Investigation into Reversed Phase Chromatography Peptide Separation Systems Part I: Development of a Protocol for Column Characterisation

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Abstract

A protocol was defined which utilised peptides as probes for the characterisation of reversed phase chromatography peptide separation systems. These peptide probes successfully distinguished between differing stationary phases through the probe's hydrophobic, electrostatic, hydrogen bonding and aromatic interactions with the stationary phase, in addition, to more subtle interactions such as the phase's ability to separate racemic or isomeric probes.

The dominating forces responsible for the chromatographic selectivity of peptides appear to be hydrophobic as well as electrostatic and polar in nature. This highlights the need for other types of stationary phase ligands with possibly mixed mode functionalities / electrostatic / polar interactions for peptide separations rather than the hydrophobic ligands which dominate small molecule separations. Selectivity differences are observed between phases, but it appears that it is the accessibility differences between these phases which play a crucial role in peptide separations i.e. accessibility to silanols, the hydrophobic acetonitrile / ligand layer or a thin adsorbed water layer on the silica surface.

Keywords

Peptides; Protocol; RPC; Characterisation; Column Selectivity; Stationary phase

1 Introduction

The biopharmaceutical industry is an expanding global market with a substantially increased market share over recent years [1-2]. Many pharmaceutical companies are now investing significant resources into developing biomolecules, which is a complex as well as expensive process. Peptides and proteins present a different analytical challenge compared to small molecules due to their size

and physico-chemical properties. They therefore require a different approach for method development activities. There are various publications which are intended to predict peptide retention times based primarily on hydrophobicity and sequence. The success of these predictions is based on an understanding of the role of hydrophobicity based on amino acid side chain differences as well as the secondary structure of the peptide [3-7]. However, there are currently no publications available describing a peptide-based approach for the characterisation and identification of columns with different or similar selectivity for peptide separations to assist in developing methods.

The development of purity methods that separate the active pharmaceutical ingredient from its impurities and degradation products is quite challenging and the potential presence of isomeric species via racemisation in particular can be difficult to determine. Due to their identical mass to charge ratio, isomers cannot be determined directly by mass spectrometry. Thus, screening of combinations of columns and mobile phases that provide large differences in selectivity is of critical importance for the development of purity methods for peptide-based drug products.

Chromatographic methods for biomolecules are often designed based on past experience. This lack of a systematic approach can prove time consuming and a poor use of valuable resources. This is contrary to small molecule separations where there are various articles on the retention mechanisms of small molecules using different stationary phases, therefore method development approaches and stationary phase selection can be made based on rational choice from column characterisation protocols [8-14].

Column characterisation is a process which uses well defined molecular probes, under controlled chromatographic conditions, which allows for direct comparison between different stationary phases, manufacturer and batches of columns. For small molecule work there are many reversed phase chromatography (RPC) characterisation protocols which have resulted in several databases, the largest being Snyder's Hydrophobic Subtraction Model in the PQRI database and the Tanaka and extended protocols by Euerby *et al*. in the ACD database which are both freely available on the internet [9-13]. Lesellier *et al*. alternatively use supercritical fluid chromatography (SFC) to characterise reversed phase stationary phases based on linear solvation energy relationships (LSER) [14]. The different protocols can provide information on the hydrophobicity, steric interactions, hydrogen bonding capability and ion exchange capacity of a column. It is, however, unclear how relevant column characterisations based on small molecules are for the selection of columns for peptides and proteins. This was confirmed by Hodges's *et al*. who concluded that there was very little correlation between the small molecule PQRI database and the retention of a range of peptides. The peptides in the study contained a high proportion of glycine residues to prohibit any "nearest neighbouring effects" in order to gain a greater fundamental understanding of interactions

between each amino acid and the stationary phase, and to minimise any higher order structure such as alpha helix and beta sheets [15-16]. Our study extends on Hodge's initial work to investigate more biologically active peptides which would have potential interferences from "nearest neighbour effects".

This first paper in a series will describe the design of a peptide-based characterisation protocol known as the Peptide RPC Column Characterisation Protocol which will investigate the factors contributing to the selectivity of reversed phase separation systems and assist in the development of rational method development strategies for the separation of peptides using mobile phases selected to meet industry best practices. This currently includes stationary phases but will also eventually include mobile phase composition. This will increase the understanding surrounding selectivity of peptide separations with general mechanisms investigated but also more specifically looking at separations involving degradation and isomeric species. The characterisation protocol can be adapted to develop a column characterisation database similar to that available for small molecule reversed phase chromatography. A database will facilitate the identification of backup columns for existing methodologies and highlight complementary stationary phase combinations with large selectivity differences for method development purposes.

2 Experimental

2.1 Chemicals and Reagents

All water and acetonitrile used were of LC-MS grade and supplied by Sigma Aldrich (Poole, UK). The compounds used in the system suitability test (SST) and the mobile phase additives (ammonium formate, formic acid and trifluoroacetic acid) were also supplied by Sigma Aldrich. 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.

2.2 Chromatographic Conditions

LC separations were performed on a Shimadzu Nexera X2 UHPLC system (Duisberg, 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 [17]. The software used to control the LC system was LabSolutions (Version 5.86).

2.2.1 Peptide RPC Column Characterisation Protocol Conditions

The test conditions utilised both low and intermediate pH to affect selectivity. The low pH mobile phases were prepared as follows A1: 0.1% v/v formic acid in water and B1: 0.1% v/v formic acid in acetonitrile. Formic acid was dispensed using a 1000 µL variable pipette where accuracy was confirmed at each preparation of mobile phase. The gradient at low pH was as follows: 5-45 %B over 40 minutes, where it was held at 45%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 intermediate pH mobile phase was prepared as follows A2: 20 mM ammonium formate in water and B2: 20 mM ammonium formate in acetonitrile/water (80:20 w/w). Solutions were prepared from a 200 mM stock buffer solution (native pH 6.45). The gradient at intermediate pH was as follows: 5.6-61.9 %B over 40 minutes, where it was held at 61.9%B for 2 minutes, before dropping back to initial conditions in 0.1 minutes. The system re-equilibrated for 12 minutes (equivalent to 10 column volumes). Initial investigations utilised a B solvent consisting of 90% acetonitrile, however, the resulting solution was initially turbid with the 200 mM ammonium formate which would become homogeneous upon sufficient mixing. However, in order to increase the ruggedness of the protocol, the B solvent was changed to 80% acetonitrile and the %B/min change adjusted accordingly to the percentages described in this paper to maintain selectivity.

Separations were performed at 40 °C and 0.3 mL/min flow rate. A wavelength of 215 nm was used with background correction at 360 nm, with bandwidths of 8 and 100 nm, respectively. Selected ion monitoring (SIM) in positive ESI mode was used to track the peaks based on the z=2 charge, which was the most abundant ion for the majority of the peptides. Isomers were tracked based on *m/z* and peak area using different concentration in the same sample.

The programmable autosampler was used to prepare the peptide mixtures within the autosampler, allowing for minimal waste of sample. These test mixtures contained two reference peptides, which covered the range in hydrophobicities (i.e. a weakly and strongly retained peptide). Each peptide mixture contained between 5-7 peptides (see Table 3 for a list of the peptides). Samples were stored in the autosampler at 10 °C.

So as to investigate whether TFA masked certain interactions, the mobile phases were as follows A3: 0.1% v/v TFA in water and B3: 0.1% v/v TFA in MeCN. TFA was dispensed from 1 mL aliquots. The same gradient was used as formic acid on the columns described in Section 2.3, whilst all other

operating parameters were as described above. The procedure was applied to a reduced number of peptides which were found to be the most discriminating for column characterisation.

2.2.2 System Suitability Test Conditions

The SST mixture contained water or uracil (dead time marker), benzylamine (changes in silanophilic activity and negative charge), benzene sulfonic acid (changes in positive charge), benzyl alcohol, caffeine (changes in hydrogen bonding), phenol (changes in phenolic interactions [18]), toluene, butyl benzene and pentyl benzene (changes in hydrophobicity). 0.1% formic acid v/v in water was assigned to Line A1 and 0.1% formic acid v/v in acetonitrile was assigned to Line B1. A gradient of 5%B at 0 minutes increased to 100% over 20 minutes, where it was held for 2 minutes before dropping to initial conditions in 0.1 minute. The column was re-equilibrated for 12 minutes.

Separations were performed at 40 °C and 0.3 mL/min flow rate. Wavelengths of 215 and 254 nm were used with background correction at 360 nm, and bandwidths of 8 and 100 nm, respectively.

2.3 Columns

All columns assessed were new as supplied by the manufacturer and were standardised in the 150 x 2.1 mm column format, with particle size (d_p) varying between 1.7 to 3 µm (Table 1). The peak apex of a water injection was used as the dead time marker for each column [17]. All stationary phases were assessed using Tanaka and extended characterisation protocols which are well described in literature [9,19] and can be accessed via the free ACD website [11]. The integrity of the stationary phases was ensured before and after usage by an SST (Section 2.2.2) which assessed the column for changes in hydrophobicity, hydrogen bonding, positive charge and negative charge (silanol activity).

The selection of the stationary phases was based on prior knowledge of the columns and it was presumed would offer a wide range in selectivity to be representative of a larger collection of stationary phases, ideal for a characterisation database. Some columns possessed a large positive charged surface whilst others had a high degree of residual silanol groups in order to characterise a wide array of column functionalities. Some of the columns chosen would not normally have been selected when developing state of the art peptide methods but were essential to establish a database.

An array of C18 phases were selected which differed by base silica (i.e. Acquity BEH C18, Acquity CSH C18 and Acquity HSS C18). In addition to selecting columns which could offer different selectivity, it is of interest to investigate the subtleties between similar phases to be able to identify back up

columns. The ligand density was also evaluated which could impact on selectivity by changing the architecture of the particle and accessibility to the silica surface (i.e. Acquity HSS C18 SB and Acquity HSS T3).

A series of alkyl lengths were also evaluated to assess the effect of varying hydrophobicity where observed selectivity differences can possibly be explained as a function of chain length differences (i.e. Acquity BEH C4, Acquity BEH C8 and Acquity BEH C18).

A commonly used range of columns include polar embedded group (PEG) and phenyl phases, which can offer alternative selectivity. A carbamate ligand (Acquity BEH Shield RP18) and an amide ligand (Polar Amide C18) were selected to represent two styles of PEG functionalities and synthetic routes, where the amide phase is prepared via a two-step synthetic route which generates positive character to the phase due to residual amino groups, whilst the carbamate is a one-step synthetic route thus can be considered neutral. The phenyl phases (i.e. Acquity CSH Fluoro Phenyl, Acquity CSH Phenyl Hexyl, Ascentis Express Biphenyl and Fortis Diphenyl) vary by alkyl linker (i.e. C3-C6), fluoro-substitutions and the number of aromatic rings, which all impact on the available interactions with probes.

The final phase selected to develop the characterisation protocol was the Acclaim Mixed Mode WCX, which is a weak cation exchange phase based on a carboxylic acid moiety. This phase can offer hydrogen bonding interactions at low pH with the protonated carboxylic acid moiety and substantial electrostatic interactions at intermediate pH via the negatively charged dissociated carboxylic acid. Both types of interactions might provide selectivity differences for polar and charged peptide species.

2.4 Software and Calculations

Principal Component Analysis (PCA) was performed using SIMCA (Version 14.1, Umetrics, Umeå, Sweden) and Origin (Version OriginPro 2016, OriginLab, Northampton, MA, USA). The variables within the PCA were all autoscaled, in order to give each variable the same importance. 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 and Discussion

3.1 Rationale for Design of Peptide

Bovine GLP-2, which is similar to Human GLP-2, was selected as the base sequence to probe the stationary phases (Table 2). It is relevant to the biopharma industry, has a typical chain length and possesses typical degradation sites. Additionally, Bovine GLP-2 is an attractive probe as it does not contain cysteine which is likely to limit the stability of peptides due to sulfide bridge formation or shuffling [20].

The peptide chain contains 33 amino acids, and has the propensity to form secondary structures such as alpha helix or beta sheets. The length of the peptides was reduced to 15 and 18 amino acids to reduce the probability for a secondary structure in an aqueous environment [21]. This length is similar to the length typically obtained by proteolytic digests of proteins [16]. A lack of secondary structures was confirmed using circular dichroism in organic / aqueous conditions and temperature corresponding to the chromatographic conditions where the characteristic absorbances were absent for any higher order structure [22].

Bovine GLP-2 can be viewed as having a more hydrophilic segment (amino acids 1-15, in Table 2) and a more hydrophobic segment (16-33). The use of the two base sequence segments instead of the single peptide would minimise the probability of secondary structures and allow for a selection of peptide probes to be synthesised which potentially could be explained in terms of retention and selectivity. In total, 26 rationally designed peptide probes were synthesised to characterise the peptide separation system. The altered position in the peptide sequence and physico-chemical properties (i.e. logP, pI and net charge) can be seen in Table 3.

The peptide chain contained several sites for common degradation, which include racemisation, isomerisation, oxidation and deamidation. Racemisation can occur at the chiral centre of the α carbon forming the D-isomer, where the rate of racemisation can depend on reaction parameters such as pH, temperature and solvents. This is particularly prevalent for serine and histidine which are known to be susceptible to racemisation during the synthetic process [23-24]. The sequence provided four racemisation sites for serine or histidine (Peptide Numbers 2, 6, 7 and 14). Aspartic acid is also known to undergo racemisation in addition to isomerisation to the isoaspartate, thus a further eleven analogues were synthesised to probe the separation of the racemic and isomeric degradants (Peptide Numbers 3, 4, 5, 9, 10, 11, 12, 16, 17, 18 and 19).

Oxidation can be explored via the methionine residue at position 10 in the chain (Peptide Number 8). It can readily oxidise during the synthetic process or during storage into two diastereoisomeric sulfoxide species by means of the lone pair of electrons on the sulfur in a 1:1 ratio.

The loss of an amide functionality from a peptide is known as deamidation. This is observed for the amino acids glutamine and in particular asparagine [25-26], especially when adjacent to an amino acid such as glycine, serine or threonine. A cyclic succinimide intermediate forms between the peptide bond and the asparagine side chain, which can ring open to form both the D- and the L-forms of aspartate or isoaspartate via hydrolysis. A series of deamidation, racemisation and isomerisation were explored using Peptide Numbers 9-12.

The more hydrophobic peptide chain was typically used to investigate specific modifications at certain points of the peptide chain, with the intention of gaining a greater understanding of the peptide-column interactions by examining a series of modifications (Peptide Numbers 13,15,20-26).

Steric interactions are particularly subtle, but are still an important modification to investigate. Racemisation can be seen as a form of steric interaction. Other steric interactions probed include substituting leucine (Peptide Number 13) for valine and isoleucine (Peptide Number 20 and 21), which differs by a methylene group and branching and by switching the order of two amino acids (Peptide Number 15).

Changing the charge of the peptide can have a substantial effect on its retentivity and can be affected by different stationary phase, mobile phase composition and pH. It is possible to investigate the effect of a small change in polarity and basicity with the same charge by modifying arginine (side chain pK_a 12.48, Peptide Number 13) in position 20 with a lysine (side chain pK_a 10.35, Peptide Number 25). The effect of changing from a neutral amino acid to a positive species can also be investigated with the addition of lysine in place of leucine in position 26 in Bovine GLP-2 (16-33) (Peptide Number 26). An increase in negative charge was probed by the addition of an aspartic acid in various positions along the chain (Peptide Numbers 4, 5, 9, 10, 11, 12, 16, 17, 18 and 19).

Other mechanisms of interest were aromatic and phenolic effects, which were investigated by exchanging leucine for phenylalanine, tryptophan or tyrosine (Peptide Numbers 22, 23 and 24, respectively). Small molecules are highly influenced by aromatic and phenolic effects, however, it is uncertain how the change of a single amino acid in a peptide will impact on selectivity, since this is a relatively subtle modification of the peptide compared to a small molecule. Mant *et al.* did observe selectivity differences on a range of peptides similar in size which differed by one amino acid [16,27], however, their peptides had several glycine residues throughout the sequence to prevent any secondary structure. This might not be the case for biologically active peptides which typically are more heterogenic.

3.2 Rationale for Selection of Buffers

Peptide analysis at low pH typically includes either of the following mobile phase additives: phosphate salts, trifluoroacetic acid (TFA), ammonium formate, ammonium acetate or formic acid [28-29]. Phosphate salts are frequently used within the biopharmaceutical industry for peptide separations as they often provide a favourable selectivity and peak shape, however, they were not favoured in the protocol due to their lack of volatility which prohibits peak identification using MS detection. TFA is a frequently used additive as it is a good compromise, i.e. it gives both acceptable chromatographic and MS performance. It was not selected for the protocol as it was shown to mask electrostatic and more subtle polar interactions between the peptides and the stationary phases hence reducing the discriminating power of the column characterisation protocol (see section 3.4). As it can also irreparably modify the stationary phase surface, it would be impractical for the protocol, in that once exposed to TFA the column could not be used with other additives in the characterisation protocol [30]. The intention of this present study was to develop a simple and robust column characterisation protocol, by differentiating the subtle interactions of peptides with the stationary phase surface and not to develop optimum LC methods. TFA and other interesting or commonly used mobile phases will however be characterised in a following study which could potentially aid the chromatographer in the selection of the most appropriate mobile phases for method development.

The primary rationale for selecting formic acid as the mobile phase additive for the column characterisation protocol was its lack of masking subtle interactions (compared to TFA) and hence enhance the discriminating power of the protocol. Additional benefits included its ease of tracking peaks using MS detection, volatility and lack of signal suppression in positive electrospray ionisation. Formic acid would not normally be used for LC-UV due to the poor peak shapes that it typically generates but is advantageous in that it permits the user to understand the purer interactions of the stationary phase.

Historically, peptide analysis was performed at low pH in order to minimise the interaction of peptides with deprotonated residual silanols at intermediate pH which can cause detrimental band spreading and excessive tailing for ionised species. However, intermediate pH should still be considered due to the alternative selectivity which it confers by changing the physico-chemical properties of both the stationary phase and peptides.

Intermediate pH can be established using ammonium formate at its native pH (6.45). Although there is little buffering capacity at this pH it is LC-MS compatible. An alternative to the formate salt could be ammonium acetate, however, there is various anecdotal evidence which suggested that the

acetate salt produced significant adducts in the MS and ion suppression. Both salts are known to be hygroscopic, which can be problematic for consistent results. This often manifests as clumping in the salt container but is frequently ignored despite its effect on buffer concentration. A sodium chloride salt chamber was set up at 20 °C to produce a relative humidity (RH) of 75.47% ±0.14 [31]. Over 6 hours the ammonium acetate had increased in mass by 32.9% and reduced to a liquid, whereas the ammonium formate had a mass increase of 24.8% and was partially crystalline. A less exacerbated scenario was set up at ambient humidity using a magnesium chloride salt chamber (RH 33.07% ±0.18), which saw a mass increase of 7.9% for ammonium acetate and 3.3% for ammonium formate over 6 hours. A comparison of ammonium acetate and ammonium formate highlighted no difference in the degree of cluster formation in the MS, although there was some improved sensitivity achieved with the formate salt on a selection of peptides (up to 2x greater), thus ammonium formate was used to characterise the stationary phases at intermediate pH.

3.3 Normalisation and Definition of Selectivity

Two reference peptides were included in each peptide mixture in order to monitor any retention time drift and to normalise retention time. The normalisation can also minimise variation related to instrumentation, solvents, analyst and remove any contribution from the dwell volume of the system (V_d) and column volume (V_m) in order to gain greater robustness for the overall procedure (Eq. 1). Normalisation of the peptide's retention was feasible due to the fact that the peptides were focused on the top of the column [32-35].

$$t_g^* = \frac{t_g - t_{g\,Ref1}}{t_{g\,Ref2} - t_{g\,Ref1}} \qquad (1)$$

Where t_g is the retention in gradient elution, $t_{g\,Ref1}$ is the retention in gradient elution of the first eluting isomer of [Met(O)10]-Bovine GLP-2 (1-15) (Peptide Number 8a) and $t_{g\,Ref2}$ is the retention in gradient elution of [Ile26,Leu27]-Bovine GLP-2 (16-33) (Peptide Number 15) (Fig. 1). These peptides are the first and typically last eluting compounds, thus t_g^* values generated were between 0 and 1.

Selectivity in gradient chromatography (α^*) has been defined in various publications [33-34] where gradient chromatography presents a more complex situation than for isocratic chromatography in that changing eluent composition, dwell volume, column dead volume and gradient shape can all influence the selectivity. A variety of α^* measures were evaluated using the peptides with the most robust and intuitive defined equation in Eq. 2, where t_g is the retention time in gradient elution and t_m and t_d are column dead time and dwell time, respectively.

$$\propto^{*} = \frac{t'_{g2}}{t'_{g1}} \qquad (2)$$

$$t'_g = t_g - t_m - t_d \qquad (3)$$

However, the result produced by α^* was heavily dependent on the point of elution in the gradient, despite visually having a similar degree of separation. The peptides of interest would produce a reduced α^* value if retained for longer on the gradient in comparison to a less retained pair of similar separating power, thus suggesting selectivity differences. Therefore, an alternative measure was required to define selectivity.

A better representation of selectivity was delta (Δt_g^*, Eq. 4), which was the difference in normalised retention time between two peaks. Delta values were preferred over α^*, as it rectified the discrepancy to provide identical selectivity, regardless of the point of elution in the gradient. This measure allowed direct comparison between stationary phases and mobile phases irrespective of the point of elution, thus giving a purer representation of the selectivity differences. In addition, Δt_g^* also compensates for differences in dwell time (t_d).

$$\Delta t_g^* = t_{g2}^* - t_{g1}^* \qquad (4)$$

In total, 33 delta values per mobile phase were identified based on various properties, including racemisation, steric interactions, increases in positive charge or negative charge, changes due to oxidation and change in hydrophobicity / alkyl and changes in hydrogen bonding or aromatic character (Table 4). The average Δt_g (expressed in minutes) and average Δt_g^* were recorded in Table 4 for the formic acid, TFA and ammonium formate gradient conditions, to allow for better judgement of what is a large or small difference in selectivity. The sign in front of the Δt_g^* result is indicative of the elution order for the separation. The largest average difference was observed using formic acid between [Leu26]- and [Lys26]-Bovine GLP-2 (Peptide Number 13 and 26, respectively), which could be expected due to the changes in charge. The smallest average differences (i.e. Δt_g^* values close to zero) were typically racemic in nature, which highlights the difficulty in separating these closely related species. The difference in responses for the position of the racemisation also accentuates the issue of analysing racemates, where [L-Ser5] → [D-Ser5] and [L-Ser7] → [D-Ser7]-Bovine GLP-2 (1-15) differ at position 5 and 7 respectively in the residue chain, but produced some signficant differences in Δt_g^* for both formic acid and ammonium formate (Peptide Number 1, 6 and 7).

3.4 Principal Component Analysis

A significant amount of information was produced in the data matrix thus the chemometric tool Principal Component Analysis (PCA) was used to visualise trends within the data, by reducing the number of variables (i.e. delta values) to two principal components [9,36]. The delta values were all coded based on the interaction of interest (Table 4). The loading plot (Fig. 2) illustrates how the different delta values contribute to the two principal components. Delta values located close to each other have similar importance for the differentiation of objects (i.e. columns). 56% of the variation between columns were described using two principal components. The use of a third principal component increased the variation described by only 11% and did not provide any additional information, thus only two principal components were used. The corresponding score plot (Fig. 3) shows how similar or different columns are to each other. Columns located close to each other are more similar. The columns in Fig. 3 were all colour coded based on prior knowledge of stationary phase properties to gain an understanding of potential groupings. The green circles are phases with neutral character i.e. high hydrophobicity and low degree of silanophilic interactions due to greater ligand density and end-capping. The blue squares are phases with negative character, such as those with low ligand coverage or absence of end-capping and thus a high degree of silanophilic interactions. The inverted red triangles are phases with positive character i.e. presence of a positively charged group in addition to the hydrophobic ligand [11].

The Acclaim Mixed Mode WCX was excluded from the PCA despite adequate peak shape and performance under low pH conditions for all the peptides, as the carboxylic acid moiety on the mixed mode phase was ionised at pH 6.45, which resulted in poor retention of Peptides 1-12. These peptides can be considered more hydrophilic and possess a charge between -3.7 and -4.7 at the intermediate pH. The affected peptides eluted in the void under those chromatographic conditions, which would heavily skew the score and loading plots, thus the decision was made to exclude the Acclaim Mixed Mode WCX for the purpose of comparison. A biplot was created solely from formic acid data on all 14 columns, where the Acclaim Mixed Mode WCX was grouped with the negative character stationary phases and located as far from the origin as the Acquity CSH Fluoro Phenyl and consequently should display very different selectivity for appropriate peptides i.e. peptides with limited negative net charge.

The possibility of reducing the number of delta values required for the Peptide RPC Column Characterisation protocol was evaluated by performing an iterative study which removed any variable near the origin in the loading plot (i.e. considered an insignificant variable), or removal of variables with similar meanings whilst ensuring the integrity of the score plot was maintained (i.e. a maintained relative location of columns). Following a step-wise process of elimination, the number of variables within the Peptide RPC Column Characterisation Protocol was reduced from 66 to 11,

whilst still covering an array of important interactions. Six of the measurements were determined in ammonium formate, whilst the remaining five were measured in formic acid, using a total of three test mixtures (summarised in Table 5). The delta value to be measured were calculated from the same test mixture to increase the robustness of the procedure.

A biplot of the final Peptide RPC Column Characterisation Protocol peptides (i.e. a combined score and loading plot, Fig. 4) highlighted the influence an observation has on the position of the stationary phase. Regions of electrostatic interactions can be deciphered, where the phases with negative character in the upper quadrants (e.g. Acquity HSS C18-SB and Acquity BEH C4) are dominated by the positive charge delta values, and the phases with positive character in the lower quadrants (e.g. Polaris Amide C18 and Acquity CSH Phenyl Hexyl) are correlated with the negative charge delta values. There is also an intermediate region of low electrostatic interactions for the stationary phases with a neutral character which have low silanophilic activity due to high ligand density and extensive end-capping.

In order to investigate the hypothesis that TFA masks certain interactions between the column and the peptide, a study was conducted where 0.1% v/v formic acid was substituted with 0.1% v/v TFA in both the aqueous and organic phase on the reduced number of delta values. 13 columns were tested using a simplified and more robust version of the protocol described in reference [37] which used 8 of the 11 probes to increase the reliability of the methodology. Distinct groups can be observed in the formic acid biplot plot Fig. 5(A) with 87% of the variability described, where the position of the columns can be rationalised based on what is known about the column characters. With TFA, it is no longer possible to see these distinct groupings, and only 68% of the variability is described, which suggests a less clear structure for that dataset (Fig. 5(B)). This would appear to confirm the hypothesis that TFA will mask peptide-column interactions and thus columns become more similar. Stationary phases such as the Acquity HSS C18-SB with no end-capping and low surface coverage appear similar to columns with end-capping and positive charge like Acquity CSH Fluoro Phenyl and Polaris Amide C18 which are very different. As such, to describe the interactions of the columns, it is important to assess each column using formic acid, rather than TFA. A further evaluation of TFA as an additive will be described in a subsequent article where mobile phases are characterised.

3.5 Peak Capacity

Large selectivity differences on their own are not sufficient for generating a good chromatographic method. Chromatographic efficiency is also required, which is measured by peak capacity in gradient

elution. Peak capacity is defined as the total number of peaks which can be resolved (R_s = 1) within a chromatogram, and is conventionally measured by dividing the gradient time by the average peak width. However, this measurement often over estimates the performance of the column, thus the sample peak capacity (PC^{**}) approach was used (Eq. 5) to determine the peak capacity for the fraction of the gradient used to separate all the compounds of interest [23].

$$PC^{**} = \frac{t_{g\,max} - t_{g\,min}}{W_{ave}} + 1 \qquad (5)$$

Where $t_{g\,min}$ and $t_{g\,max}$ are the retention time of the first and last eluting peptide. w_{ave} is the average width at base for all peaks that are separated except the diastereoisomeric oxidised methionine probe which is typically unresolved on most of the stationary phases.

The PC^{**} was measured for both the formic acid, ammonium formate and TFA gradient conditions on the reduced number of delta values. The greater performance, as shown by increased PC^{**} values, was typically achieved using intermediate pH (Table 6 and Fig. 6). Formic acid characteristically provides poorer performance, whilst TFA usually produced good values of peak capacity. Despite poorer performance, the formic acid was still within 28% on average of the peak performance produced in TFA and 41% in ammonium formate.

Poor peak shape at low pH is often attributed to silanophilic interactions, however, with the increase in modern silicas which do not possess a significant degree of acidic silanols, this cannot be the cause of exaggerated peak shapes for basic species. McCalley *et al*. hypothesised that poor peak shape can often be ascribed to overloading for basic species due to mutual repulsion effects between adsorbed ions of the same charge [28,38-39]. This effect was increased further when low ionic strength mobile phases are used. Further research by McCalley, which used positively charged peptide probes also supported this theory where he compared the responses of four multiply positively charged peptides in phosphate buffer, formic acid and TFA. The phosphate buffer gave significantly better peak shape and chromatographic performance compared to the formic acid due to a significantly higher ion strength which reduces mutual repulsion. TFA also gave improved peak shape compared to formic acid, however, in this case it was also accredited to the ion pairing effects of TFA which could reduce overloading by partially neutralising the net charge of the peptides and thereby removing the repulsion effects [28].

Although formic acid can result in poor peak shape, this was not the case for the Acquity CSH range of stationary phases which were specifically designed to provide an improved peak shape for basic species due to the presence of a small permanent positive character on the surface of the phase

[40]. The Polaris Amide C18, which also possessed a positive character, additionally provided good chromatographic performance in formic acid.

3.6 Assessment of Peptide – Column Interactions

3.6.1 Hydrophobic interactions

Hydrophobic interaction, which is the primary retention mechanism in reversed phase chromatography, could be evaluated by investigating the effect of probes possessing differing hydrophobicity (i.e. changing leucine to valine) and by changing the hydrophobicity of the stationary phase (i.e. different alkyl length).

Mant *et al*. deduced the order of hydrophobicity for amino acids when there are no "nearest neighbour" effects [27]. A purely hydrophobic interaction without "nearest neighbour" effects would therefore suggest that the elution order in this study should be Val<Tyr<Ile<Leu<Phe<Trp, i.e. Peptide 20<24<21<13<22<23. This was, however, not the case as seen in Fig. 7. One explanation can be that the peptides in the current study lack a secondary structure in the mobile phase as shown by CD but form a secondary structure in the hydrophobic acetonitrile layer on the stationary phase resulting in another elution order.

[Leu26]- and [Val26]-Bovine GLP-2 (16-33) (Peptides 13 and 20) differ by just one methyl group, which represents a subtle change in the peptide's overall hydrophobicity, however, when compared on a range of C18 phases (Acquity BEHs, HSSs and CSHs) there were substantial selectivity differences between the two probes (Fig. 7 (A), (D) and (G)). This is in agreement with Mant *et al.* who witnessed greater retention for a peptide modified with a leucine compared to one modified with a valine [27]. [Ile26,Ile27]- and [Leu26,Ile27]-Bovine GLP-2 (16-33) (Peptides 21 and 13, respectively) only differ by the position of a methylene group on the side chain, however, they have substantial selectivity differences on the C18 phases during gradient elution. Literature suggests it is reasonable to expect retention differences between these two peptides, as the β-branched chain on the isoleucine is closer to the peptide backbone, thus less able to interact with the stationary phase [27]. This is again in agreement with the previous study, where the peptide modified with leucine had greater retention than the peptide modified with isoleucine.

[Ile26,Leu27]- possesses the same overall hydrophobicity as [Leu26,Ile27]-Bovine GLP-2 (16-33) (Peptides 15 and 13, respectively), but have substantial selectivity differences on the C18 phases during gradient elution (Fig. 7 and 8), which suggests an alternative mechanism. One possible explanation could be that the change in position of the methyl group changes the shape of the

peptide in the hydrophobic acetonitrile layer on the stationary phase and that results in differences in the interactions which can take place [41-43].

The more hydrophobic molecules [Phe26]-, [Trp26]- and [Tyr26]- (Peptides 22, 23 and 24) were then compared against [Leu26]-Bovine GLP-2 (16-33) (Peptide 13). Based on retention data obtained by Mant *et al.*, although they possess bulkier, aromatic side chains, a hydrophobic retention mechanism should preferentially retain [Phe26]- and [Trp26]- over [Leu26]-Bovine GLP-2 (16-33) (Fig. 7). [Tyr26]-Bovine GLP-2 (16-33) should be the least retained of the four peptides described. However, on all of the C18 phases, [Phe26]-, [Trp26]- and [Tyr26]- eluted earlier, which confirms that a hydrophobic retention mechanism must be acting in combination with an alternative mechanism.

The same phenomenon was experienced on the Acquity BEH alkyl range of stationary phases, where a lack of pure hydrophobic mechanism was observed (Fig. 7 (A), (B) and (C)). There was a subtle change in elution order for the Acquity BEH C8 and C4, however, where [Ile26]- and [Phe26]- (Peptides 21 and 22) switched in elution order. One possible explanation could be that the aromatic groups change the shape of the peptide in the adsorbed acetonitrile layer and thereby expose other groups which can participate in polar / electrostatic interactions [41-43].

There are subtle selectivity differences between the stationary phases, however, to a large extent, the type and length of the ligand (C4-C18) does not appear to be critical for the separation of these probes.

3.6.2 Electrostatic Interactions

The addition of negative charge was evaluated using peptides [L-Asp11]- and [Asn11]-Bovine GLP-2 (1-15) (Peptides 9 and 1 respectively). Their retentions were compared on stationary phases with negative and positive character (Fig. 9) where in formic acid, both sets of columns eluted the asparagine peptide before the aspartic acid variant. In ammonium formate, however, the elution order was reversed, and the aspartic acid eluted first. Under intermediate pH conditions (pH 6.45) the aspartic acid peptide has a net charge of -4.7 whilst the asparagine peptide has a net charge of -3.7, and as such, [L-Asp11]- would be expected to elute last on the phases with positive character due to enhanced electrostatic interactions. However, as the more acidic species [L-Asp11]- eluted first it suggests that despite having a greater negative charge than [Asn11]-, it is the position of the charge and accessibility in the adsorbed peptides secondary structure that is important rather than the overall net charge of the peptide hence the more charged / hydrophilic [L-Asp11]- elutes first.

The addition of positive charge was then investigated using the probes [Lys26]- and [Leu26]-Bovine GLP-2 (16-33) (Peptides 26 and 13, respectively). [Lys26]- eluted first on all stationary phases and mobile phases, however, there was increased retention under ammonium formate conditions due to increased electrostatic interactions. The difference is most pronounced for the Acclaim Mixed Mode WCX which contains a carboxylic acid functionality. At pH 6.45, the carboxylic acid is deprotonated and thus the positively charged [Lys26]- becomes strongly retained. Due to repulsion, columns with a positive character (the CSH series and the Polaris Amide C18 columns) elute [Lys26]- earlier at low pH than columns with a negative character (i.e. more accessible silanol groups).

These interactions are confirmed by the position of the stationary phases and the delta values within the biplot (Fig. 4).

3.6.3 Hydrogen Bonding Interactions

Prior knowledge of the stationary phases suggested those phases which are likely to form hydrogen bonding interactions are the Polaris Amide C18 via the amide functionality, the Acquity BEH Shield RP-18 via the embedded carbamate moiety, the Acquity BEH C4 and Acquity HSS C18-SB phases via the short alkyl ligand and low ligand surface coverage respectively which both promote greater accessibility to the non end-capped silanol groups on the silica surface and the Acclaim Mixed Mode WCX via the carboxylic acid moiety. The Acquity BEH C18 was used as a reference since it is based on hybrid silica, is end-capped and thus should have minimal potential to form hydrogen bonds. The peptides [Phe26]- and [Tyr26]-Bovine GLP-2 (16-33) (Peptides 22 and 24) were assessed for hydrogen bonding interactions with the stationary phases as they only differ due to the presence of the hydroxyl group on the tyrosine. The phenylalanine derivative eluted last on all of the stationary phases and mobile phases although with greater retentivity in ammonium formate for the tyrosine variant (data not shown). The trend seems to suggest that polarity is more dominant than hydrogen bonding capabilities. The extra retentivity in ammonium formate of the tyrosine peptide could indicate that the stationary phase is becoming more polar. The lack of hydrogen bonding could be due to the position of the amino acid change in the peptide residue. It is possible hydrogen bond interactions could be more pronounced if tyrosine was located closer to the terminal amino acids.

There are large selectivity differences observed between the two peptides considering only a 16 Da difference in their ~2300 Da structures, which indicates the addition of the hydroxyl group has caused some significant changes in the interactions within the chromatographic system.

The position of the Acquity BEH Shield RP-18 at the origin in the score plot is highly suggestive that the carbamate group masks any underlying silanol groups and the carbamate is not involved in any pronounced hydrogen bonding interactions with [Tyr26]-Bovine-GLP-2 (16-33).

3.6.4 Aromatic Interactions

Phenyl containing phases (Acquity CSH Phenyl Hexyl, Fortis Diphenyl and Ascentis Biphenyl) and the pentafluorophenyl phase (Acquity CSH Fluoro Phenyl) were compared against the Acquity BEH C18 and CSH C18 to assess for potential aromatic interactions. The probes used were [Leu26]-, [Phe26]-, [Trp26]- and [Tyr26]-Bovine GLP-2 (16-33) (Peptides 13, 22, 23 and 24, respectively).

The CSH phases all possessed the same elution order in both formic acid and ammonium formate, highlighting minimal aromatic retention which suggests the stationary phase ligand becomes less important for these separations (Fig. 10).

The diphenyl and biphenyl phases on the other hand were able to resolve the aromatic species and also had different elution orders compared to the CSH phases. [Phe26]- elutes after [Leu26]- and there is a significantly larger retention of [Phe26]- at mid pH suggesting that this is due to electrostatic interaction and not due to π-π interactions. The diphenyl and biphenyl phases suggest a more negative character due to accessible silanol groups whereas the CSH phases have a more positive character due to positively charged groups in the stationary phase, as indicated within the PCA (Fig. 4 biplot).

There is the potential that the acetonitrile within the mobile phase could reduce any subtle aromatic interactions of the peptides with the stationary phase due to competing π-π interactions. The elution order based on hydrophobicity alone using Hodges's work would suggest [Tyr26]- elute first, then [Leu26]-, [Phe26]- and then finally [Trp26]-Bovine GLP-2 (16-33) [27]. However, this was not the case here where [Trp26]- typically eluted before [Phe26] and [Leu26]-Bovine GLP-2 (16-33). This is highly suggestive that an alternative retention mechanism must be introduced, such as the formation of a second order structure of the peptide in the stationary phase exposing certain functional groups and hiding others.

3.6.5 Interactions Related to Degradation of Peptides

Purity methods developed for biopharmaceutical peptides should be able to separate degradation products. Consequently, an important aspect of the protocol was to evaluate selectivity for common degradants and racemisation products. Racemisation and isomeric products are typically

challenging to separate since these peptides are diastereoisomers with very similar physiochemical properties. An investigation of the retention order of D- and L-forms of racemates indicate that the retention order is often the same at both low and mid pH. This was the case for 78% of the 117 combinations of delta values and columns in the current study. A comparison of which pH typically generates a higher delta value for racemates did not show any trend. In 54% of the cases mid pH gave a larger delta value. A similar result was obtained for Asp / isoAsp isomeric delta values, where 73% of the 26 combinations often gave the same elution order in both low and mid pH. However, there was a trend where low pH gave the large delta value (85% of the combinations).

The biplot (Fig. 4) was used to identify phases which were deemed chromatographically similar and dissimilar in order to assess their ability to separate racemates. The Acquity CSH Phenyl Hexyl and Acquity HSS C18-SB were selected as phases with large differences in selectivity for the separation of [D-Ser16]- and [L-Ser16]-Bovine GLP-2 (16-33) (Peptide Numbers 14 and 13, respectively), whilst the Acquity HSS T3 and Acquity BEH C18 were compared as phases which provide similar selectivity, i.e. these phases are located at the extremes and close to the origin when projected on to a line through the origin and $\Delta(14,13)$ in the bi-plot (Fig. 4).

The diverse columns with formic acid (Fig. 11 (A) and (B)) exhibit a difference in the degree of resolution, where there is coelution but a switch in elution order on the Acquity HSS C18-SB and resolution achieved on the Acquity CSH Phenyl Hexyl. By switching to ammonium formate (Fig. 11 (E) and (F)), baseline resolution was achievable on the Acquity CSH Phenyl Hexyl, whilst the Acquity HSS C18-SB has switched elution order. For difficult separations such as resolving racemic species, it is essential to have large peak capacity which is typical for mobile phases such as ammonium formate, whereas solvents which have low ionic strength, such as formic acid, typically failed to resolve the two species due to poor peak shape. For an explanation of the poor peak shape, see Section 3.5.

In Fig. 11 (C), (D), (G) and (H), the Acquity HSS T3 and Acquity BEH C18 all produced similar looking chromatograms irrespective of stationary or mobile phase, with similar selectivity and normalised retention times.

This comparison has provided an early indication that this protocol could show differentiation between the phases, even for challenging separations such as resolving racemic species. The example above was selected using $\Delta(14,13)$. In order to select columns from the biplot (Fig. 4) likely to give large differences in selectivity for any racemates the selection would have been Acquity BEH C8, Acquity HSS C18-SB, Acquity CSH Fluoro Phenyl, Acquity CSH Phenyl Hexyl and Polaris Amide C18

[Met(O)10]- and [Met10]-Bovine GLP-2 (1-15) (Peptide Numbers 8 and 1, respectively) were compared to investigate the effect of oxidation. The more hydrophilic, oxidised methionine eluted first on all the stationary phases irrespective of the mobile phase conditions, with ample degree of separation achieved between the two species in either formic acid or ammonium formate gradient conditions.

4 Conclusion

A protocol for the characterisation of stationary phases using peptides as probes was successfully developed. The protocol utilised two gradient mobile phase systems at low and intermediate pH to cover different degrees of ionisation of both peptides and stationary phases.

The peptides were rationally designed in order to systematically change characteristics deemed important for retentivity and selectivity, including hydrophobicity, aromaticity, degree of hydrogen bonding, electrostatic interactions, steric interactions and important degradation pathways. The peptides mixtures were injected onto 14 different stationary phases possessing different chromatographic characteristics, which were grouped into neutral phases (i.e. phases with high ligand density and a large degree of end-capping), negative character phases (i.e. phases with a reduced ligand coverage or no end-capping present) and finally positive character phases (i.e. phases with a positively charged functional group in addition to the RP ligand). Peptides were identified using SIM, or where differentiation by mass was not possible (i.e. isomers) different peak area ratios were used.

A range of delta values were calculated which covered the interactions of interest to assess selectivity differences. The data was analysed using PCA, which highlighted groupings of stationary phases resembling the three groups described. The number of delta values and peptides were systematically reduced whilst ensuring that the integrity of the score plot was maintained.

Further evaluation of the data indicated that the most crucial interactions between stationary phases and peptides are hydrophobic in nature, in combination with polar interactions. These results, which are based on biologically relevant peptides, suggest that it is polar interactions and the second order structure of the adsorbed peptide in the stationary phase that to a large extent contribute to the selectivity and the differences seen between columns. This was highlighted by the large selectivity differences exhibited for peptides with a similar degree of hydrophobicity. Another interesting observation was the large selectivity differences exhibited for racemisation species, which only differ by the orientation of one of the amino acids.

This first evaluation of the selectivity of peptides will be further investigated and expanded in a series of articles. The robustness of the protocol will be assessed using systematic changes to the methodology and practical constraints introduced to ensure the integrity of the methodology. The protocol will also be applied to a greater array of stationary phases from different column manufacturers to build a database for rational stationary phase selection for peptide separations which will be validated with the analysis of tryptic digests of biologically active proteins. In addition, the results from the database will be compared against small molecule column characterisation data to ascertain any correlation between the peptide probes and conventional small molecule protocols. The intention is also to utilise these peptides for the characterisation of a range of mobile phases employing different pH, ion-pairing additives, organic modifiers and salts.

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7 Figure captions

Fig. 1. Example test mixture containing the two reference peptides used to normalise retention.

Fig. 2. Loading plot of all 66 delta values showing to what extent different delta values contribute to the two principal components.

Fig. 3. Score plot showing how columns are grouped based on the 66 delta values

Fig. 4. Biplot of the Peptide RPC Column Characterisation Protocol delta values on the 13 stationary phases. The different stationary phase have been grouped and colour coded based on prior knowledge of stationary phase properties.

Fig. 5. Biplots of the reduced number of delta values on the 13 stationary phases in (A) formic acid and (B) TFA. The different stationary phase have been grouped and colour coded based on prior knowledge of stationary phase properties.

Fig. 6. Sample peak capacity for each column in the score plot measured in formic acid (solid line), ammonium formate (dotted line) and TFA (dashed line). A large circle indicates a high peak capacity.

Fig. 7. Comparison of (A) Acquity BEH C18 (B) Acquity BEH C8 (C) Acquity BEH C4 (D) Acquity HSS C18 (E) Acquity HSS C18-SB (F) Acquity HSS T3 (G) Acquity CSH C18 (H) Acquity CSH Phenyl Hexyl (I) Acquity CSH Fluoro Phenyl to investigate hydrophobic interactions (Peptide 13: [Leu26,Ile27]-, 15: [Ile26,Leu27]-, 20: [Val26,Ile27]-, 21: [Ile26,Ile27]-, 22: [Phe26,Ile27]-, 23: [Trp26,Ile27]-Bovine GLP-2 (16-33)). Data with bars above the retention axis correspond to low pH whereas below the axis correspond to intermediate pH.

Fig. 8. [Leu26,Ile27]- and [Ile26,Leu27]-Bovine GLP-2 (Peptides 13 and 15) on the Acquity BEH C8 chromatographed using the ammonium formate gradient.

Fig. 9. Comparison of (A) Acquity BEH C18, (B) Acquity CSH C18 (C) Acquity CSH Phenyl Hexyl (D) Acquity CSH Fluoro Phenyl (E) Polaris Amide C18 (F) Fortis Diphenyl (G) Ascentis Express Biphenyl (H) Acclaim Mixed Mode WCX (I) Acquity BEH C4 (J) Acquity HSS C18-SB to investigate electrostatic interactions (Peptide 1: [Asn11]-, 9: [L-Asp11]-Bovine GLP-2 (1-15), 13: [Leu26]-, 26: [Lys26]-Bovine GLP-2 (16-33)). Data with bars above the retention axis correspond to low pH whereas below the axis correspond to intermediate pH.

Fig. 10. Comparison of (A) Acquity BEH C18 (B) Fortis Diphenyl (C) Ascentis Express Biphenyl (D) Acquity CSH Fluoro Phenyl (E) Acquity CSH C18 (F) Acquity CSH Phenyl Hexyl to investigate aromatic interactions (Peptide 13: [Leu26]-, 22: [Phe26]-, 23: [Trp26]-, 24: [Tyr26]-Bovine GLP-2 (16-33)). Data with bars above the retention axis correspond to low pH whereas below the axis correspond to intermediate pH.

800 Fig. 11. Chromatograms of (13) [L-Ser16]- and (14) [D-Ser16]-Bovine GLP-2 (16-33) on phases
801 predicted to be similar or dissimilar based on the biplot in Fig. 4. From L-R: Acquity HSS C18-SB,
802 Acquity CSH Phenyl Hexyl, Acquity HSS T3, Acquity BEH C18, A-D in formic acid, E-H in ammonium
803 formate.

Table 1

Stationary phase used in the development of the Peptide RPC Column Characterisation Protocol and their physical parameters

Manufacturer	Column	Pore Size (Å)	Particle Size (µm)	Description
Waters	Acquity UPLC BEH C4	300	1.7	A non endcapped, trifunctional C4 alkyl ligand bonded to ethyl bridged silica hybrid material
Waters	Acquity BEH C8	130	1.7	An endcapped, trifunctional C8 alkyl ligand bonded to ethyl bridged silica hybrid material
Waters	Acquity UPLC BEH C18	130	1.7	An endcapped, trifunctional C18 alkyl ligand bonded to ethyl bridged silica hybrid material
Waters	Acquity BEH Shield RP18	130	1.7	An endcapped, monofunctional alkyl ligand with an embedded carbamide functionality bonded to ethyl bridged silica hybrid material
Waters	Acquity CSH C18	130	1.7	An endcapped, trifunctional C18 alkyl ligand bonded to ethyl bridged silica hybrid material which possesses a low level positive surface charge
Waters	Acquity CSH Fluoro Phenyl	130	1.7	A non endcapped, trifunctional pentafluorophenyl moiety ligand bonded to ethyl bridged silica hybrid material which possesses a low level positive surface charge
Waters	Acquity CSH Phenyl Hexyl	130	1.7	An endcapped, 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 HSS C18	100	1.8	An endcapped, trifunctional C18 alkyl ligand
Waters	Acquity HSS C18 SB	100	1.8	A non endcapped, trifunctional C18 alkyl phase with low ligand density
Waters	Acquity HSS T3	100	1.8	An endcapped, trifunctional, 100% aqueous compatible C18 alkyl phase with reduce ligand density
Agilent	Polaris Amide C18	180	3	An endcapped, monofunctional alkyl ligand with an embedded amide functionality which possesses positive character due to residual amino groups related to a two step synthetic process
Supelco	Ascentis Express Biphenyl	90	2.7	An endcapped, superficially porous particle with a biphenyl ligand
Fortis	Fortis Diphenyl	100	1.7	An endcapped, diphenyl ligand
Thermo	Acclaim Mixed Mode WCX	120	3	A mixed mode ligand composed of an alkyl chain with carboxylic acid terminus

Table 2

Peptide sequence of Bovine GLP-2

Amino Acid #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Bovine GLP-2	H	A	D	G	S	F	S	D	E	M	N	T	V	L	D	S	L	A	T	R	D	F	I	N	W	L	I	Q	T	K	I	T	D

Table 3

List of peptides used to develop the Peptide RPC Column Characterisation protocol and their rationale*

Peptide Number	Peptide	Rationale	pI	LogP	Net Charge		Sequence																																	
					pH 2.5	pH 6.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1	Bovine GLP-2 (1-15)	Original sequence	3.9	-12.18	1.2	-3.7	H	A	D	G	S	F	S	D	E	M	N	T	V	L	D																			
2	[D-His1]-Bovine GLP-2 (1-15)	Racemisation	3.9	-12.18	1.2	-3.7	h	A	D	G	S	F	S	D	E	M	N	T	V	L	D																			
3	[D-Asp3]-Bovine GLP-2 (1-15)	Racemisation	3.7	-12.18	1.2	-3.7	H	A	d	G	S	F	S	D	E	M	N	T	V	L	D																			
4	[L-isoAsp3]-Bovine GLP-2 (1-15)	Isomerisation	3.7	-12.18	1.2	-3.7	H	A	iD	G	S	F	S	D	E	M	N	T	V	L	D																			
5	[D-isoAsp3]-Bovine GLP-2 (1-15)	Racemisation / Isomerisation	3.7	-12.18	1.2	-3.7	H	A	id	G	S	F	S	D	E	M	N	T	V	L	D																			
6	[D-Ser5]-Bovine GLP-2 (1-15)	Racemisation	3.9	-12.18	1.2	-3.7	H	A	D	G	s	F	S	D	E	M	N	T	V	L	D																			
7	[D-Ser7]-Bovine GLP-2 (1-15)	Racemisation	3.9	-12.18	1.2	-3.7	H	A	D	G	S	F	s	D	E	M	N	T	V	L	D																			
8	[Met(O)10]-Bovine GLP-2 (1-15)	Oxidation	3.9	-12.18	1.2	-3.7	H	A	D	G	S	F	S	D	E	oM	N	T	V	L	D																			
9	[L-Asp11]-Bovine GLP-2 (1-15)	Deamidation / Negative charge	3.6	-11.55	1.1	-4.7	H	A	D	G	S	F	S	D	E	M	D	T	V	L	D																			
10	[D-Asp11]-Bovine GLP-2 (1-15)	Deamidation / Racemisation / Negative charge	3.6	-11.55	1.1	-4.7	H	A	D	G	S	F	S	D	E	M	d	T	V	L	D																			
11	[L-isoAsp11]-Bovine GLP-2 (1-15)	Deamidation / Isomerisation / Negative charge	3.6	-11.55	1.1	-4.7	H	A	D	G	S	F	S	D	E	M	iD	T	V	L	D																			
12	[D-isoAsp11]-Bovine GLP-2 (1-15)	Deamidation / Isomerisation / Racemisation / Negative charge	3.6	-11.55	1.1	-4.7	H	A	D	G	S	F	S	D	E	M	id	T	V	L	D																			
13	Bovine GLP-2 (16-33)	Original sequence	5.4	-10.21	2.2	0.0																S	L	A	T	R	D	F	I	N	W	L	I	Q	T	K	I	T	D
14	[D-Ser16]-Bovine GLP-2 (16-33)	Racemisation	5.4	-10.21	2.2	0.0																s	L	A	T	R	D	F	I	N	W	L	I	Q	T	K	I	T	D
15	[Ile26,Leu27]-Bovine GLP-2 (16-33)	Switch in AA sequence	5.4	-10.21	2.2	0.0																S	L	A	T	R	D	F	I	N	W	I	L	Q	T	K	I	T	D
16	[L-Asp21,Gly22]-Bovine GLP-2 (16-33)	Loss of aromatic group	5.4	-10.21	2.2	0.0																S	L	A	T	R	D	G	I	N	W	L	I	Q	T	K	I	T	D
17	[D-Asp21,Gly22]-Bovine GLP-2 (16-33)	Loss of aromatic group / Racemisation	5.4	-10.21	2.2	0.0																S	L	A	T	R	d	G	I	N	W	L	I	Q	T	K	I	T	D
18	[L-isoAsp21,Gly22]-Bovine GLP-2 (16-33)	Loss of aromatic group / Isomerisation	5.3	-12.24	2.2	0.0																S	L	A	T	R	iD	G	I	N	W	L	I	Q	T	K	I	T	D
19	[D-isoAsp21,Gly22]-Bovine GLP-2 (16-33)	Loss of aromatic group / Racemisation / Isomerisation	5.3	-12.24	2.2	0.0																S	L	A	T	R	id	G	I	N	W	L	I	Q	T	K	I	T	D
20	[Val26]-Bovine GLP-2 (16-33)	Steric / Aliphatic effect	5.4	-10.59	2.2	0.0																S	L	A	T	R	D	F	I	N	W	V	I	Q	T	K	I	T	D
21	[Ile26]-Bovine GLP-2 (16-33)	Steric / Aliphatic effect	5.4	-10.14	2.2	0.0																S	L	A	T	R	D	F	I	N	W	I	I	Q	T	K	I	T	D
22	[Phe26]-Bovine GLP-2 (16-33)	Aromatic effect	5.4	-9.88	2.2	0.0																S	L	A	T	R	D	F	I	N	W	F	I	Q	T	K	I	T	D
23	[Trp26]-Bovine GLP-2 (16-33)	Aromatic effect	5.4	-9.59	2.2	0.0																S	L	A	T	R	D	F	I	N	W	W	I	Q	T	K	I	T	D
24	[Tyr26]-Bovine GLP-2 (16-33)	Aromatic / Phenolic effect	5.4	-10.12	2.2	0.0																S	L	A	T	R	D	F	I	N	W	Y	I	Q	T	K	I	T	D
25	[Lys20]-Bovine GLP-2 (16-33)	Polarity effect	5.4	-12.09	2.2	0.0																S	L	A	T	K	D	F	I	N	W	L	I	Q	T	K	I	T	D
26	[Lys26]-Bovine GLP-2 (16-33)	Positive charge	8.2	-13.99	3.2	1.0																S	L	A	T	R	D	F	I	N	W	K	I	Q	T	K	I	T	D

*d corresponds to D-Asp, iD to L-isoAsp, id to D-isoAsp, h and s corresponds to D-His and D-Ser respectively and oM corresponds to Met(O).

Table 4

List of delta values, their rationale and the average Δt_g and Δt_g^* in formic acid, TFA or ammonium formate

Delta	Change	Rationale	Average Δt_g in FA (min)	Average Δt_g^* in FA (-)	Average Δt_g in TFA (min)	Average Δt_g^* in TFA (-)	Average Δt_g in AF (min)	Average Δt_g^* in AF (-)
Δ(2,1)	[L-His1] → [D-His1]	Steric - racemisation	0.091	0.009			0.315	0.016
Δ(3,1)	[L-Asp3] → [D-Asp3]	Steric - racemisation	-0.026	0.004			-0.076	-0.001
Δ(4,1)	[L-Asp3] → [L-isoAsp3]	Increase in negative charge	0.078	0.016			-0.263	-0.008
Δ(5,1)	[L-Asp3] → [D-isoAsp3]	Increase in negative charge	0.395	0.038			0.049	0.006
Δ(5,4)	[L-isoAsp3] → [D-isoAsp3]	Steric - racemisation	0.317	0.022			0.311	0.014
Δ(6,1)	[L-Ser5] → [D-Ser5]	Steric - racemisation	0.006	0.008			0.283	0.016
Δ(7,1)	[L-Ser7] → [D-Ser7]	Steric - racemisation	-0.619	-0.036			-0.051	0.002
Δ(8a,1)*	[Met10] → [Met(O)10]	Oxidation	-4.166	-0.289	-3.655	-0.278	-3.598	-0.157
Δ(8b,1)*	[Met10] → [Met(O)10]	Oxidation	-4.085	-0.283			-3.542	-0.154
Δ(8b,8a)*	[Met(O)10] → [Met(O)10]	Steric - racemisation	0.081	0.006			0.056	0.003
Δ(9,1)	[L-Asn11] → [L-Asp11]	Increase in negative charge	0.802	0.059	0.583	0.044	-1.142	-0.055
Δ(10,1)	[L-Asn11] → [D-Asp11]	Increase in negative charge	0.575	0.044			-1.786	-0.083
Δ(10,9)	[L-Asp11] → [D-Asp11]	Steric - racemisation	-0.228	-0.016			-0.644	-0.028
Δ(11,1)	[L-Asn11] → [L-isoAsp11]	Increase in negative charge	-1.232	-0.078			-2.569	-0.115
Δ(12,1)	[L-Asn11] → [D-isoAsp11]	Increase in negative charge	-0.648	-0.038			-2.148	-0.098
Δ(12,11)	[L-isoAsp11] → [D-isoAsp11]	Steric - racemisation	0.584	0.040			0.421	0.017
Δ(14,13)	[L-Ser16] → [D-Ser16]	Steric - racemisation	-0.100	0.007	-0.167	-0.013	0.297	0.011
Δ(15,13)	[Leu26,Ile27] → [Ile26,Leu27]	Steric – switch in amino acid sequence	0.491	0.047	0.443	0.033	1.118	0.049
Δ(16,13)	[L-Asp21,Phe22] → [L-Asp21,Gly22]	Aromatic – removal of aromatic group	-5.927	-0.404	-4.425	-0.329	-7.037	-0.312
Δ(17,16)	[L-Asp21,Gly22] → [D-Asp21,Gly22]	Steric - racemisation	0.129	-0.004			0.929	0.038
Δ(18,16)	[L-Asp21,Gly22] → [L-isoAsp21,Gly22]	Increase in negative charge	0.652	0.036			1.122	0.052
Δ(19,16)	[L-Asp21,Gly22] → [D-isoAsp21,Gly22]	Increase in negative charge	0.421	0.017			0.902	0.044
Δ(19,18)	[L-isoAsp21,Gly22] → [D-isoAsp21,Gly22]	Steric - racemisation	-0.231	-0.019			-0.220	-0.009
Δ(20,13)	[Leu26] → [Val26]	Alkyl – removal of $-CH_2$	-2.025	-0.117			-1.745	-0.075
Δ(21,13)	[Leu26] → [Ile26]	Alkyl – change of $-CH_3$ position	-0.924	-0.033			-0.456	-0.018
Δ(22,13)	[Leu26] → [Phe26]	Aromatic – addition of aromatic group	-0.803	-0.023			-0.338	-0.014
Δ(23,13)	[Leu26] → [Trp26]	Aromatic – addition of aromatic group	-1.201	-0.056			-0.962	-0.039
Δ(23,22)	[Phe26] → [Trp26]	Aromatic – addition of aromatic group	-0.398	-0.032			-0.624	-0.025
Δ(24,13)	[Leu26] → [Tyr26]	Phenolic – addition of hydroxyl group	-3.648	-0.236	-2.791	-0.210	-3.835	-0.164
Δ(24,22)	[Phe26] → [Tyr26]	Phenolic – addition of hydroxyl group	-2.844	-0.212			-3.497	-0.151
Δ(24,23)	[Trp26] → [Tyr26]	Phenolic – addition of hydroxyl group	-2.447	-0.180			-2.873	-0.126
Δ(25,13)	[Arg20] → [Lys20]	Change in polarity	-0.767	-0.012			-0.116	-0.004
Δ(26,13)	[Leu26] → [Lys26]	Increase in positive charge	-8.213	-0.587	-5.926	-0.457	-6.773	-0.279

*a and b corresponds to the first and last 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 5

Final test mixtures used for the Peptide RPC Column Characterisation Protocol

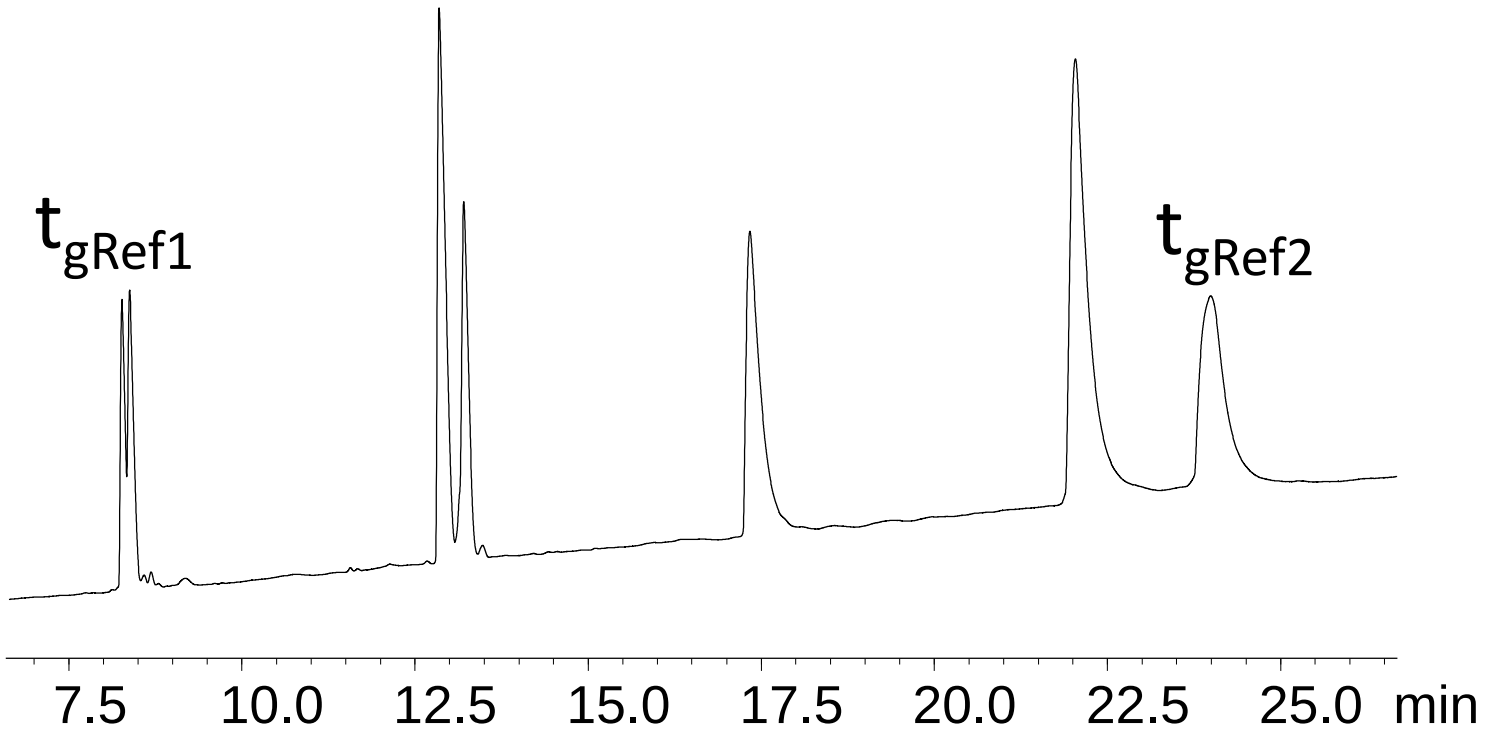
Test Mixture	Delta	Measured in Formic Acid	Measured in Ammonium Formate
TM1	Δ(8a,1)*	✓	
TM1	Δ(9,1)	✓	✓
TM1	Δ(15,13)		✓
TM1	Δ(16,13)	✓	
TM1	Δ(24,13)		✓
TM2	Δ(10,9)		✓
TM2	Δ(26,13)	✓	✓
TM3	Δ(3,1)		✓
TM3	Δ(14,13)	✓	

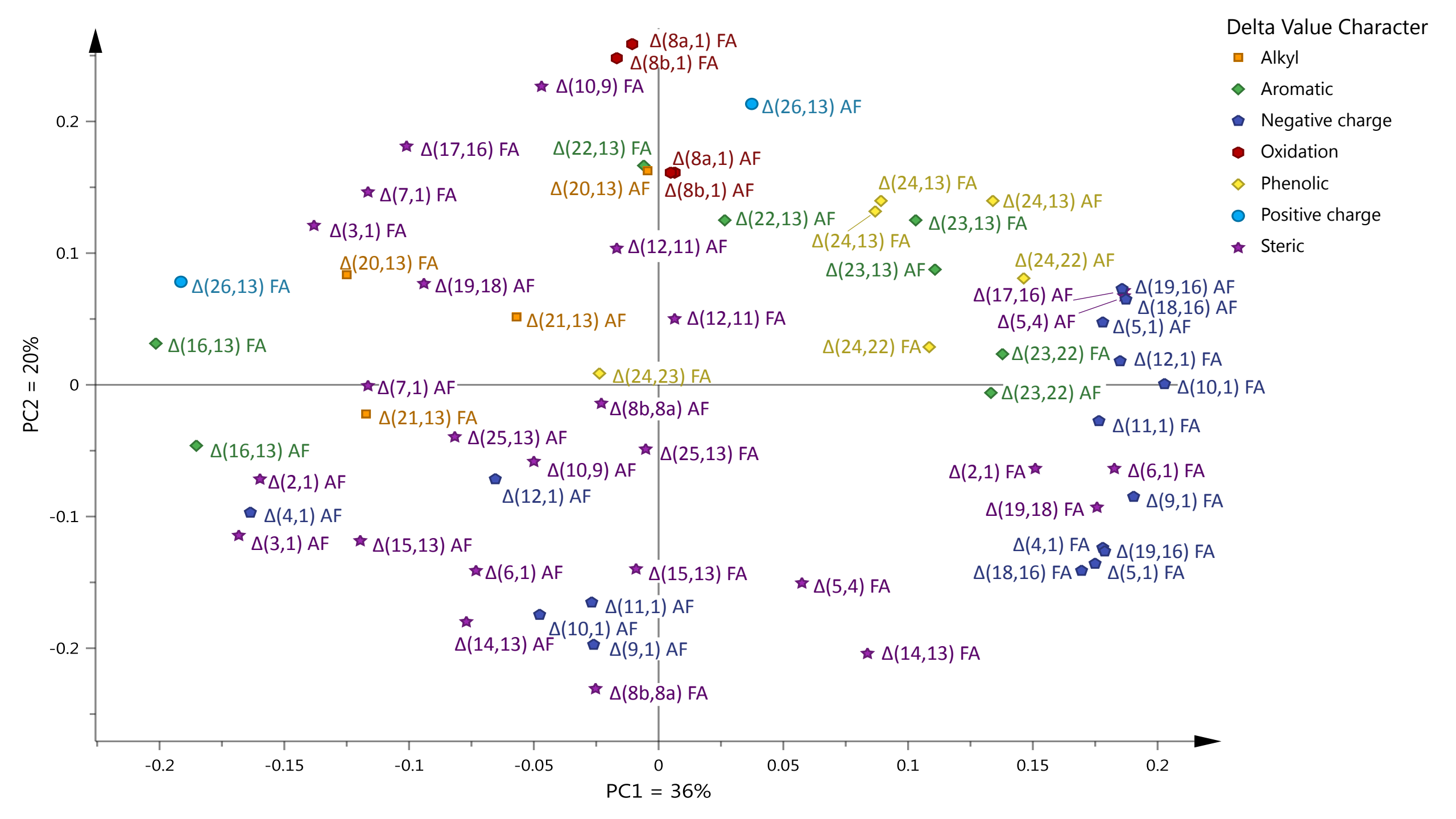
*a corresponds to the first eluting isomer of [Met(O)10]-Bovine GLP-2 (1-15)

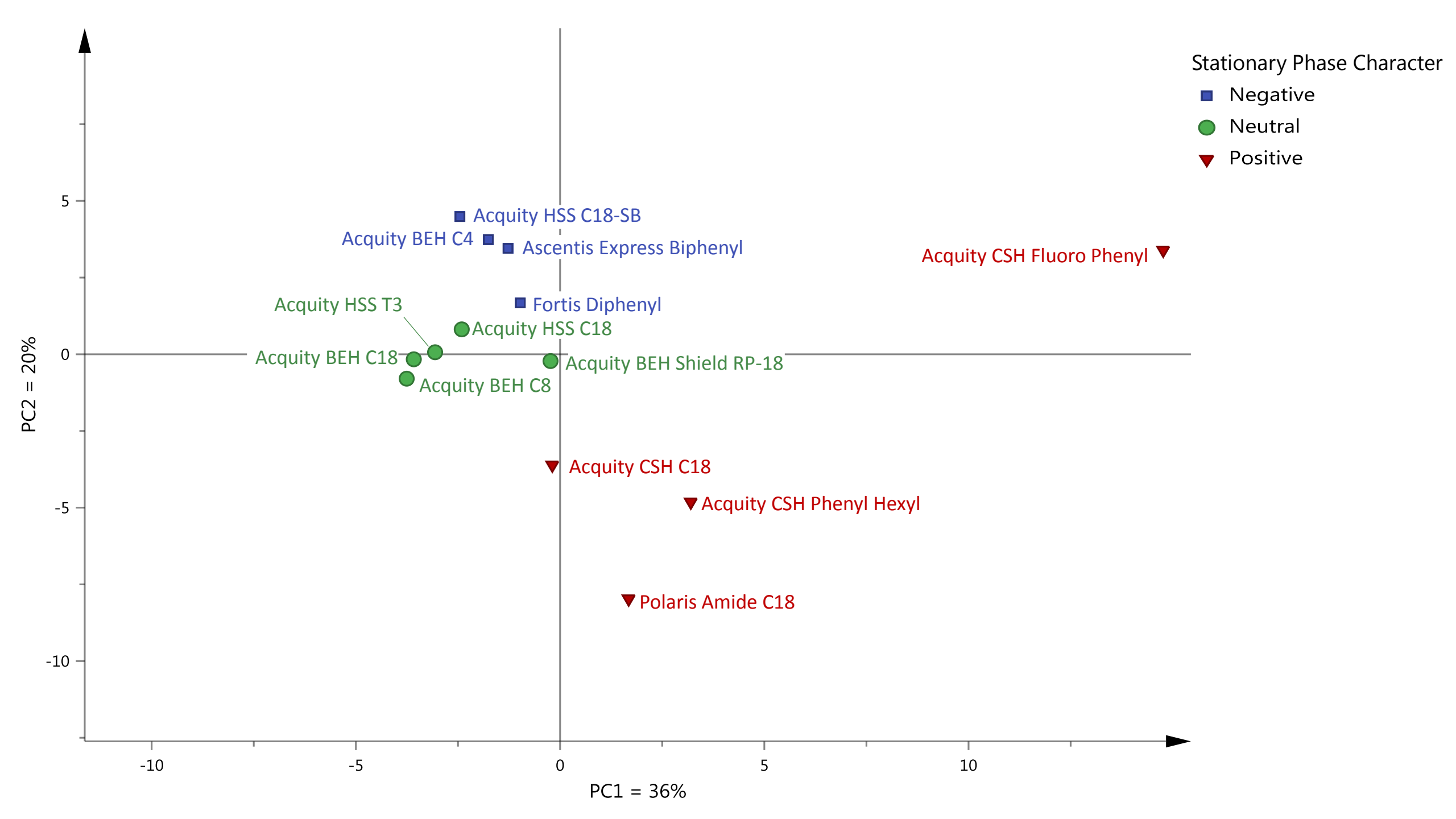
Table 6

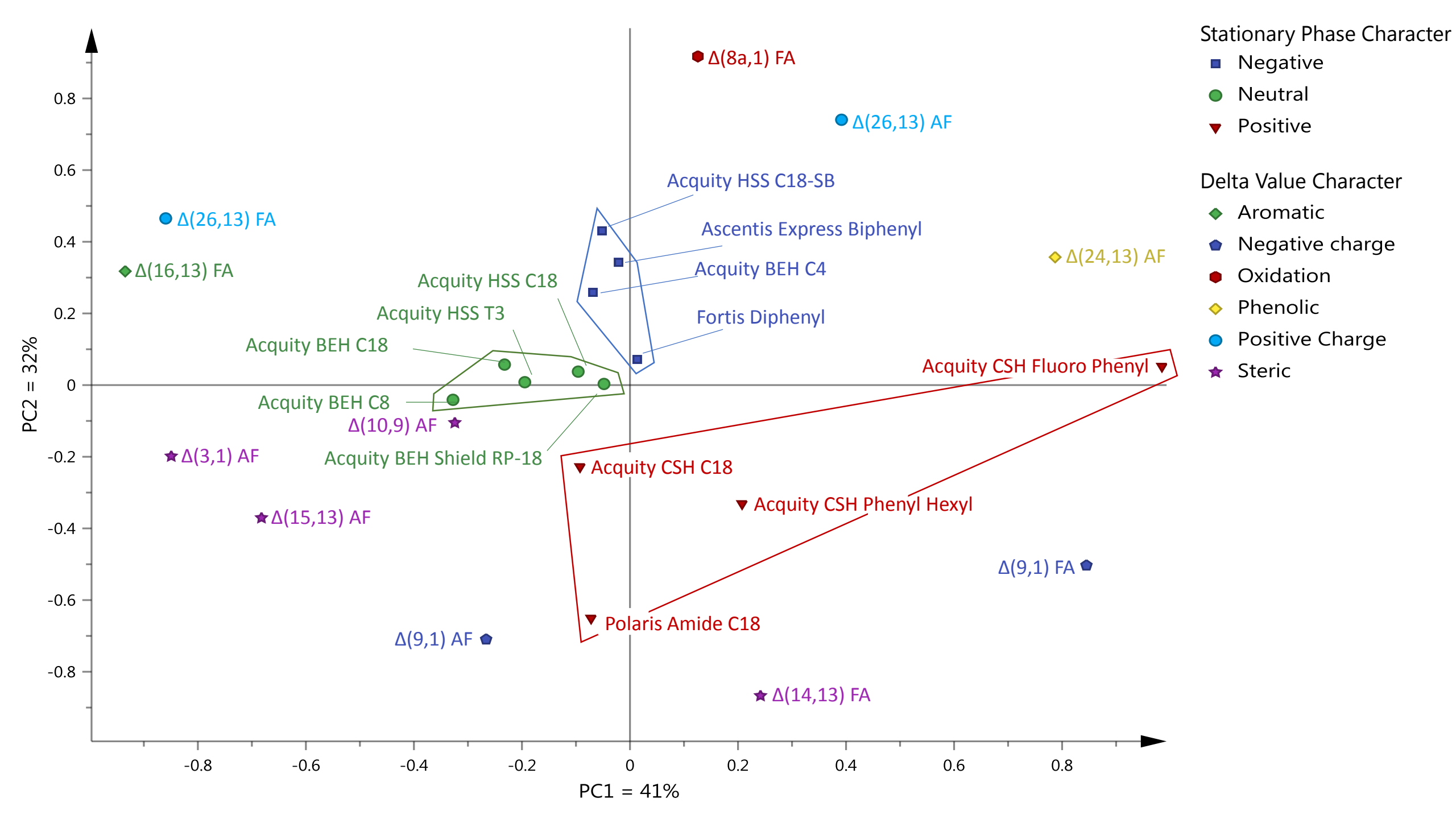
Peak capacity measured for each stationary phase using both the formic acid, TFA and ammonium formate gradients

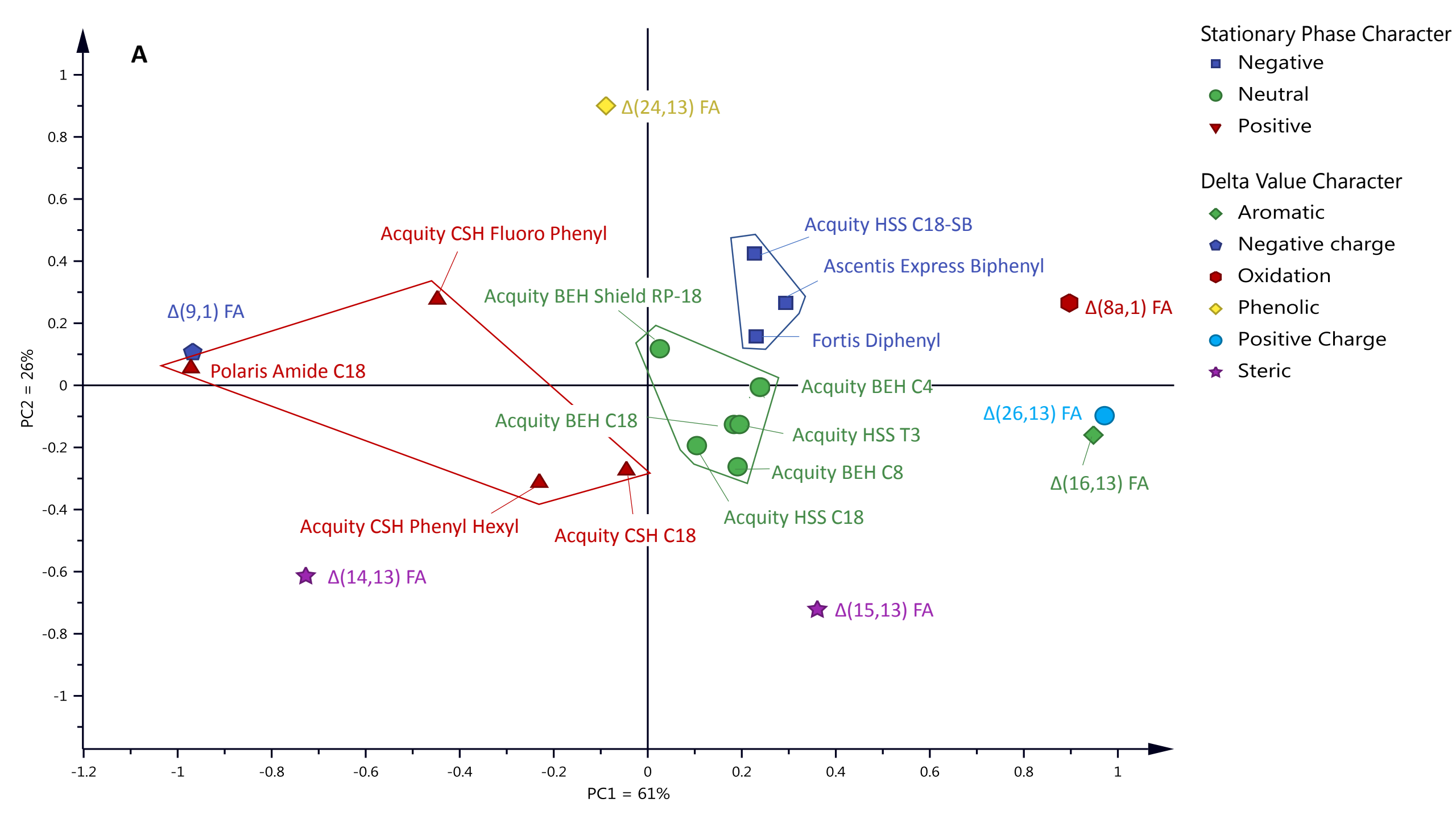
PC^{**}	Acquity BEH C4	Acquity BEH C8	Acquity BEH C18	Acquity BEH Shield RP-18	Acquity CSH C18	Acquity CSH Fluoro Phenyl	Acquity CSH Phenyl Hexyl	Acquity HSS C18	Acquity HSS C18-SB	Acquity HSS T3	Ascentis Express Biphenyl	Fortis Diphenyl	Polaris Amide C18
Formic Acid	90	121	123	137	146	78	108	80	108	64	66	66	79
Trifluoroacetic Acid	172	137	174	168	173	140	147	142	68	81	129	130	108
Ammonium Formate	129	212	222	229	196	124	180	173	81	183	193	122	96

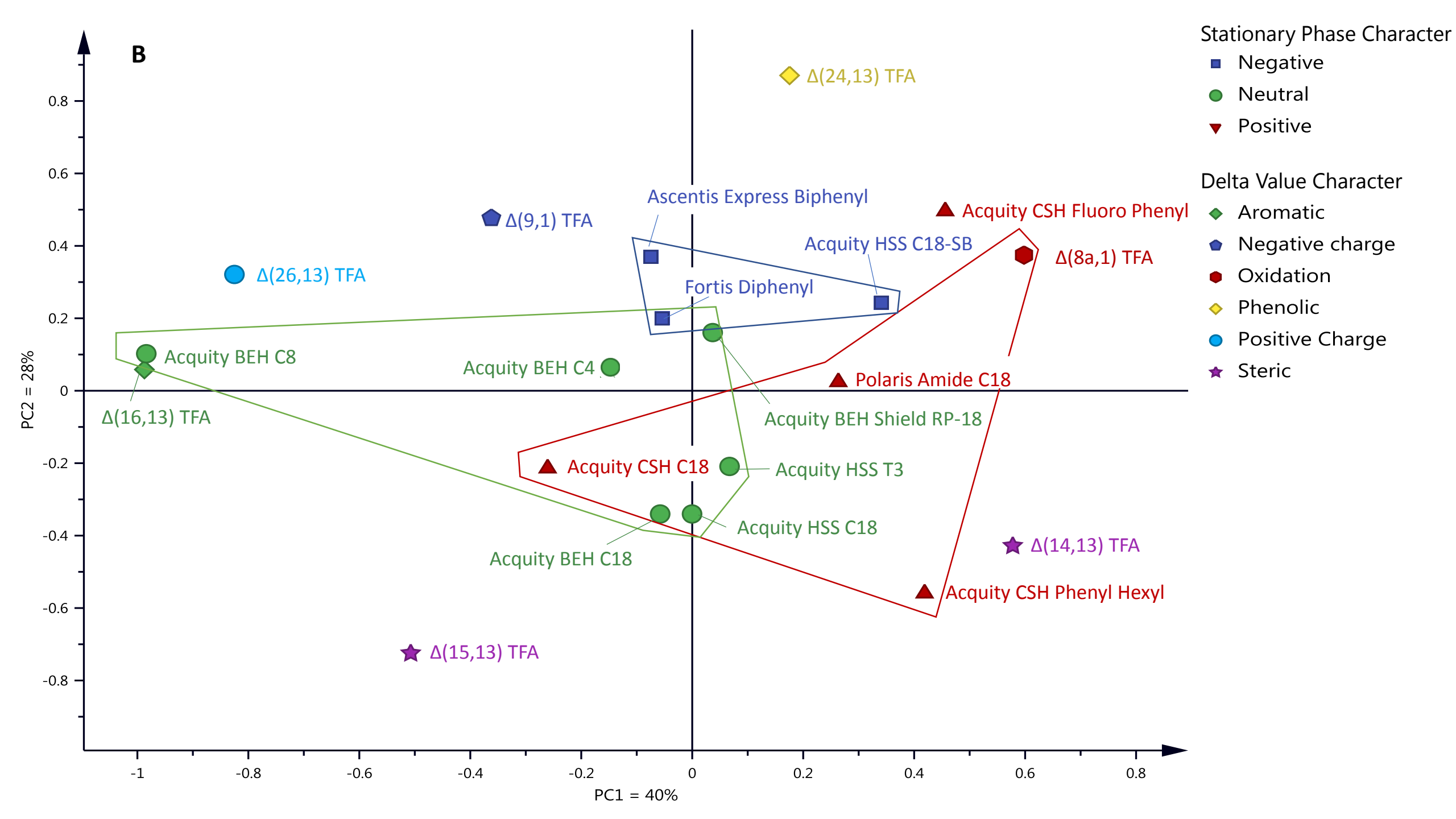

t_{gRef1}
t_{gRef2}
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
min

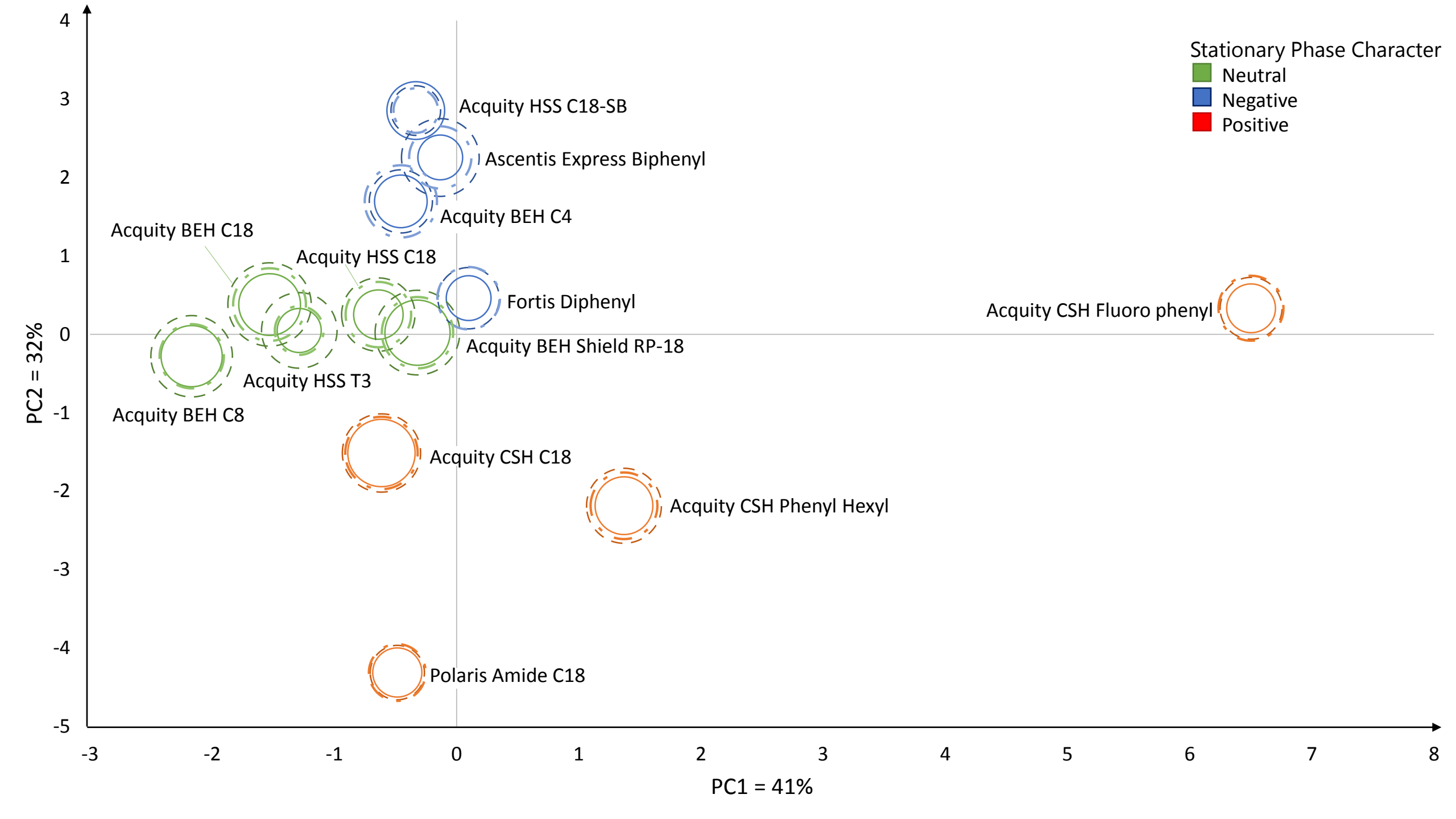

Delta Value Character
Alkyl
Aromatic
Negative charge
Oxidation
Phenolic
Positive charge
Steric
Δ(8a,1) FA
Δ(8b,1) FA
Δ(10,9) FA
Δ(26,13) AF
Δ(17,16) FA
Δ(22,13) FA
Δ(8a,1) AF
Δ(20,13) AF
Δ(8b,1) AF
Δ(24,13) FA
Δ(24,13) AF
Δ(7,1) FA
Δ(22,13) AF
Δ(23,13) FA
Δ(3,1) FA
Δ(24,13) FA
Δ(12,11) AF
Δ(20,13) FA
Δ(23,13) AF
Δ(24,22) AF
Δ(26,13) FA
Δ(19,18) AF
Δ(17,16) AF
Δ(19,16) AF
Δ(18,16) AF
Δ(21,13) AF
Δ(12,11) FA
Δ(5,4) AF
Δ(5,1) AF
Δ(16,13) FA
Δ(24,22) FA
Δ(23,22) FA
Δ(12,1) FA
Δ(24,23) FA
Δ(7,1) AF
Δ(23,22) AF
Δ(10,1) FA
Δ(8b,8a) AF
Δ(21,13) FA
Δ(11,1) FA
Δ(25,13) AF
Δ(16,13) AF
Δ(25,13) FA
Δ(2,1) FA
Δ(6,1) FA
Δ(10,9) AF
Δ(2,1) AF
Δ(12,1) AF
Δ(19,18) FA
Δ(9,1) FA
Δ(4,1) AF
Δ(4,1) FA
Δ(3,1) AF
Δ(15,13) AF
Δ(19,16) FA
Δ(18,16) FA
Δ(5,1) FA
Δ(6,1) AF
Δ(15,13) FA
Δ(5,4) FA
Δ(11,1) AF
Δ(10,1) AF
Δ(14,13) AF
Δ(9,1) AF
Δ(14,13) FA
Δ(8b,8a) FA
0.2
0.1
0
-0.1
-0.2
PC2 = 20%
-0.2
-0.15
-0.1
-0.05
0
0.05
0.1
0.15
0.2
PC1 = 36%

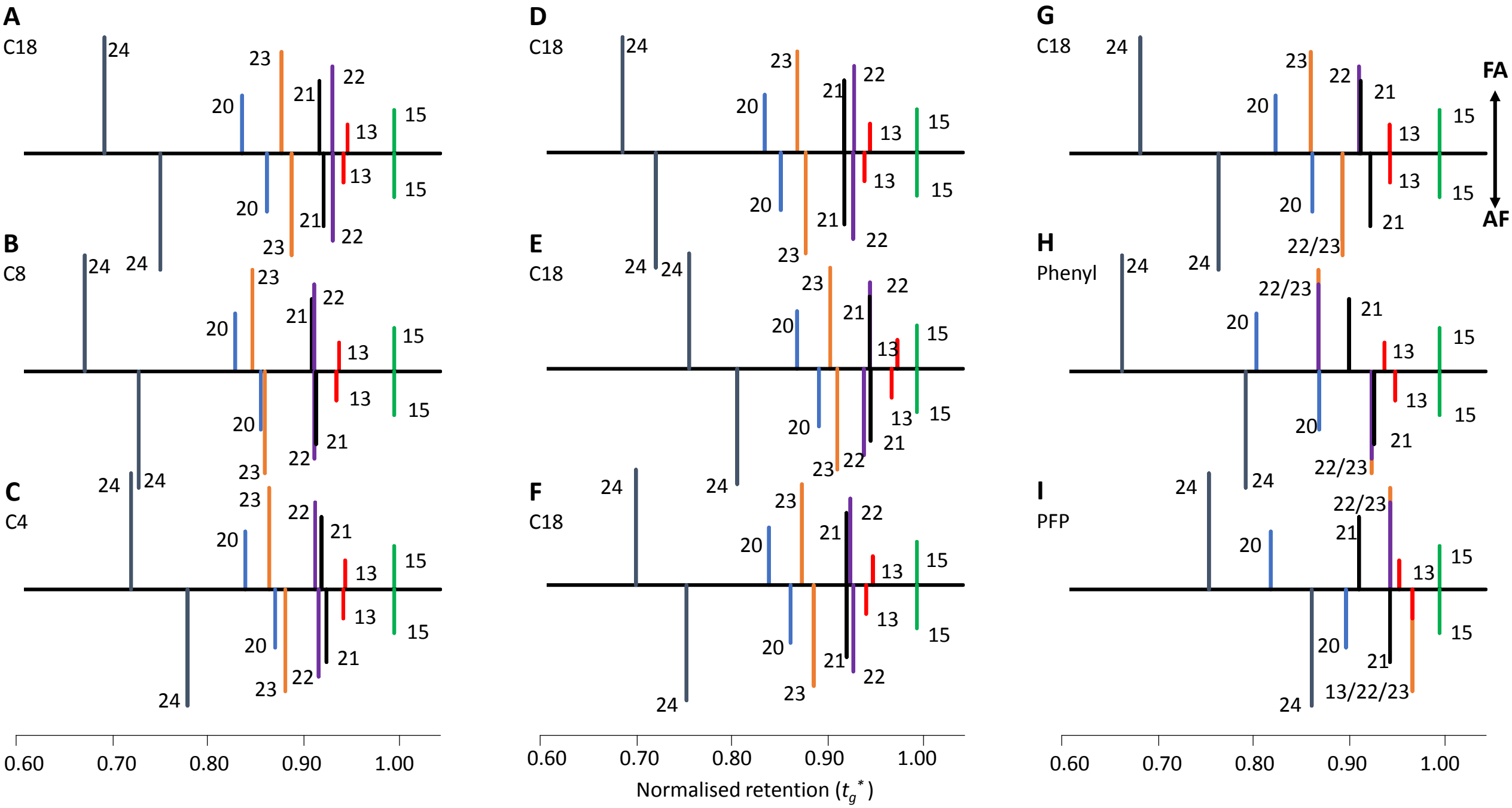

Stationary Phase Character
Negative
Neutral
Positive
Acquity HSS C18-SB
Acquity BEH C4
Ascentis Express Biphenyl
Acquity CSH Fluoro Phenyl
Acquity HSS T3
Fortis Diphenyl
Acquity HSS C18
Acquity BEH C18
Acquity BEH Shield RP-18
Acquity BEH C8
Acquity CSH C18
Acquity CSH Phenyl Hexyl
Polaris Amide C18
PC2 = 20%
PC1 = 36%
5
0
-5
-10
-10
-5
0
5
10

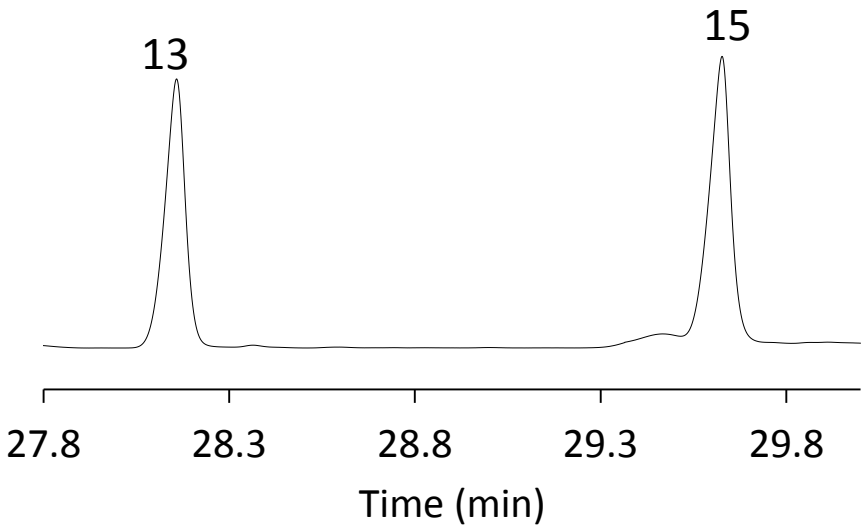

Stationary Phase Character
Negative
Neutral
Positive
Delta Value Character
Aromatic
Negative charge
Oxidation
Phenolic
Positive Charge
Steric
Δ(8a,1) FA
Δ(26,13) AF
Acquity HSS C18-SB
Ascentis Express Biphenyl
Acquity BEH C4
Fortis Diphenyl
Δ(26,13) FA
Δ(16,13) FA
Acquity HSS C18
Acquity HSS T3
Acquity BEH C18
Acquity BEH C8
Δ(10,9) AF
Δ(3,1) AF
Acquity BEH Shield RP-18
Δ(24,13) AF
Acquity CSH Fluoro Phenyl
Acquity CSH C18
Acquity CSH Phenyl Hexyl
Δ(15,13) AF
Δ(9,1) FA
Polaris Amide C18
Δ(9,1) AF
Δ(14,13) FA
PC2 = 32%
PC1 = 41%
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8

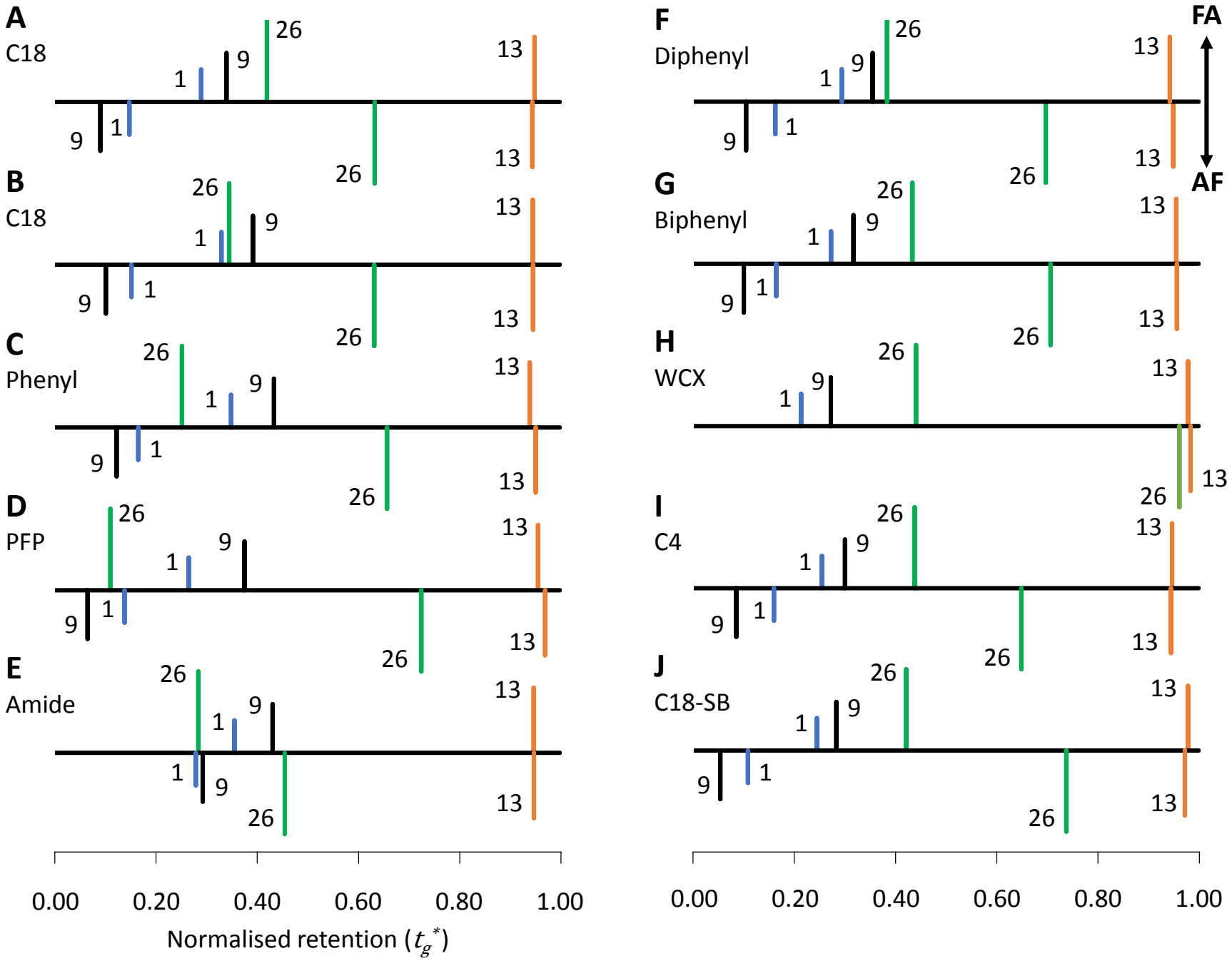

A
Δ(24,13) FA
Acquity CSH Fluoro Phenyl
Acquity HSS C18-SB
Ascentis Express Biphenyl
Acquity BEH Shield RP-18
Δ(8a,1) FA
Δ(9,1) FA
Fortis Diphenyl
Polaris Amide C18
Acquity BEH C4
Δ(26,13) FA
Acquity BEH C18
Acquity HSS T3
Acquity BEH C8
Δ(16,13) FA
Acquity HSS C18
Acquity CSH Phenyl Hexyl
Acquity CSH C18
Δ(14,13) FA
Δ(15,13) FA
PC2 = 26%
PC1 = 61%
Stationary Phase Character
Negative
Neutral
Positive
Delta Value Character
Aromatic
Negative charge
Oxidation
Phenolic
Positive Charge
Steric

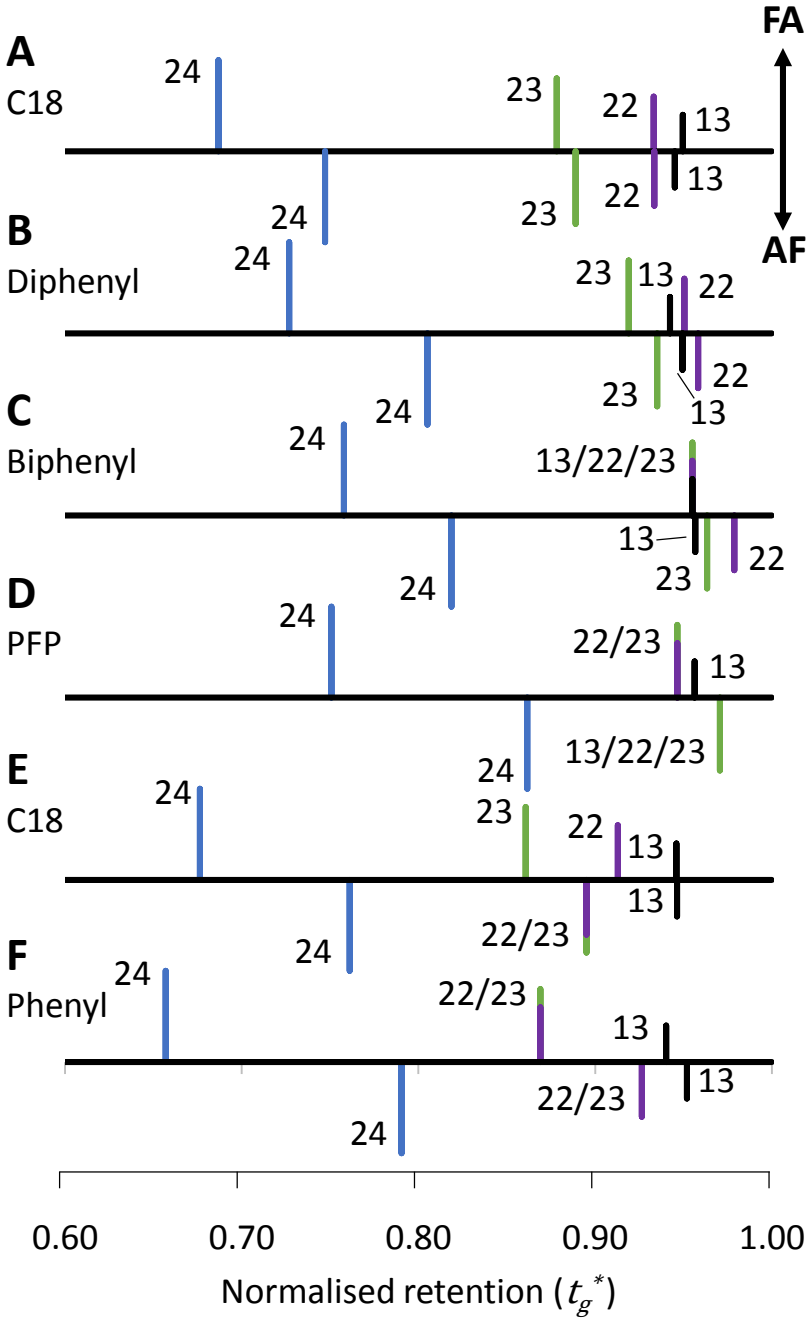

B
Δ(24,13) TFA
Ascentis Express Biphenyl
Δ(9,1) TFA
Acquity CSH Fluoro Phenyl
Acquity HSS C18-SB
Δ(8a,1) TFA
Δ(26,13) TFA
Fortis Diphenyl
Acquity BEH C8
Acquity BEH C4
Polaris Amide C18
Δ(16,13) TFA
Acquity BEH Shield RP-18
Acquity CSH C18
Acquity HSS T3
Acquity HSS C18
Acquity BEH C18
Δ(14,13) TFA
Acquity CSH Phenyl Hexyl
Δ(15,13) TFA
PC2 = 28%
PC1 = 40%
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
Stationary Phase Character
Negative
Neutral
Positive
Delta Value Character
Aromatic
Negative charge
Oxidation
Phenolic
Positive Charge
Steric

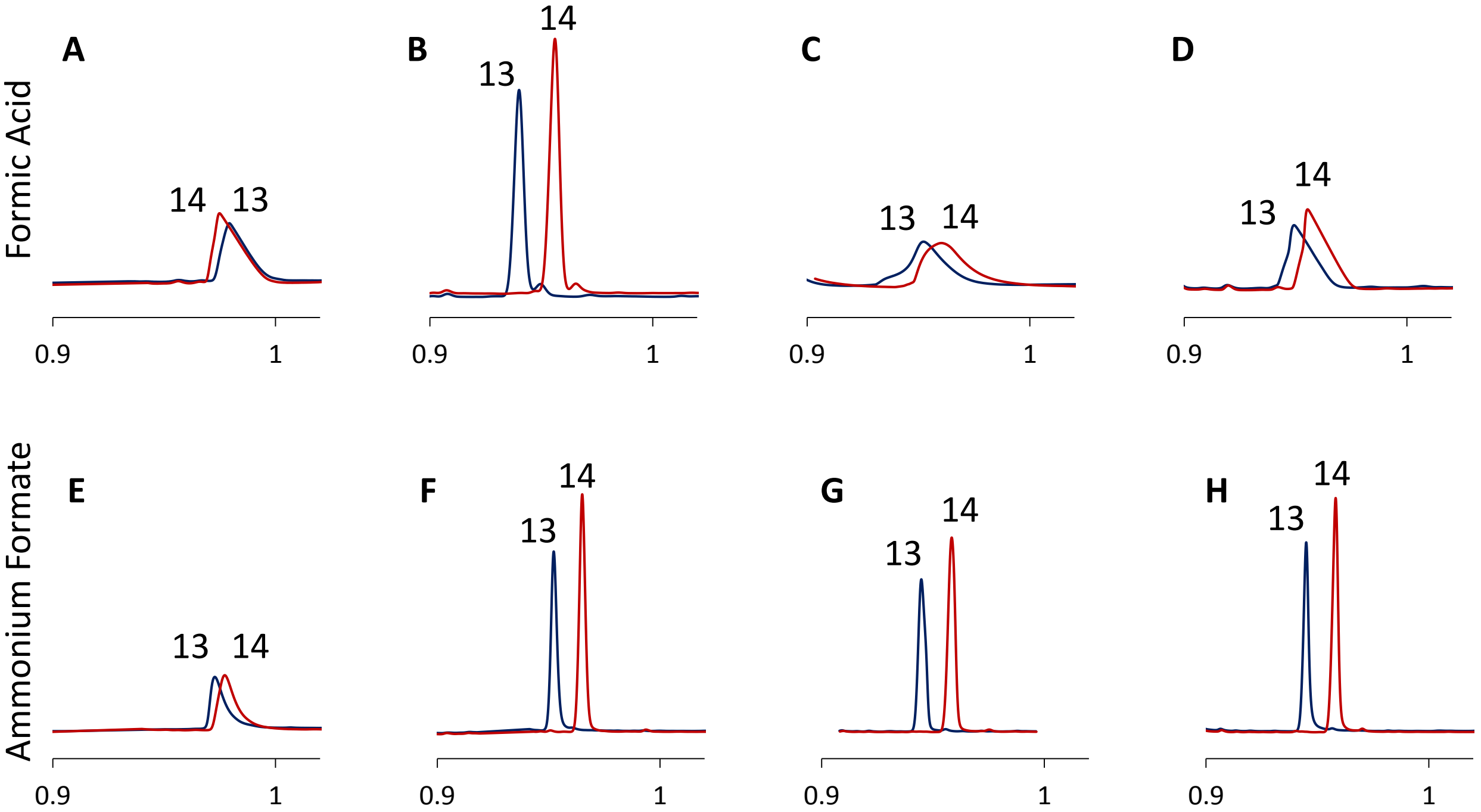

Stationary Phase Character
Neutral
Negative
Positive
Acquity HSS C18-SB
Ascentis Express Biphenyl
Acquity BEH C4
Acquity BEH C18
Acquity HSS C18
Fortis Diphenyl
Acquity CSH Fluoro phenyl
Acquity BEH Shield RP-18
Acquity HSS T3
Acquity BEH C8
Acquity CSH C18
Acquity CSH Phenyl Hexyl
Polaris Amide C18
PC2 = 32%
PC1 = 41%
4
3
2
1
0
-1
-2
-3
-4
-5
-3
-2
-1
0
1
2
3
4
5
6
7
8


A
C18
B
C8
C
C4
D
C18
E
C18
F
C18
G
C18
H
Phenyl
I
PFP
FA
AF
22/23
13/22/23
0.60
0.70
0.80
0.90
1.00
Normalised retention (t_g^*)


13
15
27.8
28.3
28.8
29.3
29.8
Time (min)


A
C18
B
C18
C
Phenyl
D
PFP
E
Amide
F
Diphenyl
G
Biphenyl
H
WCX
I
C4
J
C18-SB
FA
AF
1
9
26
13
0.00
0.20
0.40
0.60
0.80
1.00
Normalised retention (t_g^*)


A
C18
B
Diphenyl
C
Biphenyl
D
PFP
E
C18
F
Phenyl
FA
AF
24
23
22
13
13/22/23
22/23
0.60
0.70
0.80
0.90
1.00
Normalised retention (t_g^*)


Formic Acid
Ammonium Formate
A
B
C
D
E
F
G
H
13
14
0.9
1