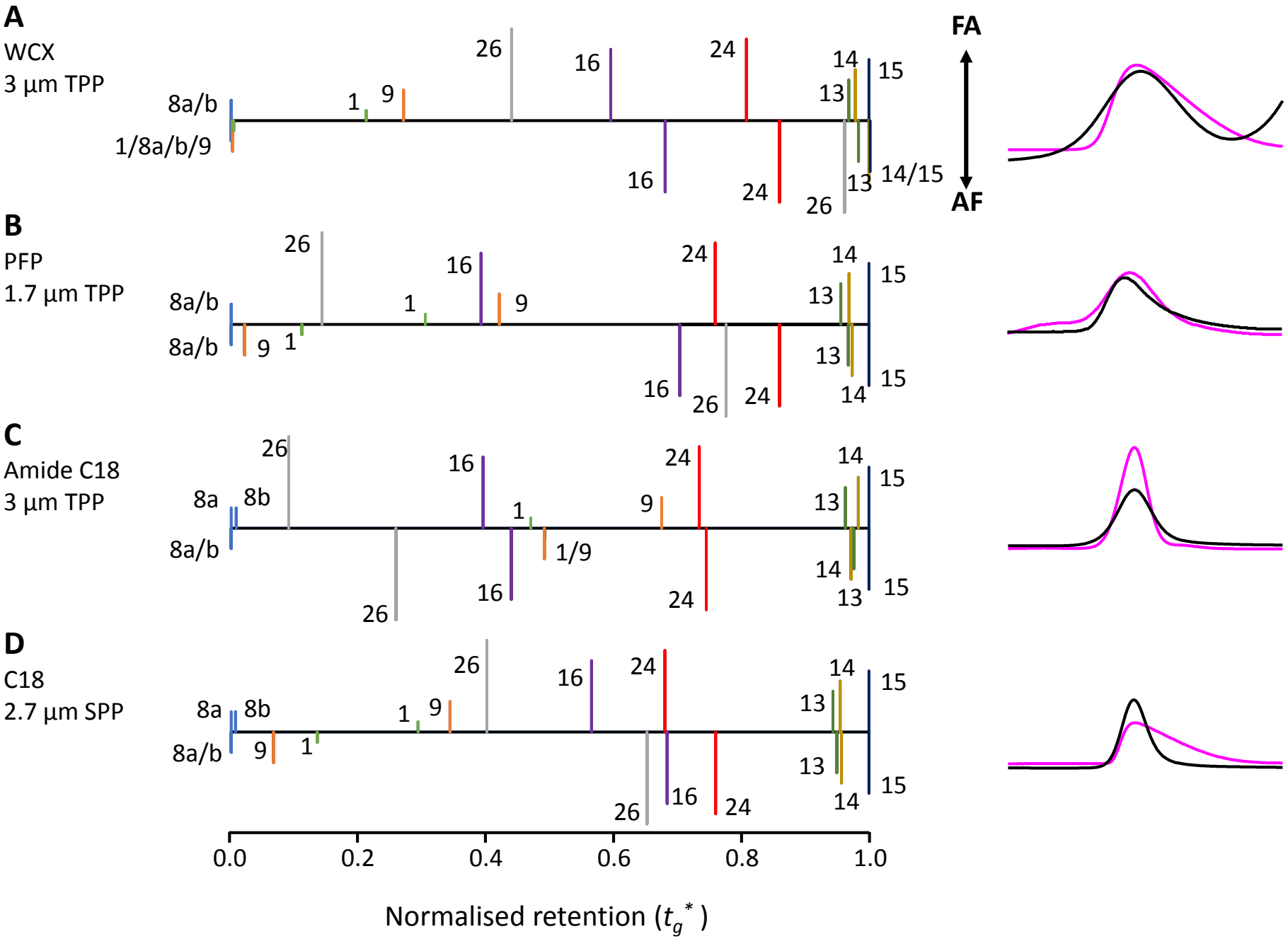

A
WCX
3 µm TPP
B
PFP
1.7 µm TPP
C
Amide C18
3 µm TPP
D
C18
2.7 µm SPP
FA
AF
0.0
0.2
0.4
0.6
0.8
1.0
Normalised retention (t_g^*)

Fig 2

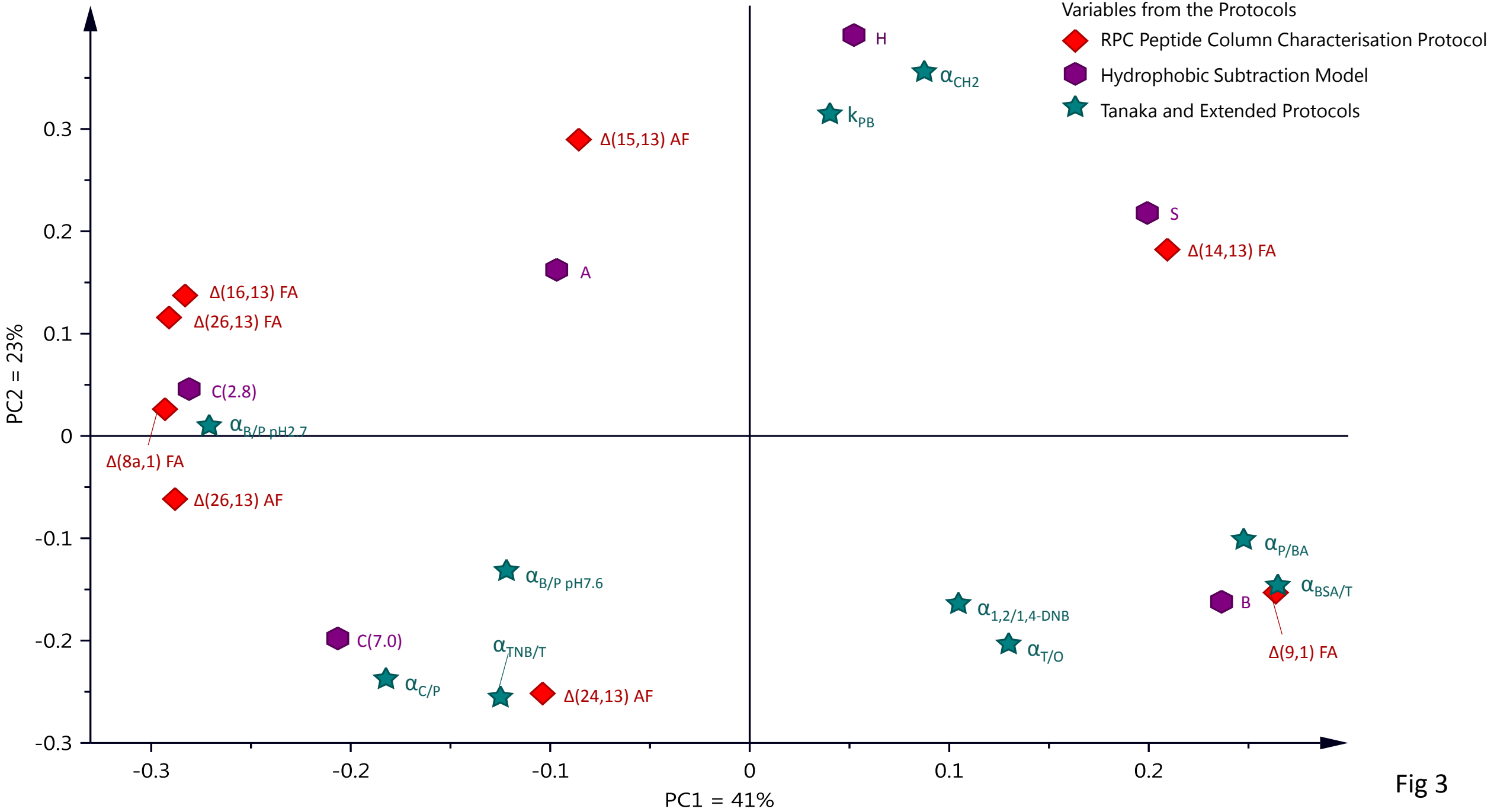

Variables from the Protocols
RPC Peptide Column Characterisation Protocol
Hydrophobic Subtraction Model
Tanaka and Extended Protocols
H
α_{CH2}
k_{PB}
Δ(15,13) AF
S
Δ(14,13) FA
A
Δ(16,13) FA
Δ(26,13) FA
C(2.8)
$\alpha_{B/P\ pH2.7}$
Δ(8a,1) FA
Δ(26,13) AF
$\alpha_{P/BA}$
$\alpha_{BSA/T}$
B
$\alpha_{1,2/1,4\text{-}DNB}$
$\alpha_{T/O}$
Δ(9,1) FA
$\alpha_{B/P\ pH7.6}$
C(7.0)
$\alpha_{TNB/T}$
$\alpha_{C/P}$
Δ(24,13) AF
PC2 = 23%
PC1 = 41%
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.3
-0.2
-0.1
0
0.1
0.2

Fig 3

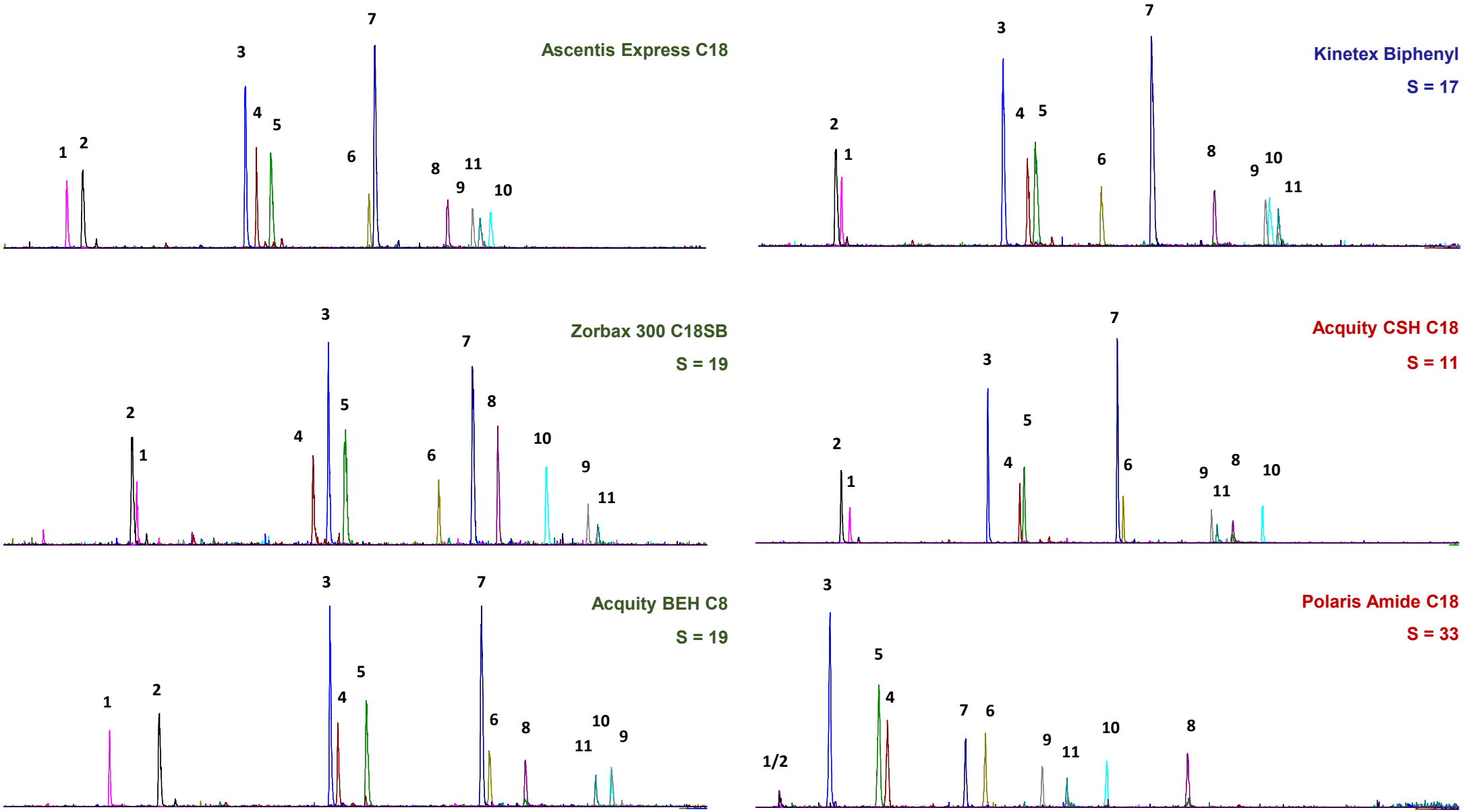
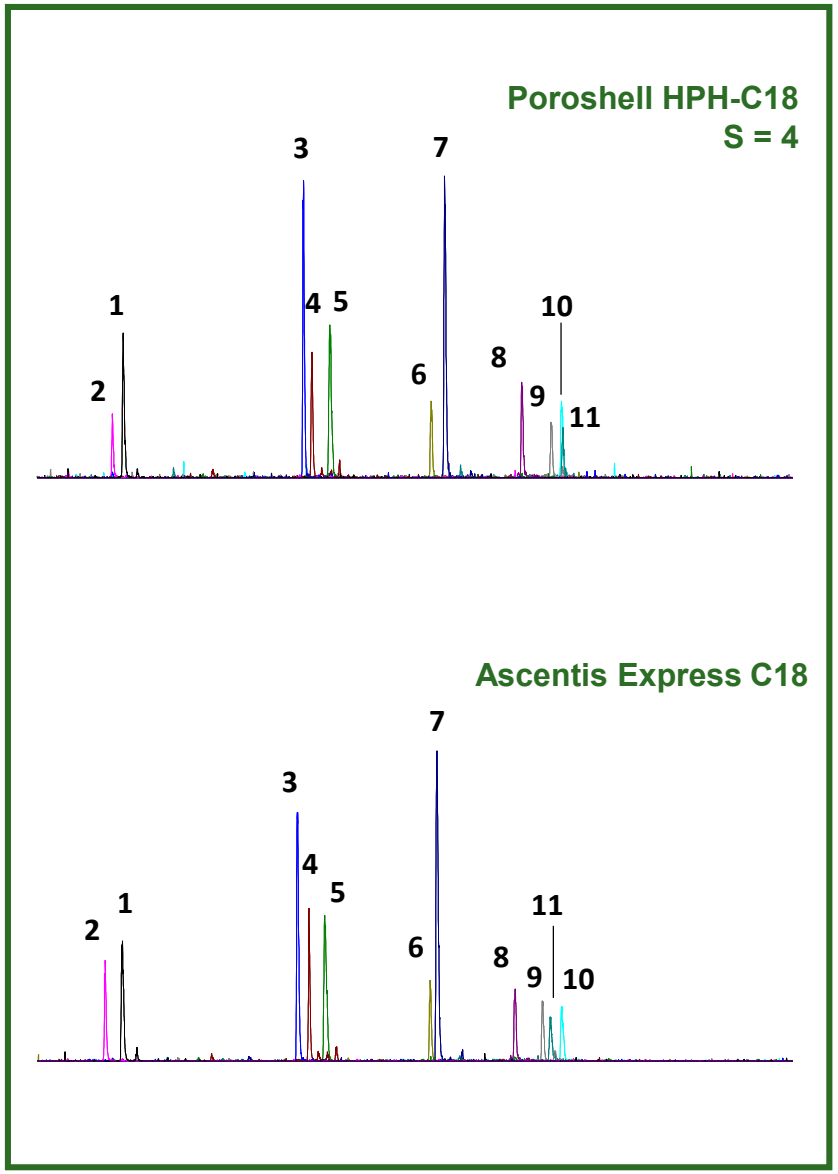
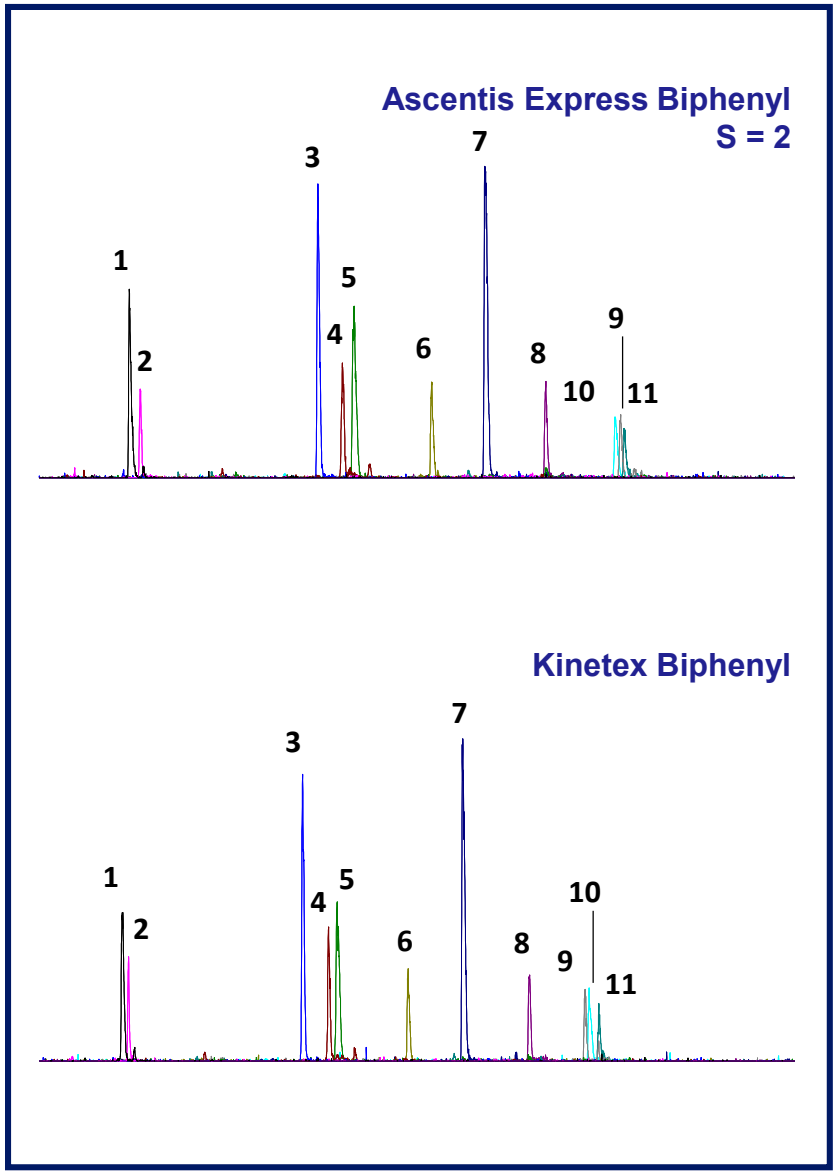

Ascentis Express C18
1
2
3
4
5
6
7
8
9
10
11
Kinetex Biphenyl
S = 17
Zorbax 300 C18SB
S = 19
Acquity CSH C18
S = 11
Acquity BEH C8
S = 19
Polaris Amide C18
S = 33
1/2

Fig 4


Poroshell HPH-C18
S = 4
Ascentis Express C18


Ascentis Express Biphenyl
S = 2
Kinetex Biphenyl

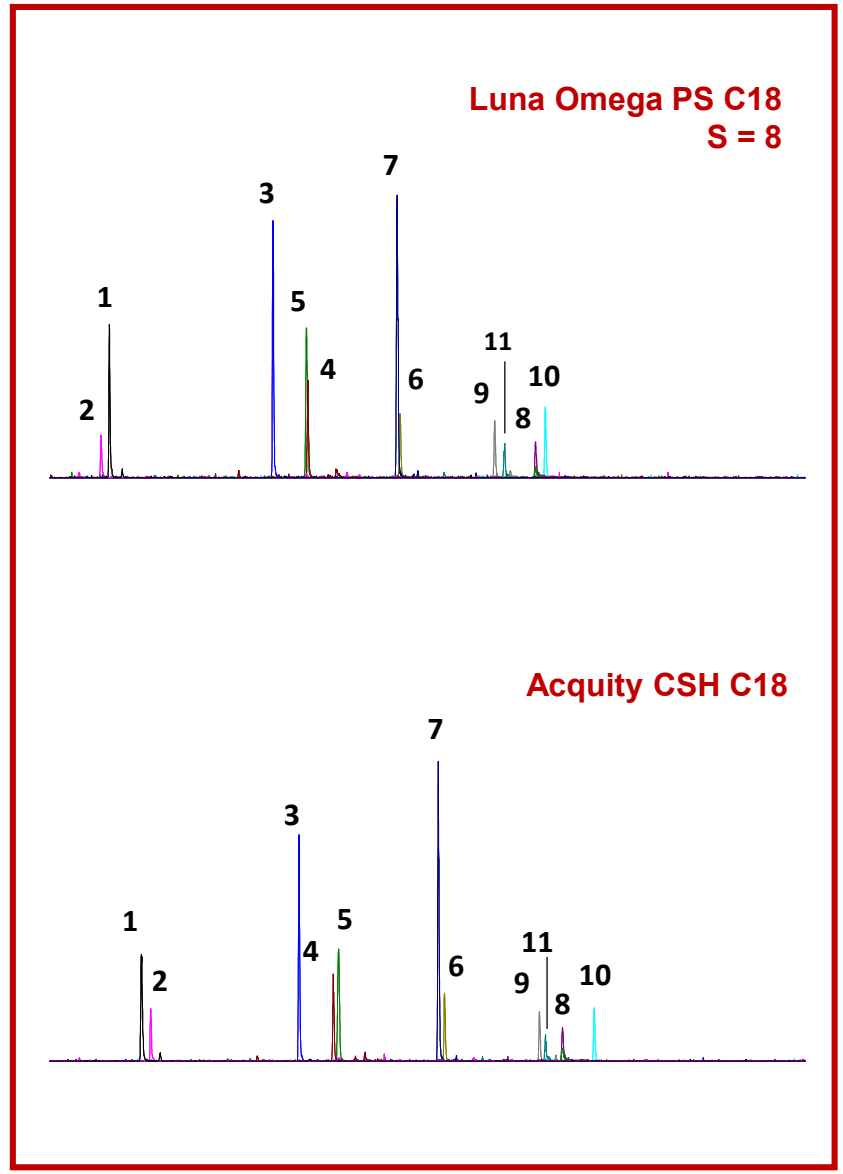

Luna Omega PS C18
S = 8
Acquity CSH C18

Fig 5

Table 1

Stationary phase characterised using the Peptide RPC Column Characterisation Protocol as described in the manufacturer

Column	Pore size (Å)	Particle size (µm)	Description as given by manufacturers	Manufacturer
Acclaim Mixed Mode WCX^{+}	120	3	A mixed mode ligand composed of an alkyl chain with carboxylic acid terminus	Thermo
Acquity UPLC Protein BEH C4	300	1.7	A non end capped, trifunctional C4 alkyl ligand bonded to ethyl bridged silica hybrid material	Waters
Acquity UPLC BEH C8*	130	1.7	An end capped, trifunctional C8 alkyl ligand bonded to ethyl bridged silica hybrid material	Waters
Acquity UPLC Peptide BEH C18*	130	1.7	An end capped, trifunctional C18 alkyl ligand bonded to ethyl bridged silica hybrid material	Waters
Acquity UPLC Peptide BEH C18	300	1.7	An end capped, trifunctional C18 alkyl ligand bonded to ethyl bridged silica hybrid material	Waters
Acquity UPLC BEH Shield RP18*	130	1.7	An end capped, monofunctional alkyl ligand with an embedded carbamide functionality bonded to ethyl bridged silica hybrid material	Waters
Acquity UPLC Peptide CSH C18*	130	1.7	An end capped, trifunctional C18 alkyl ligand bonded to ethyl bridged silica hybrid material which possesses a low level positive surface charge	Waters
Acquity UPLC 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	Waters
Acquity UPLC CSH Phenyl Hexyl	130	1.7	An end capped, trifunctional C6 alkyl chain with a terminal phenyl functionality bonded to ethyl bridged silica hybrid material which possesses a low level positive surface charge	Waters
Acquity UPLC HSS C18*	100	1.8	An end capped, trifunctional C18 alkyl ligand	Waters
Acquity UPLC HSS C18 SB*	100	1.8	A non end capped, trifunctional C18 alkyl phase with low ligand density	Waters
Acquity UPLC HSS T3*	100	1.8	An end capped, trifunctional, 100% aqueous compatible C18 alkyl phase with reduce ligand density	Waters
AdvanceBio Peptide Map^{+}	120	2.7	An end capped, densely bonded C18 alkyl ligand on superficially porous particle	Agilent
Ascentis Express Biphenyl*	90	2.7	An end capped, superficially porous particle with a biphenyl ligand	Supelco
Ascentis Express C8*	90	2.7	An end capped, superficially porous particle with a C8 alkyl ligand	Supelco
Ascentis Express C18*	90	2.7	An end capped, superficially porous particle with a C18 alkyl ligand	Supelco
Ascentis Express F5^{+}	90	2.7	An end capped, superficially porous particle with a pentafluorophenylpropyl ligand	Supelco
Ascentis Express Phenyl-Hexyl*	90	2.7	An end capped, superficially porous particle with a C6 alkyl chain and terminal phenyl functionality	Supelco
Ascentis Express RP-Amide*	90	2.7	An end capped, superficially porous particle with a polar embedded amide group in the alkyl ligand	Supelco
BIOshell A160 Peptide C18^{+}	160	2.7	A non end capped, superficially porous particle with C18 alkyl ligand	Supelco
BIOshell A160 Peptide CN^{+}	160	2.7	A non end capped, superficially porous particle with a diisopropylcyanopropyl ligand	Supelco
Chromolith Performance RP-18e*			An end capped, monolithic C18 alkyl ligand	Merck
Cortecs UPLC T3	120	1.6	An end capped, trifunctional, 100% aqueous compatible C18 alkyl phase with reduce ligand density on a superficially porous particle	Waters

Fortis Diphenyl*	100	1.7	An end capped, diphenyl ligand	Fortis
Kinetex Biphenyl*	100	2.6	An end capped, superficially porous particle with a biphenyl ligand	Phenomenex
Kinetex C8*	100	2.6	An end capped, superficially porous particle with a C8 alkyl ligand	Phenomenex
Kinetex C18*	100	2.6	An end capped, superficially porous particle with a C18 alkyl ligand	Phenomenex
Kinetex Evo C18*	100	2.6	An end capped, superficially porous organo-silica particle with ethane crossing linking and C18 alkyl ligand	Phenomenex
Kinetex F5*	100	2.6	An end capped, superficially porous particle with a pentafluorophenyl ligand	Phenomenex
Luna Omega C18*	100	1.6	An end capped, C18 alkyl ligand	Phenomenex
Luna Omega Polar C18*	100	1.6	An end capped, C18 alkyl ligand with polar modified particle surface	Phenomenex
Luna Omega PS C18*	100	1.6	An end capped, C18 alkyl ligand with a positive charge on the surface of the particle	Phenomenex
PLRP-S	100	3	A hydrophobic styrene /divinylbenzene polymeric particle with no bonded phase	Agilent
Polaris 3 Amide C18*	180	3	An end capped, monofunctional alkyl ligand with an embedded amide functionality	Agilent
Poroshell 120 Bonus-RP*	120	2.7	A triple end capped, superficially porous particle with a polar group embedded in the alkyl ligand	Agilent
Poroshell 120 PFP*	120	2.7	An end capped, superficially porous particle with a pentafluorophenyl ligand	Agilent
Poroshell 120 Phenyl Hexyl*	120	2.7	A double end capped, superficially porous particle with C6 alkyl chain and terminal phenyl functionality	Agilent
Poroshell 120 SB-AQ*	120	2.7	A non end capped, superficially porous particle with C18 alkyl ligand	Agilent
Poroshell HPH-C8	120	2.7	A double end capped, superficially porous particle with C8 alkyl ligand	Agilent
Poroshell HPH-C18*	120	2.7	A double end capped, superficially porous particle with C18 alkyl ligand	Agilent
Zorbax SB-C8*	80	1.8	A non end capped, C8 alkyl ligand with diisopropyl sterically protected siloxane bonds	Agilent
Zorbax SB-C18*	80	1.8	A non end capped, C18 alkyl ligand with diisobutyl sterically protected siloxane bonds	Agilent
Zorbax RRHD 300 SB-C18	300	1.8	A non end capped, C18 alkyl ligand with diisobutyl sterically protected siloxane bonds on wide pore material	Agilent

* Stationary phases compared using the Peptide RPC Column Characterisation Protocol, extended Tanaka protocol and Hydrophobic Subtraction Model

[+]Although 43 stationary phases are described in this table, only 38 could be fully characterised using the Peptide RPC Column Characterisation protocol. This was explained in Section 3.1.

Table 2

Peptide pairs used to measure delta values for column classification and rationale for their selection. The base sequences were described in *Section 2.1*. For further information regarding the selection of peptides, see References [9] and [10].

Change	Delta	Peptide Number	Net Charge		Peptide	Rationale	Average Δt_g (min)	Average Δt_g^* (-)
			FA	AF				
[Met10] → [Met(O)10]	Δ(8a,1)FA*	8a 1	1.2 1.2		[Met(O)10]-Bovine GLP-2 (1-15) [Met10]-Bovine GLP-2 (1-15)	Oxidation	-3.443	-0.288
[L-Asn11] → [L-Asp11]	Δ(9,1)FA	9 1	1.1 1.2		[L-Asp11]-Bovine GLP-2 (1-15) [L-Asn11]-Bovine GLP-2 (1-15)	Increase in negative charge - deamidation	0.655	0.057
[L-Ser16] → [D-Ser16]	Δ(14,13)FA	14 13	2.2 2.2		[D-Ser16]-Bovine GLP-2 (16-33) [L-Ser16]-Bovine GLP-2 (16-33)	Steric – racemisation	0.102	0.008
[Phe22] → [Gly22]	Δ(16,13)FA	16 13	2.2 2.2		[Gly22]-Bovine GLP-2 (16-33) [Phe22]-Bovine GLP-2 (16-33)	Aromatic – removal of aromatic group	-4.609	-0.391
[Leu26] → [Lys26]	Δ(26,13)FA	26 13	3.2 2.2		[Lys26]-Bovine GLP-2 (16-33) [Leu26]-Bovine GLP-2 (16-33)	Increase in positive charge	-6.664	-0.565
[Leu26,Ile27] → [Ile26,Leu27]	Δ(15,13)AF	15 13		0 0	[Ile26,Leu27]-Bovine GLP-2 (16-33) [Leu26,Ile27]-Bovine GLP-2 (16-33)	Steric – switch in amino acid sequence	0.853	0.047
[Leu26] → [Tyr26]	Δ(24,13)AF	24 13		0 0	[Tyr26]-Bovine GLP-2 (16-33) [Leu26]-Bovine GLP-2 (16-33)	Aromatic and phenolic – addition of phenolic group	-3.657	-0.172
[Leu26] → [Lys26]	Δ(26,13)AF	26 13		1 0	[Lys26]-Bovine GLP-2 (16-33) [Leu26]-Bovine GLP-2 (16-33)	Increase in positive charge	-5.956	-0.284

*a corresponds to the first eluting isomer of [Met(O)10]-Bovine GLP-2 (1-15)

NB The sign prior to the Δt_g^* in mins indicates the elution order e.g. [Leu26] → [Lys26] with a Δt_g of -8.213 means that [Lys26] elutes earlier than [Leu26]

Table 3

The regression coefficients between specific delta values and terms from the Hydrophobic Subtraction Model or extended Tanaka protocol.

Delta Value	Compared Against	Interaction Description with Respect to the Analyte	Regression Coefficient (R^2)
Δ(9,1) FA	$\alpha_{(BSA/T)}$	Changes in negative charge	0.75 (0.25)*
Δ(9,1) FA	B	Changes in negative charge	0.55 (0.20)*
Δ(14,13) FA	S	Steric	0.32 (0.25)*
Δ(24,13) AF	$\alpha_{(C/P)}$	Hydrogen bonding	0.13 (0.10)*
Δ(26,13) FA	$\alpha_{(B/P)\ pH\ 2.7}$	Changes in positive charge at low pH	0.55 (0.45)*
Δ(26,13) FA	C(2.8)	Changes in positive charge at low pH	0.73 (0.36)*
Δ(26,13) AF	$\alpha_{(B/P)\ pH\ 7.6}$	Changes in positive charge at intermediate pH	0.15 (0.36)*
Δ(26,13) AF	C(7.0)	Changes in positive charge at intermediate pH	0.41 (0.41)*

* The most extreme column (Polaris Amide C18) was removed and in general this reduced the correlation between the different probes.