Investigation into Reversed Phase Chromatography Peptide Separation Systems Part II: An Evaluation of the Robustness of a Protocol for Column Characterisation

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Abstract

The robustness of the Peptide Reversed Phase Chromatography (RPC) Column Characterisation Protocol was evaluated using reduced factorial design, to ascertain the degree of control required for parameters including temperature, flow rate, dwell volume, a systematic shift in the gradient, amount of formic acid in the aqueous and organic, pH of the ammonium formate and amount of acetonitrile in the strong solvent (%MeCN). All levels were deemed acceptable within reasonable tolerances except the %MeCN in the strong solvent, where a loss of MeCN resulted in an unacceptable variation. Mitigations have been introduced to ensure the integrity of the data to allow RPC columns to be characterised using peptides as probes, with the definitive protocol described. In addition, the instrument and column batch to batch variability were assessed with good reproducibility.

Keywords

Robustness; Factorial Design; Peptides; Column Characterisation; RPC Stationary Phases; Principal Component Analysis

1 Introduction

The selection of appropriate stationary phases is a key element of method development, however, with the plethora of phases available from many different column manufacturers, it is difficult to make a rational decision as to which column to start with. For small molecules, there are various strategies including the Tanaka and its extended protocol, Snyder's Hydrophobic Subtraction Model and Lesellier's Linear Subtraction Energy Relationships (LSER) which have characterised reversed phase (RP) stationary phases, with the results available in databases which are free to access [1-5]. These databases allow the end user to establish which phases are chromatographically different for

a diverse range of phases used for method development purposes, or alternatively, select phases which are chromatographically similar, in order to select “back-up” columns.

Until recently, there has not been a corresponding characterisation protocol for RP columns suitable for peptide separations, however, the Peptide RPC Column Characterisation Protocol was developed to address this deficiency [6]. A range of 26 specifically designed peptides with different physico-chemical properties were synthesised to assess prominent interactions such as hydrophobicity, hydrogen bonding, electrostatic interactions and aromatic character as well as reflect typical paths for degradation of peptides. These 26 peptides were evaluated on 14 stationary phases that possessed different properties based on prior knowledge of the column chemistry using gradient chromatography at low and intermediate pH. Selectivity was measured using the difference in normalised retention (Δt_g^*), where a total of 66 delta values were produced to probe different types of interactions which were critically assessed using principal component analysis (PCA). The mobile phases selected were formic acid (~pH 2.5) and ammonium formate at (~pH 6.45, native pH) to evaluate the purer interactions of the stationary phase. The rationale for mobile phase selection can be observed in reference 6. The stationary phases were categorised into three distinct groupings to describe the prominent interactions; neutral (phases which possess a high degree of ligand density and / or end capping), negative / polar (phases which either contain a negatively charged moiety in the ligand, capable of silanophilic interactions due to a light bonding / lack of end capping, or functionalities which are capable of forming polar interactions such as hydrogen bonding) and positive character(phases which contain a positively charged moiety). The total of peptides required was reduced from 26 to 11 which generated 11 delta values to describe changes in positive / negative charge, hydrophobicity, phenolic character, aromatic character as well as steric changes, racemisation, deamidation and oxidation (Table 1 and Table 2). A smaller study was conducted to observe the effect of TFA instead of formic acid as the low pH additive, as TFA is quite frequently used for peptide analysis. The resulting data caused the distinct categories produced via the formic acid / ammonium formate biplot to lose their structure which suggests a loss of information when using TFA. This is as hypothesised as TFA acts as an ion pair therefore the character of the stationary phase is of less importance, thus emphasising the necessity to conduct a column characterisation protocol using an appropriate additive like formic acid. An iterative study was conducted which appraised the 66 delta values to determine if the total number could be reduced whilst still fully describing the stationary phases.

In order for the Peptide RPC Column Characterisation protocol to be truly valuable, the robustness must be considered [7]. Robustness is defined by the ICH guidelines as “a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an

indication of its reliability during normal usage" [8,9]. The robustness of the protocol was assessed using a reduced factorial design often referred to as a DoE (design of experiments), which is one of the most commonly used tools for the assessment of robustness both within academia and the pharmaceutical industry. In this methodology, small systematic changes reflecting the expected experimental errors associated with the procedure are explored [9-11]. Alternative methods such as one factor at a time (OFAT) could be used, which looks to vary one variable whilst maintaining all other parameters of interest. However, it can often prove more resource intensive (i.e. time, materials etc) and can fail to estimate the interaction between different variables. A considerable amount of information can be derived from DoE with statistical significance, which cannot be obtained via the OFAT approach [12]. The 11 peptide delta values were used as responses to create the DoE models which highlighted the degree of deviation the systematic changes created from the nominal centre point i.e. the original method conditions. This data informs what variable(s) are statistically significant and their practical relevance on the result, thus highlighting what operating parameters must be carefully controlled in order to maintain a robust methodology. It is integral that the robustness of the procedure be considered before a larger set of stationary phases are evaluated which can be included into a column characterisation database for individuals to rationally select stationary phases for peptide / protein separations. A similar approach was used to assess the robustness of the Tanaka and extended protocols in order to ensure the integrity of the column database [10].

This paper aims to gain a greater understanding of the crucial factors which could impact on method robustness, which not only includes performing a DoE on LC parameters used in the protocol, but also establishes the robustness of mobile phase pH switching and sample load. Finally, the practical operating limits of the LC parameters used in the protocol will be established in order to maintain acceptable repeatability and reliability of the integrity of the peptide-based column characterisation database. This paper is part of a series of articles which are designed to interrogate the factors which influence the peptide separation system, where firstly the stationary phases were evaluated before investigating the effect of different mobile phase additives to aid the chromatographer in making rational decisions relating to their chromatographic separation.

2 Experimental

2.1 Chemicals, Reagents and Instrumentation

All chemicals used are described in reference 6. Unless otherwise stated all LC separations were performed on a Shimadzu Nexera X2 UHPLC system where modules were previously described [6].

The base sequence for each peptide can be located in Table 2, and further description of the peptide probes described in reference 6.

2.2 Factorial Design

The Peptide RPC Column Characterisation protocol conditions were described in reference 6. The new test mixtures with each specific column load can be found in Table 3. Each test mixture contained the two peptides required to calculate a precise delta value. This sometimes meant that there was duplication of peptides in multiple test mixtures. This was to ensure the delta values were well described and increase method robustness as small degrees of retention drift could impact on subtle interactions such as racemisation. The peptides selected were based on the iterative process of elimination conducted within reference 6, and contain both subtle and more significant changes to evaluate common degradation and specific interactions to describe the column for characterisation. The delta values, which are a measure of the selectivity in gradient elution, are used as the input for the DoE.

The factors evaluated in the DoE were summarised in Table 4, including the different ±1 levels. The factors investigated were selected as the most likely sources of error, where the levels were ascertained to reflect the random variation one could expect (i.e. at least 3x the expected standard deviation). Error propagation calculations based on instrument specifications and qualification data were employed in the estimation of standard deviation [13].

Modde Pro software (see Section 2.4) was employed to create and evaluate reduced factorial designs, which utilised eight methods with various +1 or -1 levels for the different variables, and three repeat methods for the central nominal conditions to ascertain the reproducibility of the procedure (Tables 5 and 6 for formic acid and ammonium formate gradients, respectively). Different batches of solvents were produced for the nominal conditions. The software created a random run order to remove any bias within the results.

2.3 Instrument Variability

Instrumental variability was compared between three additional LC instruments; Waters H-Class, Waters I-Class (Milford, Ma, USA) and Agilent 1290 binary system (Waldbronn, Germany) using the same batches of mobile and stationary phase. The dwell volumes varied between 300 and 700 µL. The Waters instruments were controlled by Empower 3 (Feature release 3) whilst the Agilent configuration was controlled by OpenLab CDS (Chemstation C.01.07 SR4).

2.4 Stationary Phases

The Peptide RPC Column Characterisation protocol was developed using 14 diverse stationary phases [6] and a standardised column format of 150 x 2.1 mm. All columns were new as supplied by the manufacturer. A Phenomenex Kinetex Evo C18 (150 x 2.1 mm, 100 Å, 2.6 µm) was used for the reduced factorial design experiments whilst the instrument variation was performed on a Waters Acquity HSS C18 (150 x 2.1 mm, 130 Å, 1.8 µm). Column batch to batch variability was assessed on six different batches of Ascentis Express C18 with six differing base silicas and four differing silanes (150 x 2.1 mm, 90 Å, 2.7 µm). Loading studies were performed on a Phenomenex Kinetex C18 (150 x 2.1 mm, 100 Å, 2.6 µm). The peak apex of a water injection was used as the dead time marker for each column [14].

2.5 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. Reduced factorial design was performed using Modde Pro (Version 12.0.1. Umetrics, Umeå, Sweden). 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 Principle Component Analysis and Factorial Design

The robustness of each delta value (Table 4) was assessed by fitting a first order polynomial model to the data obtained for the formic acid and ammonium formate designs (Table 5 and 6). The typical Δt_g and Δt_g^* values obtained on the Kinetex Evo C18 can be seen in Table 1 under the nominal centre point conditions.

The quality of the model is measured using a regression coefficient from fitting the model (R2) and one from a cross validation of the model (Q2) [11,15]. For a good model both values are close to 1. However, in a robustness evaluation an ideal outcome would be a poor model, i.e. low R2 and Q2 values. The effect of a ±1 change of the different factors should, for a robust methodology, correspond to the experimental noise at nominal conditions.

The average R2 and Q2 for the delta values in formic acid were 0.761 (standard deviation SD 0.208) and -0.133 (SD 0.151), respectively, whilst for the ammonium formate delta values, R2 and Q2 measured 0.750 (SD 0.080) and -0.200 (SD 0.000), respectively. The centre point experiments (N9-11) provide an indication into the reproducibility of the procedure, where the average difference for the delta probes was 0.001 (ranged from 0.000 to 0.004 for the relevant delta values). The result gives an early indication that the Peptide RPC Column Characterisation protocol should be robust.

The method robustness can be evaluated further by comparing coefficient plots which evaluates the individual effect of each parameter on the robustness (Fig. 1 and 2). Each variable was scaled and centred in the coefficient plots, so that they were comparable. The height of the bar gives the degree of the effect whilst the error bar indicates the 95% confidence interval which highlights statistically significant parameters when the error bar does not cross zero.

3.1.1 Temperature

Temperature had no discernible effect on any of the responses in ammonium formate and the majority in formic acid, however, there was a statistical response for Δ(8a,1) and Δ(16,13) in formic acid. The height of the bars however would indicate that the practical significance on these two delta results would be minimal. Although for these probes it has a minimal effect, it is recommended that the actual temperature of the column is determined, as it is known that the column oven design and even column position within the oven can change the actual temperature within the column.

This can be achieved by injecting caprylophenone (detection at 250 nm) onto a column using a premixed mobile phase of MeCN/H_2O (45:55 w/w) to eliminate instrument to instrument %MeCN variations. The column oven temperature is changed in 5 °C intervals over the range 30-60 °C. The same column is then tested by immersing it in a water bath with 30 cm of 0.12 mm tubing prior to the column for thermostating. This is then assumed to be an accurate measure of the temperature of the column. The temperature of the water bath should be determined using a calibrated / certified thermometer with an appropriate accuracy e.g. ±0.5 °C between 30 to 60 °C.

The sample is injected at 30 to 60 °C in 5 °C intervals in order to construct a plot (retention time – system dead time) *vs.* temperature plot in order to determine any deviation in temperature for the LC system (i.e. ΔT at a certain retention time). System dead time is subtracted from the retention time to allow for comparison between the ovens and is determined by the retention time of water (detection at 215 nm), where the column is replaced with a union. This procedure should be followed for any new type of column oven design, and once the actual temperature of the column is

196 determined, the Peptide RPC Column Characterisation Protocol can be adapted to compensate for
197 any deviation in temperature.

198

199 **3.1.2 Systematic Shift in the Gradient Composition**

200 The responses in formic acid and ammonium formate were all unaffected by the systematic shift in
201 the gradient. This systematic shift in the gradient assumes the same degree of error will apply across
202 the length of the gradient.

203

204 **3.1.3 Flow rate**

205 A change of flow rate also corresponds to a change in gradient slope. Flow rate only presented a
206 small statistical response for Δ(9,1) in formic acid, whilst all other responses in both ammonium
207 formate and formic acid were unaffected. Similar to temperature, the actual practical effect of this
208 variable would be quite minimal, thus this parameter can be assumed to be robust within the
209 methodology.

210

211 **3.1.4 Dwell Volume**

212 Dwell volume was statistically insignificant for all responses in either mobile phase, indicating this
213 variable does not impact on the robustness of either the formic acid or ammonium formate
214 gradient. This is to a large extent due to the normalisation of the retention times which removes the
215 effect of dwell volume, allowing direct comparison between different instrumentation. The dwell
216 volume range selected for evaluation (100 - 500 µL) should cover UHPLC instrumentation.

217

218 **3.1.5 Amount of Formic Acid**

219 Differences in formic acid levels could impact on the robustness of the protocol as different levels
220 would result in a different pH which would affect the overall net charge on the peptides. However,
221 differences in formic acid volume were observed to be insignificant, the Δ(9,1) and Δ(16,13) values
222 exhibited a very small statistical significance but this was deemed to be of little practical relevance. It
223 is recommended though that formic acid volumes should be dispensed volumetrically from a pre-
224 calibrated pipette which is checked each time a solvent is prepared to ensure the integrity of the
225 chromatographic results.

226

3.1.6 Ammonium Formate Stock Solution pH

The stationary phase environment at intermediate pH is somewhat unpredictable due to the range of pK_a values for the residual silanols [16,17]. It is believed the majority of silanols should be ionised at pH 6.45 (the native pH of ammonium formate), however, this uncertainty can potentially lead to a greater degree of variation in results and hence can contribute to the lack of robustness. The ammonium formate can also be a source of error, where the age of the buffer, its storage environment and its resultant pH range were investigated. The pH was measured for 16 different 200 mM solutions where the average pH was 6.45 (SD 0.03). The levels in the DoE (pH 6.45 ±0.06) were set based on a 99% CI based on triplicate determinations of the pH. The age of the ammonium formate did not appear to greatly affect the overall pH of the solution, where the pH measured was within the range tested in the DoE. There was, however, a change in pH based on salts which were inappropriately stored, which resulted in a lower pH for a poorly capped container which indicates a loss of ammonia. This could impact on the degree of silanol ionisation and hence affect retention and the delta values. Ideally, the formate salt should be stored in a desiccator to reduce water uptake and firmly replacing the cap should reduce the risk of ammonia loss.

The responses in ammonium formate were all stable within the upper and lower pH limits in the DoE, with no statistical significance. The pH of the stock buffer solution should be measured using appropriately calibrated standards to ensure the pH is within this range to ensure the integrity of the protocol. It is also advised that if the ammonium formate exhibits any considerable signs of hygroscopicity in addition to changes in pH then it should not be used.

To avoid microbial growth which could contaminate the LC system and potentially block the column inlet frit, causing split peaks and higher back pressures, it is recommended to limit the storage of stock buffer solution to 4 months at 5 °C.

3.1.7 MeCN Composition in the B Solvent

The selectivity of Δ(3,1), Δ(9,1), Δ(10,9) and Δ(24,13) were particularly susceptible to the change in acetonitrile content in the B solvent in the ammonium formate gradients. The effect was actually significant enough that it would have practical relevance, unlike previous variables, and thus warranted further investigation.

As described in the previous publication which defined the Peptide RPC Column Characterisation Protocol, PCA is used in order to visualise similarities and differences between delta values and columns in so called biplots [3,18]. The delta values for the ammonium formate and formic acid DoE's were included in a PCA to evaluate the robustness of the method on the protocols ability to

differentiate differing stationary phases from one another and to see what limits of the proposed MeCN levels had on the integrity of the results. As can be seen in the resulting biplot (Fig. 3), most of the DoE runs are clustered close to the origin except for a small subset of runs which are located further down in the lower left quadrant. Further evaluation indicated the subset was pulled away by the -1 level for the MeCN content in the B solvent.

A loss of MeCN could possibly be expected by evaporation of solvent in the mobile phase reservoirs over a period of time. An evaporation study was therefore performed to ascertain what could be reasonably lost via the LC solvent caps. An Agilent Valve cap (Waldbronn, Germany) and a SCAT safety cap (Mörfelden-Walldorf, Germany) were compared against a closed cap used for solvent storage. There was 0.00% loss in weight for the closed cap over 30 days, suggesting acetonitrile is not lost during storage, however, it was calculated that losses of 0.04 and 0.03% for the Agilent and SCAT caps, respectively, could be expected per day, which could prove practically problematic.

In order to combat this, the approach was taken to change the B solvent from 20 mM ammonium formate in MeCN/H_2O (80:20 w/w) to 100% MeCN. The gradient was adjusted accordingly to achieve the same volume fraction of MeCN and compared against the original method, with similar chromatographic results, regardless of the reduced buffer concentration in the B solvent (the ammonium formate concentration is reduced from 18 to 12 mM during the part of the gradient where peptides typically elute).

3.2 Instrument Variability

The ability to successfully translate LC methodologies between differing LC instrumentation is extremely important for the widespread acceptance of the protocol. This is particularly important with respect to gradient chromatography where the contribution of column volume and dwell volume has been shown to cause considerable selectivity differences [19].

The three peptide mixtures were injected onto an Acquity HSS C18 column on three different instruments, Agilent 1290, Waters Acquity H- and I-class ranging in dwell volume between 300 and 700 µL, which was larger than the range assessed within the DoE. The same batches of mobile phases and column were used on all the comparisons to remove any variation attributed to mobile phase preparation differences and understand the instrument contribution to the study.

The delta values were recorded for each instrument and compared, after which the data was analysed by PCA and placed in the biplot (Fig. 3). The three instruments are circled within a 95%

confidence limit. This variation is comparable to what can be expected from the DoE thus indicating results obtained on different type of instruments should be comparable.

3.3 Column Batch to Batch Variability

The column batch to batch variability was assessed using six Ascentis Express C18 columns. All columns differed by silica, whilst three columns contained the same batch of silane with three additional silane batches used for the remaining columns.

All batch to batch columns were tested using the new protocol with the reduced number of probes (i.e. removal of probes susceptible to changes in MeCN) on the same occasion and mobile phases to remove their contribution to any variability.

The batch to batch observations can be seen encircled within the biplot (Fig. 4) where the scatter seen is due to the batch to batch variation as the data was collected on the same day using the same instrument and solvents to eliminate their contribution from the results. The results are also in keeping with previous batch to batch studies performed on other columns using various protocols [9,20-26]. This highlights that any deviation between columns within the biplot is caused by selectivity differences, thus it is feasible to distinguish stationary phases which are chromatographically similar or dissimilar using this approach.

3.4 Slow Equilibration

Slow equilibration of certain RP materials when changing from intermediate to low pH has been well documented by Snyder *et al.* It is exhibited by a steady retention time drift on changing the mobile phase pH. It is believed that approximately 40% of all commercially available stationary phases exhibited some form of slow equilibration [27]. The exact mechanism for this phenomenon is not known, but it is speculated that with the advent of modern silica with low surface charge, changes in pH can require significant time to re-equilibrate which is displayed as retention drift for ionisable species.

A selection of C18 type phases were assessed using the peptides as probes to determine any practical constraints for the Peptide RPC Column Characterisation protocol, which utilises both low and intermediate pH.

The peptide test mixture was repeatedly injected on a C18 stationary phase using the formic acid gradient conditions with consistent results (Fig. 5(A)). The phase was then exposed to the ammonium formate gradient conditions, which saw quick equilibration of the peptide mixture within

duplicate injections, suggesting consistent results can be achieved when moving from low to intermediate conditions.

However, when the same column was then re-exposed to the formic acid gradient, it failed to yield results comparable to those prior to exposure to intermediate pH (slow reduction in retention – see Fig. 5(B)). The retention times for all peaks had increased, but were consistently decreasing in retention between injections.

Literature has suggested static equilibration can restore a column which exhibits slow equilibration [27-29], however, even an overnight static equilibration in low pH conditions failed to restore this stationary phase to its original chromatographic retentivity (Fig. 5(C)).

This phenomenon is not exclusive to just formic acid, but between any switch from intermediate or high pH to low pH for ionisable species. The issue would also not be corrected with the use of TFA, as the ion pairing effect of this additive would mean dedicated columns would be required which would be impractical for column characterisation [30]. Although there are stationary phases which have been devised to combat this slow equilibration issue such as the Acquity CSH range of phases [31], there are a number of commercially available columns which do exhibit this phenomenon. As such, the decision was made to characterise each stationary phase initially using the formic acid gradient, before testing at intermediate pH using ammonium formate in order to avoid any detrimental retention drifts or the necessity for excessive equilibration times.

3.5 Loadability

Chromatographic efficiency can be highly susceptible to analyte overloading which contributes to poor peak shape, especially for ionisable species. The permissible load before chromatographic performance is affected is often substantially lower for peptides and protein separations [31]. A significant amount of literature is available on the subject of loadability of basic species, and rationalising overloading effects [32-34].

A stock solution of [L-Asp3]-Bovine GLP-2 (1-15) (1 mg/mL) underwent a series of dilutions using DMSO/H_2O (80:20 v/v). Each solution was reproducibly injected onto the Kinetex Evo C18 (150 x 2.1 mm, 2.6 µm) using the formic acid gradient chromatographic conditions. The low ionic strength of formic acid is a worst-case scenario; thus, it was selected to observe the effects of loadability and overloading.

Eight dilutions were made in total (four shown for simplicity in Fig. 6) with the resulting chromatograms overlaid. The hydrophilic peptide, which has a net charge of +1.2 at pH 2.5,

displayed the characteristic “shark fin” peak shape with increased load for a positively charged species in acidic conditions i.e. a typical right-angled front and extreme tailing. The apex of the peak is used to measure the retention time decrease with increased load on the column and as such, would affect the normalised retention times used to calculate delta values. The degree to which this effect occurs could be different for each peptide, hence the load for each peptide must be well described in order to maintain consistent delta values. The peak shape for this study is not of critical concern as it is the retention time which must be consistent, hence the necessity to keep the load constant. When devising chromatographic methods, it would be crucial to select mobile phases which can provide better peak shape, with the biopharmaceutical industry typically utilising phosphate salt based systems.

The loading profile could also be different depending on the type of stationary phase used. For example, the Acquity CSH range of stationary phases were optimised to provide improved peak shape and efficiency for basic species to provide linear isotherms, rather than typical Langmuir isotherm (Fig. 6). Similar to the slow equilibration effect, overloading behaviour is thought to be due to variations in the surface charge, where the balanced surface charge of the CSH range counteracts that issue to produce symmetrical, efficient peaks [31].

The sample solubility also is critical when it comes to sample load. The net charge of the hydrophilic peptides at pH 2.5 and 6.5 are +1.1 to +1.2 and -4.7 to -3.7, respectively. The hydrophobic peptides have a net charge of +2.2 at pH 2.5, whilst at pH 6.5 the net charge is 0.0. A pI of 0.0 may highlight potential solubility problems and the possibility of precipitation and clogging of the inlet frit. Pressure increases and decreased column performance were observed after prolonged exposure to intermediate pH conditions. Replacement of the inlet frit and scanning electron microscopy proved that particulates had been deposited onto the frit. Hence, in order to minimise the likelihood of this happening the load of the peptides was reduced and inline filters installed between the injector and the column.

3.6 Mitigating Action: Adjustment of the Peptide RPC Column Characterisation Protocol

The ideal scenario from the reduced factorial design is to find that none of the parameters assessed are statistically or practically relevant. However, if there are parameters which are practically relevant, mitigation must be put in place to reduce the effect.

The tolerances for the systematic shift in the gradient, the flow rate and the dwell volume in both the formic acid and ammonium formate DoE’s, and the pH of ammonium formate were all acceptable. Despite being deemed statistically significant, the practical relevance of the temperature

limits in both DoE's, and the % formic acid in both the aqueous and organic was negligible, thus the method for characterisation using formic acid can be considered robust within the stated limits.

The concentration of acetonitrile in the B solvent for the ammonium formate gradient, however, has been shown to be a statistically significant result with practical implications. The decrease in concentration had ramifications on four of the six delta values, leading to the removal of the sensitive Δ(3,1), Δ(9,1) and Δ(10,9) probes from the characterisation. The removed delta values were probes for racemisation, which was still represented by Δ(9,1) and Δ(14,13) in formic acid. In addition, the integrity of the score plot was also assessed without the affected probes, with consistent results for the stationary phases.

It is believed the robustness will actually be significantly better than shown in this study since the mitigations introduced (Table 7) will reduce variation further. The updated Peptide RPC Column Characterisation protocol is described in Appendix I.

4 Conclusion

The robustness of the Peptide RPC Column Characterisation protocol was assessed using reduced factorial design and PCA, with various factors systematically altered to deduce the impact on subtle changes to the protocol. The results indicate the formic acid gradient can be seen to provide robust results within the given tolerances of this study. The ammonium formate gradient, however, required mitigation to improve robustness in regards to the concentration of acetonitrile in the B solvent. All other parameters assessed did not influence the robustness.

The sample load for the columns was also determined and the potential impact on switching between low and intermediate pH for certain commercially available stationary phases was evaluated. Both studies had ramifications for the protocol and mitigation was put in place to address both phenomena.

In addition, the instrument variability on three different LC configurations and column batch to batch variability was assessed to ascertain the degree of variability which could be expected. Both the LC and column variability were minimal and highlights that the differences between stationary phases observed using the Peptide RPC Column Characterisation protocol are caused by chromatographic selectivity differences, rather than random error.

A number of modifications were suggested in order to improve robustness, thus moving forward, the results should provide even greater reliability and reproducibility than shown in this study. This will offer greater confidence in characterising different stationary phases using peptides as probes

and distinguishing their selectivity differences, thus allowing complementary stationary phases to be selected for method development or similar columns for back-up methods.

5 Acknowledgments

With thanks to Shimadzu for the supply of the Nexera X2 instrument and mass spectrometer and to Agilent, Fortis, Phenomenex, Supelco and Waters for the supply of stationary phases. Finally, special thanks to Waters for performing the SEM experiments.

Appendix I: Description of the Peptide RPC Column Characterisation Protocol for 150 x 2.1 mm Column Formats

If different column dimensions are employed, it is recommended that the user employs method translation tools [19].

Parameter	Protocol
Mobile Phase	A1: 0.1% (±0.005%) v/v formic acid in water – Add 1.000 mL formic acid to 999.0 g (±0.01 g) water B1: 0.1% (±0.005%) v/v formic acid in acetonitrile – Add 1.000 mL formic acid to 785.2 g (±0.01 g) acetonitrile A2: 20 mM Ammonium formate in water – Add 100.0 g (±0.01 g) 200 mM ammonium formate stock solution to 900.0 g (±0.01 g) water B2: Acetonitrile
Stock Buffer	200 mM Ammonium formate pH 6.45 (±0.06) – Dissolve 1.261 g in 100.0 g (±0.01 g) water and measure the pH using an appropriately calibrated probe
Gradient	Time / %B: 0.0 / 4.5; 40.0 / 50.0; 42.0 / 50.0; 42.1 / 4.5; 54.1 / 4.5
Flow Rate	0.3 mL/min (±0.005 mL/min)
Column Oven Temperature	40 °C (±2 °C)
Autosampler Temperature	Recommend 10 °C
Wavelength	215 nm Ref 360 nm (band width 8 and 100 nm)
MS	Selected Ion Monitoring (z=2)
Dwell Volume	100 – 500 µL
Sample Concentration & Diluent	0.25 mg/mL in DMSO/H_2O (80:20 v/v)

The run order is of great importance and should be first assessed at low pH then intermediate pH.

Test Mixture	Peptide Number	Peptide	Rationale	*m/z*	Load (µg)
TM1	1	Bovine GLP-2 (1-15)	Original sequence	820	0.250
	8a	[Met(O)10]-Bovine GLP-2 (1-15)	Oxidation	828	0.250
	9	[L-Asp11]-Bovine GLP-2 (1-15)	Deamidation / Negative charge	820	0.125
	13	Bovine GLP-2 (16-33)	Original sequence	1069	0.250
	15	[Ile26,Leu27]-Bovine GLP-2 (16-33)	Switch in AA sequence	1069	0.075
TM2	8a	[Met(O10]-Bovine GLP-2 (1-15)	Oxidation	828	0.250
	13	Bovine GLP-2 (16-33)	Original sequence	1069	0.250
	15	[Ile26,Leu27]-Bovine GLP-2 (16-33)	Switch in AA sequence	1069	0.075
	24	[Tyr26]-Bovine GLP-2 (16-33)	Phenolic effect	1076	0.125

	26	[Lys26]-Bovine GLP-2 (16-33)	Positive charge	1094	0.250
TM3	8a	[Met(O)10]-Bovine GLP-2 (1-15)	Oxidation	828	0.250
	13	Bovine GLP-2 (16-33)	Original sequence	1069	0.250
	14	[D-Ser16]-Bovine GLP-2 (16-33)	Racemisation	1069	0.125
	15	[Ile26,Leu27]-Bovine GLP-2 (16-33)	Switch in AA sequence	1069	0.075
	16	[L-Asp21,Gly22]-Bovine GLP-2 (16-33)	Loss of aromatic group / Racemisation	1024	0.125

434

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

435

436

437

6 Reference

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7 Figure Captions

Fig. 1. Coefficient plots for each delta response for the different variables based on formic acid data.

Fig. 2. Coefficient plots for each response for the different variables based on ammonium formate data.

Fig. 3. A biplot of the columns used to develop the Peptide RPC Column Characterisation protocol in addition to the instrument variability (yellow triangles) and the robustness (light blue diamonds) results to assess the variability of the protocols using the 11 delta value responses.

Fig. 4. Biplot of the batch to batch reproducibility performed on six Ascentis Express C18 columns on the reduced number of delta values, indicating the variability which could be expected between different batches of silica and silanes. Fig. 4 is mirrored in comparison to Fig. 3 due to one of the features of PCA where a very small difference in data often result in an axis being mirrored. The pattern and groupings are, however, the same in both figures.

Fig. 5. Chromatograms demonstrating the effect of slow equilibration. (A) the original chromatogram in formic acid prior to exposure to ammonium formate at intermediate pH, (B) re-evaluation of the same column in formic acid after exposure to intermediate pH (C) re-evaluation after a static equilibration in formic acid to attempt to restore the original chromatography

Fig. 6. Overlaid peak profile of the hydrophilic peptide [D-Asp3]-Bovine GLP-2 (1-15) of differing loads (0.031 – 2.000 µg) using the formic acid gradient conditions. The peak demonstrated the characteristic Langmuir's isotherm with significant peak tailing with increased sample load

Table 1

The peptides used to calculate the delta values in (A) formic acid and (B) ammonium formate, with the accompanying rationale and typical Δt_g and Δt_g^* for the Kinetex Evo C18 under the nominal conditions

(A)

Change	Delta	Peptide Number	Peptide	Rationale	Δt_g	Δt_g^*
[Met10] → [Met(O)10]	Δ(8a,1)	8a 1	[Met(O)10]-Bovine GLP-2 (1-15) [Met10]-Bovine GLP-2 (1-15)	Oxidation	-3.840	-0.286
[L-Asn11] → [L-Asp11]	Δ(9,1)	9 1	[L-Asp11]-Bovine GLP-2 (1-15) [L-Asn11]-Bovine GLP-2 (1-15)	Increase in negative charge	0.729	0.054
[L-Ser16] → [D-Ser16]	Δ(14,13)	14 13	[D-Ser16]-Bovine GLP-2 (16-33) [L-Ser16]-Bovine GLP-2 (16-33)	Steric - racemisation	0.171	0.013
[Phe22] → [Gly22]	Δ(16,13)	16 13	[Gly22]-Bovine GLP-2 (16-33) [Phe22]-Bovine GLP-2 (16-33)	Aromatic – removal of aromatic group	-5.302	-0.395
[Leu26] → [Lys26]	Δ(26,13)	26 13	[Lys26]-Bovine GLP-2 (16-33) [Leu26]-Bovine GLP-2 (16-33)	Increase in positive charge	-7.611	-0.566

(B)

Change	Delta	Peptide Number	Peptide	Rationale	Δt_g	Δt_g^*
[L-Asp3] → [D-Asp3]	Δ(3,1)	3 1	[D-Asp3]-Bovine GLP-2 (1-15) [L-Asp3]-Bovine GLP-2 (1-15)	Steric - racemisation	0.064	0.003
[L-Asn11] → [L-Asp11]	Δ(9,1)	9 1	[L-Asp11]-Bovine GLP-2 (1-15) [L-Asn11]-Bovine GLP-2 (1-15)	Increase in negative charge	1.399	0.063
[L-Asp11] → [D-Asp11]	Δ(10,9)	10 9	[D-Asp11]-Bovine GLP-2 (1-15) [L-Asp11]-Bovine GLP-2 (1-15)	Steric - racemisation	-0.581	-0.026
[Leu26,Ile27] → [Ile26,Leu27]	Δ(15,13)	15 13	[Ile26,Leu27]-Bovine GLP-2 (16-33) [Leu26,Ile27]-Bovine GLP-2 (16-33)	Steric – switch in amino acid sequence	1.053	0.047
[Leu26] → [Tyr26]	Δ(24,13)	24 13	[Tyr26]-Bovine GLP-2 (16-33) [Leu26]-Bovine GLP-2 (16-33)	Aromatic and phenolic – addition of phenolic group	-3.959	-0.179
[Leu26] → [Lys26]	Δ(26,13)	26 13	[Lys26]-Bovine GLP-2 (16-33) [Leu26]-Bovine GLP-2 (16-33)	Increase in positive charge	-6.526	-0.295

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

The peptide test mixtures, rationale and sample load

Test Mixture	Peptide Number	Peptide	Rationale	Load (µg)
TM1	1	Bovine GLP-2 (1-15)	Original sequence	0.250
	8a	[Met(O)10]-Bovine GLP-2 (1-15)	Oxidation	0.250
	9	[L-Asp11]-Bovine GLP-2 (1-15)	Deamidation / Negative charge	0.125
	13	Bovine GLP-2 (16-33)	Original sequence	0.250
	15	[Ile26,Leu27]-Bovine GLP-2 (16-33)	Switch in AA sequence	0.075
	16	[L-Asp21,Gly22]-Bovine GLP-2 (16-33)	Loss of aromatic group / Racemisation	0.125
	24	[Tyr26]-Bovine GLP-2 (16-33)	Aromatic / Phenolic effect	0.125
TM2	8a	[Met(O10]-Bovine GLP-2 (1-15)	Oxidation	0.250
	9	[L-Asp11]-Bovine GLP-2 (1-15)	Deamidation / Negative charge	0.125
	10	[D-Asp11]-Bovine GLP-2 (1-15)	Deamidation / Racemisation / Negative charge	0.250
	13	Bovine GLP-2 (16-33)	Original sequence	0.250
	15	[Ile26,Leu27]-Bovine GLP-2 (16-33)	Switch in AA sequence	0.075
	26	[Lys26]-Bovine GLP-2 (16-33)	Positive charge	0.250
TM3	1	Bovine GLP-2 (1-15)	Original sequence	0.250
	3	[D-Asp3]-Bovine GLP-2 (1-15)	Racemisation	0.125
	8a	[Met(O)10]-Bovine GLP-2 (1-15)	Oxidation	0.250
	13	Bovine GLP-2 (16-33)	Original sequence	0.250
	14	[D-Ser16]-Bovine GLP-2 (16-33)	Racemisation	0.125
	15	[Ile26,Leu27]-Bovine GLP-2 (16-33)	Switch in AA sequence	0.075

Table 4

Operating parameters investigated in the DoE, including the nominal conditions (0 level) and the expected deviation (±1 levels)

Parameter	-1 Level	0 Level	+1 Level
Column Temperature (°C)[1]	38	40	42
Flow Rate (mL/min)[1]	0.295	0.300	0.305
Systematic Shift in Gradient (%B)[1]	-0.4	0.0	+0.4
Dwell Volume (µL)[1]	100	300	500
Volume Formic Acid in Aqueous (% v/v)[2]	0.095	0.100	0.105
Volume Formic Acid in Organic (% v/v)[2]	0.095	0.100	0.105
pH of Ammonium Formate[3]	6.39	6.45	6.51
MeCN Composition in B (%)[3]	79.9	80.0	80.1

[1] Measured using both the formic acid and ammonium formate gradients

[2] Measured in just the formic acid gradient

[3] Measured in just the ammonium formate gradient

Table 5

The DoE design for the formic acid gradient, including the different experimental levels. N1-8 vary the ±1 levels whilst N9-11 are the nominal central conditions used to assess reproducibility

Experiment Name	Column Temperature (°C)	Systematic Shift in Gradient (%B)	Flow Rate (mL/min)	Volume of Formic Acid in Aqueous (% v/v)	Volume of Formic Acid in Organic (% v/v)	Dwell Volume (µL)
N1	-	-	-	+	+	+
N2	+	-	-	-	-	+
N3	-	+	-	-	+	-
N4	+	+	-	+	-	-
N5	-	-	+	+	-	-
N6	+	-	+	-	+	-
N7	-	+	+	-	-	+
N8	+	+	+	+	+	+
N9	0	0	0	0	0	0
N10	0	0	0	0	0	0
N11	0	0	0	0	0	0

Table 6

The DoE design for the ammonium formate gradient, including the different experimental levels. N1-8 vary the ±1 levels whilst N9-11 are the nominal central conditions used to assess reproducibility

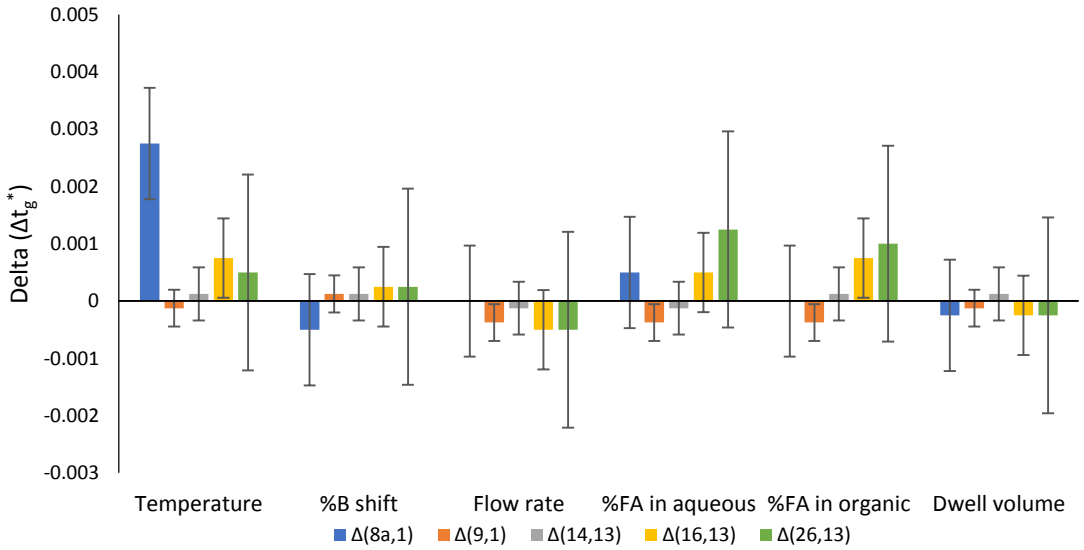
Experiment Name	Column Temperature (°C)	Systematic Shift in Gradient (%B)	Flow Rate (mL/min)	pH of Stock Ammonium Formate Solution	MeCN Composition in B (%)	Dwell Volume (µL)
N1	-	-	-	+	+	+
N2	+	-	-	-	-	+
N3	-	+	-	-	+	-
N4	+	+	-	+	-	-
N5	-	-	+	+	-	-
N6	+	-	+	-	+	-
N7	-	+	+	-	-	+
N8	+	+	+	+	+	+
N9	0	0	0	0	0	0
N10	0	0	0	0	0	0
N11	0	0	0	0	0	0

Table 7

Mitigation to increase the robustness and reliability of the Peptide RPC Column Characterisation protocol, including the rationale for each action

Mitigating Action	**Rationale**
Prepare solvents by weight rather than volume	There are greater errors associated with glassware, thus more reproducible mobile phases can be prepared by weight.
Assess the accuracy of the pipette before each use	To ensure the pipette can accurately dispense formic acid.
Measure the pH of the stock ammonium formate solution (6.39-6.51).	The salt container should be carefully capped to avoid loss of ammonia which can result in a lower pH. In addition, measures should be put in place to reduce the effect of hygroscopicity (i.e. use a desiccator, avoid using salt which has significant clump formation).
Use 100% MeCN instead of 20 mM ammonium formate in MeCN/H_2O (80:20 w/w) in the B solvent combined with a corresponding change in gradient slope.	The loss of acetonitrile in the B solvent causes significant differences for certain delta values. Changing to 100% MeCN addresses this problem. Even with the change in ammonium formate concentration, the results are still better with this change.
Remove Δ(3,1), Δ(9,1) and Δ(10,9) measured in ammonium formate	Improve the robustness of the procedure as they were sensitive to changes in MeCN. Although these delta values had some influence within the loading plot, they can be removed with minimal effect on the score plot and the remaining probes cover the range of interactions which should be investigated.
Use reference peptides in each test mixture	Allows retention times to be normalised for direct comparison between different batches of solvent, different analysts and removes the contribution from the dwell volume and column volume.
Each test mixture should contain the two peptides used to create the delta value	Removes any random injection to injection variation of retention time in addition to fluctuations in temperature or mobile phase composition.
Characterise the stationary phase in formic acid prior to ammonium formate	Removes the effect of slow equilibration and retention drifts.
Use a specific load for each peptide	Changing the load on the column can cause changes in retention which will impact on the delta value produced.
Assess the actual temperature of the column	Column oven designs can create as much as ±5 °C difference [35,36], which can impact significantly on selectivity. Obtaining the actual temperature of the oven enables the end-user to adjust the temperature appropriately for direct comparisons of different column oven designs.

Solvent bottles should be stored correctly with a cap and stored at 5 °C. When stored on the system, a good vapour valve should be installed	Storage of capped solvent bottles in the fridge reduces microbial growth and evaporation, whilst the vapour valve prevents dust / microbes entering the chromatographic system and acetonitrile losses.
Reduce load of the peptides and add an inline filter to induce mixing and trapping of particles prior to column	The hydrophobic peptides have a pI of 0.0 in ammonium formate at pH 6.45, which could cause solubility issues such as precipitation on the frit at the head of the column. This can cause bad peak shapes, increased pressures and reduced column lifetime. By reducing the load and introducing an inline filter, it will reduce the risk for precipitation and increase the robustness of the protocol.
Use a reference column to act as a system suitability test	This provides a baseline for the instrument to detect any differences in any asymmetrical shifts in the gradient (as well as other problems).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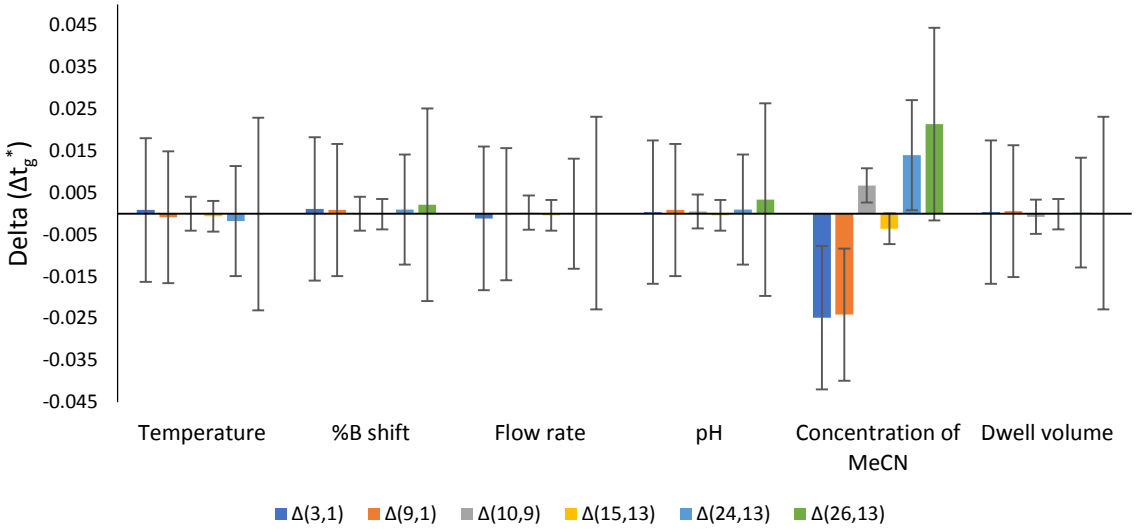
0.004
0.003
0.002
0.001
0
-0.001
-0.002
-0.003
Delta (Δt_g^*)
Temperature
%B shift
Flow rate
%FA in aqueous
%FA in organic
Dwell volume
Δ(8a,1)
Δ(9,1)
Δ(14,13)
Δ(16,13)
Δ(26,13)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Temperature
%B shift
Flow rate
pH
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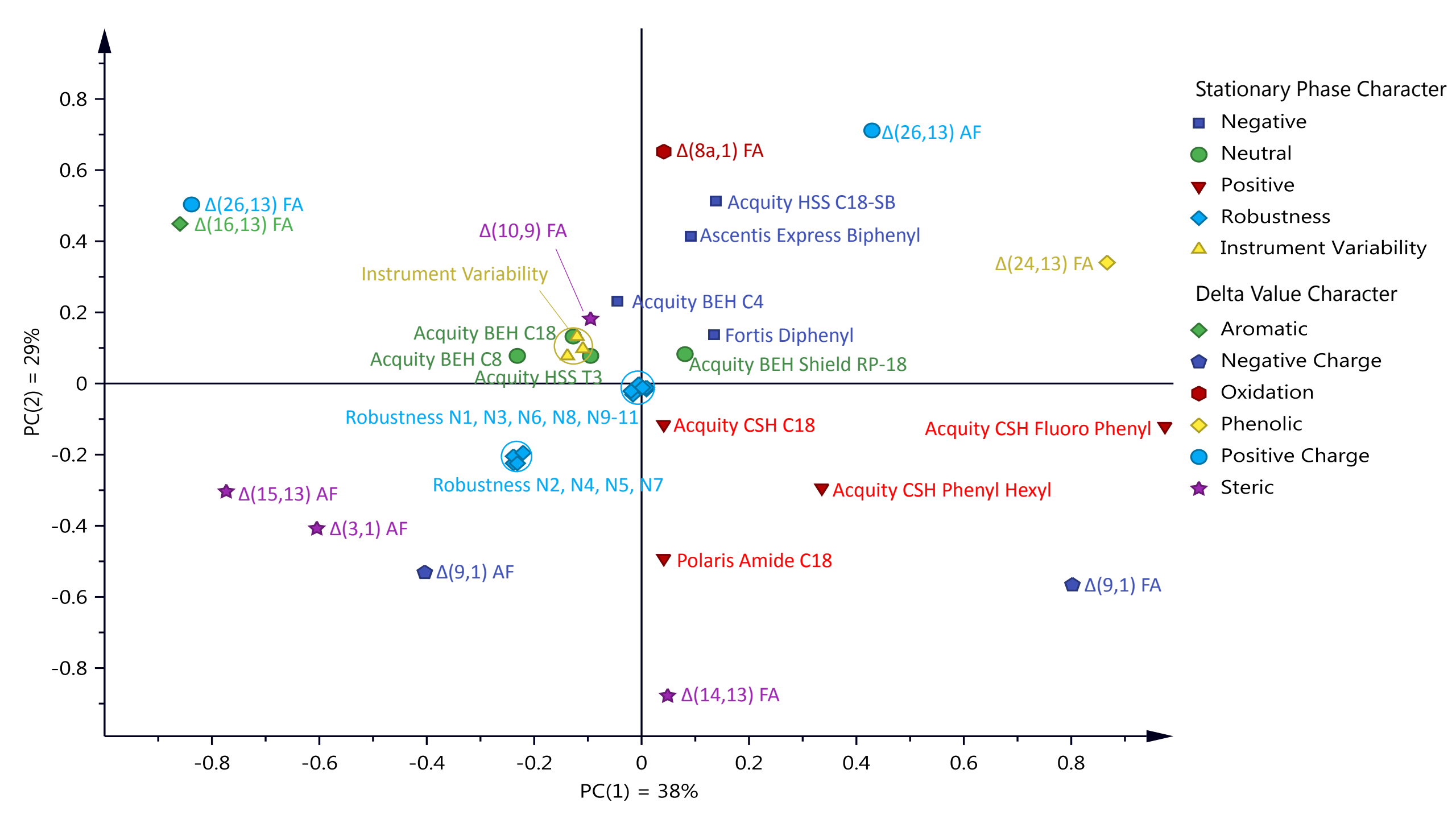
Δ(26,13)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Δ(8a,1) FA
Δ(26,13) FA
Δ(16,13) FA
Acquity HSS C18-SB
Ascentis Express Biphenyl
Δ(10,9) FA
Δ(24,13) FA
Instrument Variability
Acquity BEH C4
Acquity BEH C18
Fortis Diphenyl
Acquity BEH C8
Acquity BEH Shield RP-18
Acquity HSS T3
Robustness N1, N3, N6, N8, N9-11
Acquity CSH C18
Acquity CSH Fluoro Phenyl
Robustness N2, N4, N5, N7
Δ(15,13) AF
Acquity CSH Phenyl Hexyl
Δ(3,1) AF
Polaris Amide C18
Δ(9,1) AF
Δ(9,1) FA
Δ(14,13) FA
PC(2) = 29%
PC(1) = 38%
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
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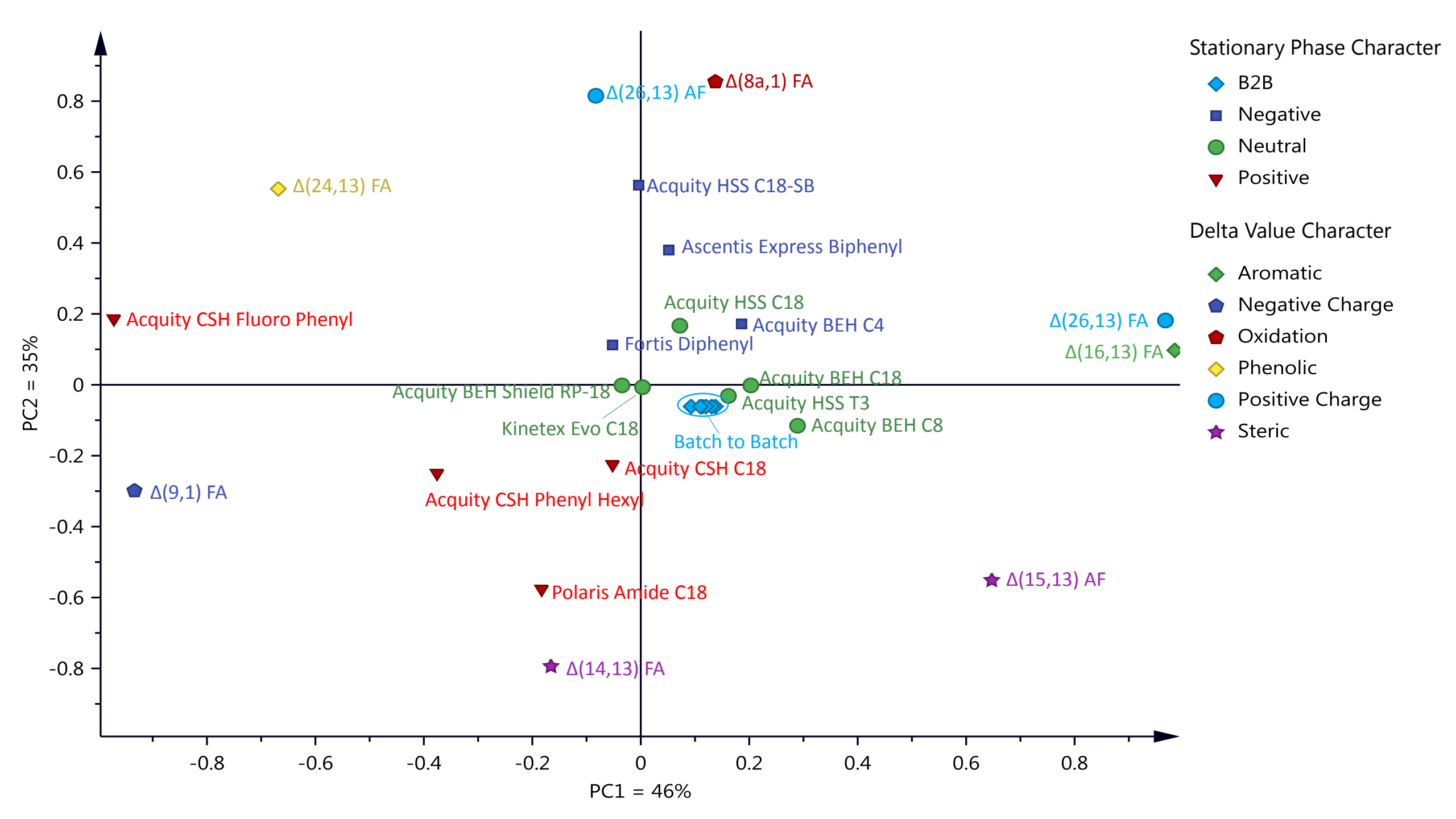
Oxidation
Phenolic
Positive Charge
Steric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Δ(8a,1) FA
Δ(24,13) FA
Acquity HSS C18-SB
Ascentis Express Biphenyl
Acquity HSS C18
Acquity CSH Fluoro Phenyl
Acquity BEH C4
Δ(26,13) FA
Fortis Diphenyl
Δ(16,13) FA
Acquity BEH C18
Acquity BEH Shield RP-18
Acquity HSS T3
Kinetex Evo C18
Batch to Batch
Acquity BEH C8
Acquity CSH C18
Δ(9,1) FA
Acquity CSH Phenyl Hexyl
Δ(15,13) AF
Polaris Amide C18
Δ(14,13) FA
PC2 = 35%
PC1 = 46%
0.8
0.6
0.4
0.2
0
-0.2
-0.4
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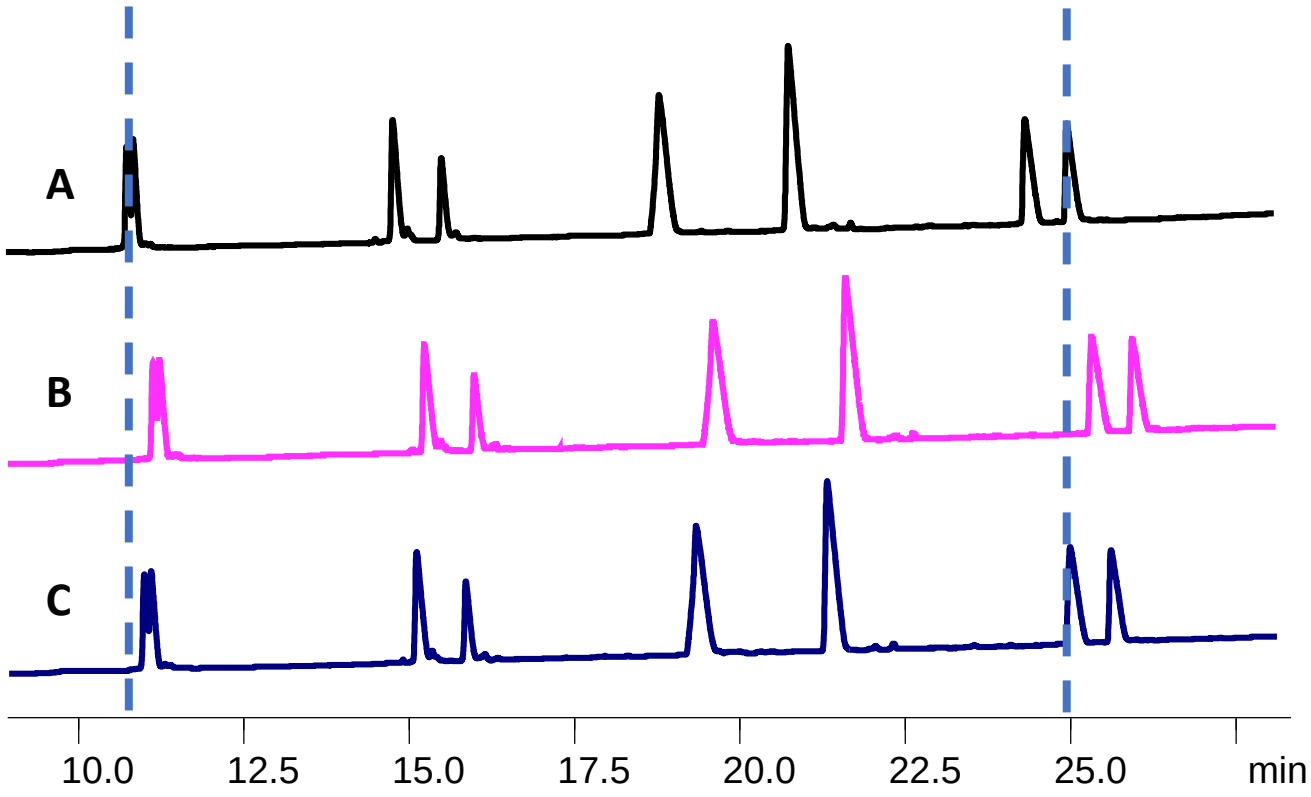
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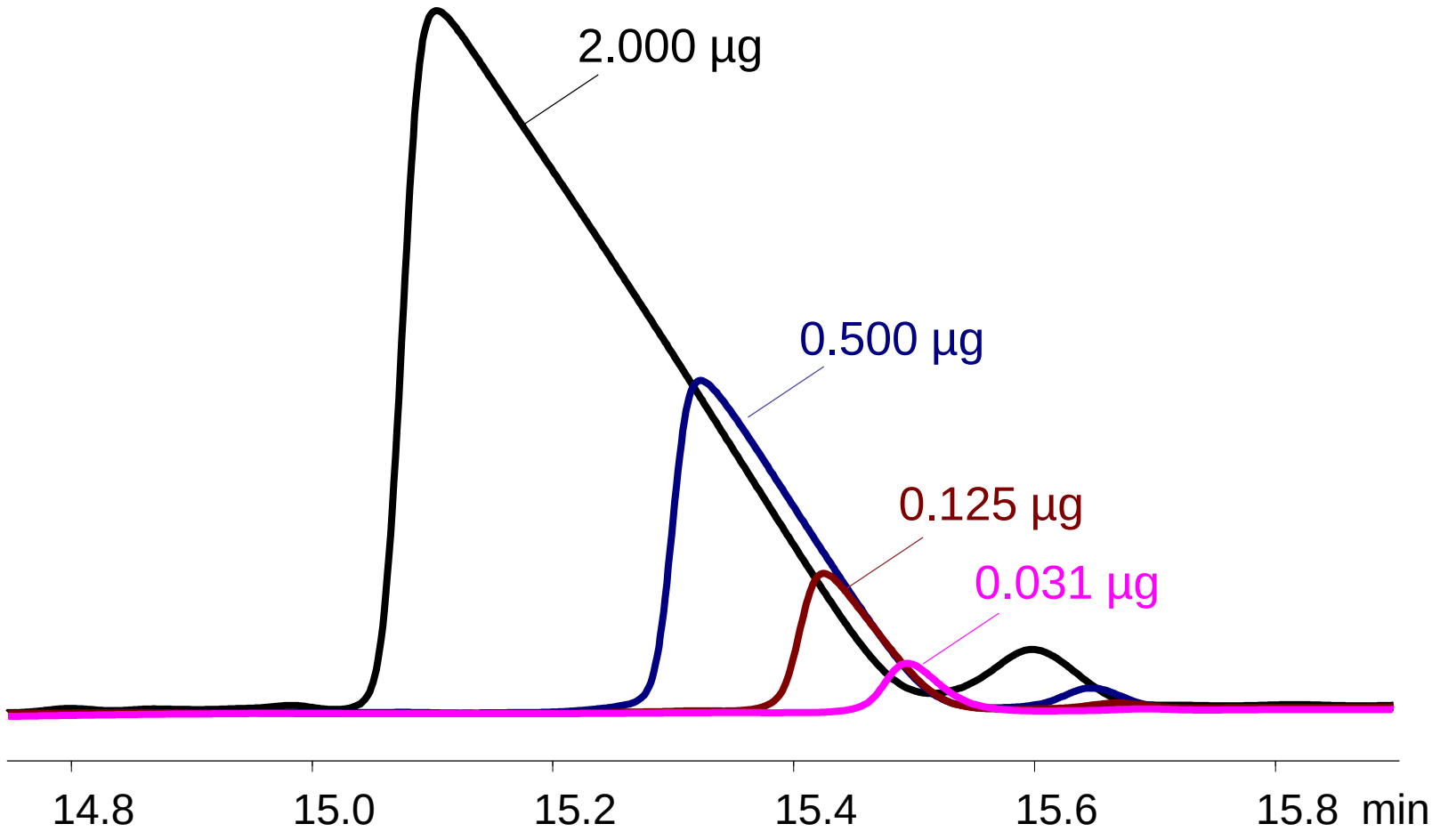
2.000 µg
0.500 µg
0.125 µg
0.031 µg
14.8
15.0
15.2
15.4
15.6
15.8
min