Characterization of Reversed-phase Liquid Chromatographic Columns Containing Positively Charged Functionality

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Abstract

To date, the most commonly used column characterization databases do not determine the relative positive charge associated with new generation RP columns, or they fail to successfully discriminate between RP columns of purportedly low level positive and neutral characters. This paper rectifies this in that it describes a convenient and robust chromatographic procedure for the assessment of the low levels of positive charge on a range of RP columns. The low degree of positive charge was determined by their electrostatic attraction towards the negatively charged 4-*n*-octylbenzene sulfonic acid (4-OBSA) relative to their retention of the hydrophobic marker toluene (Tol).

The new parameter ($\alpha_{4\text{-OBSA/Tol}}$) was determined for 15 commercially available RP-LC columns. When this was combined with existing Tanaka parameters it was possible to guide the chromatographer towards similar columns as "*backup / equivalent phases*" or dissimilar columns for exploitation in method development strategies. It should be noted that under certain chromatographic conditions the retention mechanism(s) may be too complex to allow direct location of a "*backup / equivalent*" column(s).

The $\alpha_{4\text{-OBSA/Tol}}$ results indicate that even the new generation neutral alkyl phases may exhibit a small degree of positive charge at low buffer concentrations. Mobile phases containing low %MeCN were demonstrated to promote mixed mode (anionic exchange / hydrophobic) retention whereas at high %MeCN anionic exchange retention dominated.

The measure of electrostatic repulsion between positively charged columns and positively charged bases was assessed by evaluating the relative retention of a range of bases and neutral analytes. The greatest electrostatic repulsion was observed with hydrophilic bases. While there was no correlation between the positive charge associated with the phases assessed by electrostatic attraction or repulsion, the columns could be broadly divided into three subsets (i.e., significant positive character, medium to low positive character and insignificant positive character).

Finally, the results were used to highlight the usefulness of the column characterization database containing the new anionic exchange retention parameter ($\alpha_{4\text{-OBSA/Tol}}$) for the selection of an equivalent column possessing a low level of positive character in the analysis of a real-life biopharmaceutical application.

Keywords

- Positively Charged Surface RP-LC Columns
- Modified Tanaka Chromatographic Characterization Protocol
- Anionic Exchange Retention
- Cationic Repulsion of Basic Analytes
- Similarity / Dissimilarity of Columns based on Euclidian Distances

1 Introduction

Over the last ten years RP columns possessing low levels of positive character have been made commercially available [1-4]. These types of phases have proved extremely popular in the LC-MS analysis of basic analytes due to the following reasons: 1) improved peak shape for basic analytes when low ionic strength mobile phases are employed *e.g.* 0.1% formic acid, 2) reduced column overloading phenomena, 3) minimized chromatographic hysteresis (i.e., slow equilibration) on pH switching in method development strategies [1]. In addition, these phases may impair complementary selectivity due to additional electrostatic interactions. As a result of their increased use, there is a need to be able to characterize RP columns possessing low levels of positive character, assess their mixed mode retention and to additionally be able to select chromatographically equivalent columns.

There have been several reports of RP columns exhibiting a small positive character at low pH, as demonstrated by their enhanced anion exchange retention (i.e., electrostatic attraction) of nitrate and chloride ions and enhanced cationic repulsion (i.e., ion exclusion) of protonated glycinamide as a function of the mobile phase ionic strength and flow induced streaming potential (electrokinetic property of the stationary phase) [5-10]. The small and varied positive nature of a range of 14 commercially available RP columns (pre-2008, classed as neutral by their manufacturer) was attributed to small amounts of residual basic catalyst in the columns from the alkyl silane bonding process [5].

The largest column characterization database, which employs the Hydrophobic Subtraction Model [11-14], does not include an assessment of the anionic exchange retention of RP columns. It has been reported that the cation exchange capacity value "C" at pH 2.8 for a range of RP columns was demonstrated to correlate with their positive character as measured by the anionic retention of nitrate and *p*-toluene sulfonic acid [5]. As the "C value at pH 2.8" decreased, the positive character increased. In comparison, the popular Tanaka Protocol [15-18] does determine and provide the anionic exchange retention of RP columns in its modified database [17,18], however, it has been found to be only suitable for assessing relatively large amounts of positive character. For example, it can easily discriminate between amide based polar embedded phases which possess a large degree of positive character as a result of incomplete acylation of the precursor amino silica and neutral RP phases [18,19]. In its present form, the modified Tanaka characterization does not successfully discriminate between RP columns with low levels of positive character and with those claimed to possess a neutral character.

Currently, the anionic exchange retention of RP columns is assessed in the Modified Tanaka Characterization protocol [15-18], by determining the selectivity factor ($\alpha_{BSA/Tol}$) between benzene sulfonic acid (BSA) and toluene (Tol). The former negatively charged probe electrostatically interacts with the protonated amino functionality, while Tol is used to assess the hydrophobic nature of the phase. The hydrophobicity difference between these two probes is large and there is little hydrophobic retention of the BSA compared to Tol and when there is little positive character, this results in low retention of BSA and poor discrimination between phases. Hence, we have evaluated a range of aryl sulfonic acids possessing differing log *D* values to select an appropriate negatively charged probe which would differentiate between the varying low levels of positive character on a range of 15 commercially available RP columns (post 2008). The positive character of RP phases can also be assessed using the repulsive nature of protonated basic analytes with the positive charge on the column [5-7]. Hence, the electrostatic attraction of the phases, as measured by the relative retention of the negatively charged aryl sulfonic acid to toluene, was then compared to the electrostatic

repulsive nature of the RP columns towards a range of positively charged basic analytes of differing hydrophobicity and chemical diversity.

The new anionic exchange retention parameter was then combined with the existing Tanaka characterization parameters and the chromatographic similarity / dissimilarity of the columns was assessed by the principle of Euclidian distances between the chromatographic parameters [20]. Finally, the results were used to evaluate the column characterization database containing the new parameter for the assessment of low level positive character for the selection of an equivalent column possessing a small amount of positive character in the analysis of a real-life biopharmaceutical application.

2. Experimental

2.1 Chemicals and stationary phases

Water, acetonitrile (MeCN) and all mobile phase additives used were of LC-MS grade and supplied by Sigma Aldrich (Poole, UK). All columns were new and of a 150 x 2.1 mm ID format. A brief description of each of the stationary phases can be found in Table 1. The probes were all purchased from Merck, each dissolved individually in varying proportions of MeCN/H_2O based on their hydrophobicity to a concentration of 0.5 mg/mL (see Supplementary Material 1 and 2). Solutions were stored at 4°C. Buffer solution was made to pH 2.5 by mixture of 71 mM KH_2PO_4 (9.66 g) and 29 mM H_3PO_4 (1.98 mL, 85% w/w) and made up to 1000 mL with water. For pH robustness study buffers of pH 2.4 and 2.6 were also made up by the addition of 65.24 mM KH_2PO_4 (8.88 g) and 34.76 H_3PO_4 (2.38 mL, 85% w/w) made up to 1000 mL with water and 76.04 mM KH_2PO_4 (2.35 g) with 23.96 mM H_3PO_4 (1.64 mL, 85% w/w) made up to 1000 mL with water respectively.

2.2 Liquid chromatography and experimental settings

Flow rate was maintained at 0.3 mL/min at a temperature of 40°C and detection was at 215 nm (bandwidth 8 nm) referenced at 360 nm (bandwidth 100 nm) and a sampling rate of 12.5 Hz unless otherwise stated. A water sandwich injector program [21-23] was utilised to focus the peaks on top of the column. The peak apex of an uracil injection (0.5 mg/mL) was used as the dead time marker [24]. The initial gradient scouting experiments for the selection of the negatively charged probe and the assessment of electrostatic repulsion of basic analytes were performed as follows: mobile phase line A – water, line B – MeCN and line C - 100 mM KH_2PO_4 pH 2.4 – 2.6 in water, gradient time was performed A, 90: B, 5: C, 5 to A, 30: B, 65: C, 5 over 10 minutes, with an isocratic hold at the top of the gradient for 2 minutes, before returning to the original conditions in 0.1 minutes and 4.9 minute re-equilibration (equivalent to approximately 5 column volumes). Isocratic mobile phases (2-15 mM KH_2PO_4 pH 2.4 – 2.6 in water / MeCN 35 – 65% v/v) were delivered by the quaternary pump mixing appropriate proportions of line A water, line B MeCN and line C 100 mM KH_2PO_4 pH 2.4 – 2.6 in water.

LC separations were performed using a Shimadzu Nexera X3 UHPLC system (Duisburg, Germany) equipped with two binary pumps (LC-40AD) and proportionating valves, degassers (DGU-40S), autosampler with cooling capabilities (SIL-40), column oven (CTO-40C), diode array detector (SPD-M30A), 180 µL mixer and a six-position column switching valve and communication bus module (CBM-40Lite). The LC configuration had a dwell volume of 815 µL and system retention volume of 33 µL [24].

2.2.1 Characterisation of RP columns containing low levels of positive charge

Conditions as described in section 2.2 were employed with the exception that an isocratic mobile phase containing 5 mM KH_2PO_4 pH 2.5 in water / MeCN 50:50% v/v was used (typically delivered by quaternary pump mixing of line A water, line B MeCN and line C 100 mM KH_2PO_4 pH 5 in water 25:50:5 v/v/v).

2.3 Software and calculations

Shimadzu LabSolution software (Version 5.86, Duisburg, Germany) was used for LC instrument control and data processing. Log *D* and pK_a values were predicted using ACD/Percepta (Toronto, Canada, version 2019.1.3). Corrected retention factor was used to accommodate for the dead time of the LC system, this was calculated using t_{ext} calculated from system volume [24]. Reduced factorial design calculations were performed using Design Expert (Version 11, Design Expert, Minneapolis, MN). The mobile phase calculations were performed using BufferMaker (Version 1.1.0.0, ChemBuddy, BPP Marcin Borkowski, Poland). Euclidian distances were calculated for autoscaled variables using Pythagoras' theorem and an Excel spreadsheet as described in [15]. The spreadsheet for calculating Euclidian distances can be obtained from the authors on request.

3 Results and Discussion

Small inorganic anions such as nitrate and chloride have previously been used to estimate the positive character of RP columns [5-7] and the results obtained with nitrate were shown to correlate with those of the more hydrophobic negatively charged *p*-toluene sulfonic acid [5]. The current Modified Tanaka characterization protocol utilizes the selectivity factor ($\alpha_{BSA/Tol}$) between the negatively charged benzene sulfonic acid (BSA) and the hydrophobic marker Toluene (Tol) to estimate the positive character of the column relative to its hydrophobicity. RP columns known to contain a relatively large amount of positive character typically generated $\alpha_{BSA/Tol} > 0.1$, whereas RP columns claimed to be neutral and based on pure silica, typically exhibited values < 0.1 [18].

The hydrophobicity difference between the BSA and Tol is too large, and there is minimal hydrophobic retention of BSA compared to Tol using the current chromatographic conditions [18]. When there is low positive character associated with the column there is little, if any, electrostatic attraction of BSA which results in poor discrimination between neutral phases and those possessing low levels of positive charge. Therefore, a range of aryl sulfonic acid and neutral probes of differing hydrophobicity were evaluated in order to estimate the low level positive charge on a range of newly commercialized RP LC columns (post 2008) and to evaluate their mixed mode retention with respect to electrostatic attraction of negatively charged analytes and electrostatic repulsion of positively charged analytes with the positive charge associated with the RPC column in real-life chromatographic situations.

3.1 Development of the new protocol for assessing low level positive character of RP columns via electrostatic attraction of negatively charged probes

MeCN was selected in place of methanol (MeOH) as the organic modifier to minimize any potential π : π interactions between the analytes and any aromatic functionality on the column [25] (*e.g.,* the pyridinyl moiety associated with the CSH phases [26,27]). The pH of the aqueous component of the mobile phase was set at 2.5 as aryl sulfonic acids (typically $pk_a < 0$ in water) and any amino functionality (assumed $pk_a > 4.5$ in water, see Table 1) on the RP columns should be fully ionized even in 50:50 v/v MeCN /water [28] promoting electrostatic attraction while the surface silanol groups should be in their unionized forms. Potassium phosphate was employed as the buffer due to its ubiquitous use and its good buffering capacity at pH 2.5.

Initially, three differing RP columns reported to contain either none (*i.e.*, Express C18, $\alpha_{BSA/Tol} = 0.01$), or significant amounts of positive character (*i.e.*, BEH C18 AX, $\alpha_{BSA/Tol} = 3.09$ and the Bonus RP $\alpha_{BSA/Tol} = 1.60$) were evaluated with MeCN / 5 mM KH_2PO_4 pH 2.5 gradient scouting experiments to select the most appropriate negatively charged probe from a range of sulfonic acids of differing hydrophobicity (log *D* value at pH 2.5 ranged from -3.1 to 0.9, see Supplementary Material 1). The BEH C18 AX is a mixed mode phase which possesses both RP and a strong positively charged character [26] while the positive character attributed to the Bonus RP is thought to arise from residual amino functionality as a result of incomplete acylation of the amino silica in the formation of the amide polar embedded moiety [18, 31]. 4-*n*-Octylbenzene sulfonic acid (4-OBSA $pK_a = -0.4$ in water, log *D* value at pH 2.5 of 0.9) was deemed the most suitable negatively charged probe in terms of a comparable retention to Tol (log *D* at pH 2.5 calculated as 2.6). On a RP column purported to contain no positive charge, 4-OBSA elutes before Tol, whereas if a significant positive charge is associated with the stationary phase, then the 4-OBSA is significantly retained and elutes after Tol.

The high positively charged columns were replaced with those claimed to contain lower levels of positive character (*i.e.*, PS C18) and compared to an allegedly neutral column (*i.e.*, Express C18) using a range of isocratic conditions. 4-OBSA was found to be too strongly retained using isocratic conditions suitable for eluting Tol on the highly positive columns.

As expected for ion exchange chromatography the logarithm of the retention factor of 4-OBSA was proportional to the logarithm of the buffer concentration [32]. The effect was more pronounced on the PS C18 column, while Tol was unaffected on both the PS C18 and Express C18 columns. A buffer concentration of 5 mM was selected as this produced good discrimination between the retention of 4-OBSA and Tol and between neutral columns and those possessing a lower level of positive character (see Figure 1).

Even though the Express C18 was claimed to possess a neutral character, Figure 1 highlights a small but discernible "*apparent positive character*" at low buffer concentration. This supports the findings of Marchand and Snyder [5] who reported that 11 out of 14 "*neutral*" (pre-2008) RP columns in fact carried a small but measurable positive character.

The hydrophobic marker Tol, highlighted a similar degree of hydrophobicity between the four columns (see Figure 2A-D), the two hybrid columns (i.e., BEH and CSH C18) possessed very similar hydrophobicity as expected due to similar bonding technology being employed in the preparation of both phases [1,26]. The Express C18 phase possessed the lowest

retentivity due to its superficially porous nature [33]. 4-OBSA exhibited the same dependence on the proportion of %MeCN in the mobile phase irrespective of amount of positive charge the phase possessed (see Figure 2A-D). This suggested that there is a significant hydrophobic interaction of the sulfonic acid probe at lower %MeCN (*i.e.*, 4-OBSA was able to partition well into the C18 phases).
A bigger difference in the apparent "*positive character*" was observed between the PS C18 and the CSH C18 at higher %B than was seen at lower %B (see Figure 2A & B). This may in part be due to a dissimilarity in the way the differing levels of MeCN affects the pk_a of the amino functionalities [28] on the PS C18 and CSH C18 phases respectively. Figure 2A-D demonstrated that a mobile phase containing 50% MeCN would be suitable to generate good discrimination between phases containing low and no apparent positive character at an acceptable run time and pressure.

The results highlight a similar trend for both the BEH C18 (presumed no positive charge) and the CSH C18 phase (reported low positive charge). The elution order of 4-OBSA and Tol demonstrated a switch around when low and high %MeCN conditions were compared, indicative of a similar retention mechanism. As both phases possessed very similar hydrophobic retention characteristics, the increased retention of 4-OBSA at high %MeCN levels (*i.e.*, low hydrophobic retention) compared to the BEH C18 phase may be attributed to the additional electrostatic attraction of the negatively charged 4-OBSA to the positive charged pyridinyl functionality on the CSH C18 phase [26,27]. At low %MeCN levels the CSH C18 phase exhibits both electrostatic attraction for the protonated amino functionality and the enhanced hydrophobic interaction of the 4-OBSA (*i.e.*, a mixed mode interaction) resulting in higher values for $\alpha_{4\text{-OBSA/Tol}}$.
The same observations were noted for the Express C18 (claimed neutral character) and the PS C18 (low level positive charge). For example, at high %MeCN the PS C18 exhibited electrostatic attraction of the negatively charged probe to the protonated amino functionality and minimal hydrophobic retention of 4-OBSA, whereas at low %MeCN – it exhibited both electrostatic attraction and enhanced hydrophobic interaction of 4-OBSA resulting in higher values for $\alpha_{4\text{-OBSA/Tol}}$. The similarity of the response of 4-OBSA to %MeCN suggests that all the four phases possess differing levels of positive character (PS C18 > CSH C18 > BEH C18 > Express C18).

3.2 Robustness assessment of the new protocol for assessing low level positive character on RP columns

The robustness of the selected chromatographic conditions was investigated for a positive character column (i.e., PS C18 $\alpha_{4\text{-OBSA/Tol}} = 2.32$). The investigation was conducted by a one factor at a time series of experiments as well as a two-level reduced factorial design for the following factors and levels: pH 2.5 ±0.1, amount of MeCN (%MeCN) 50 ±2% v/v, temperature (T) 40 ±2°C, buffer concentration (C) 5 ±0.5 mM and injection volume (V_{inj}) 1 ±0.1 µL. The levels correspond to twice the expected variation or twice the instrument specifications [34]. The outcome of the study is summarized in Figure 3 which highlights how $\alpha_{4\text{-OBSA/Tol}}$ is affected if a factor is set to +1, *e.g.* +0.1 pH units. The error bars ±0.03 corresponds to a 95% confidence interval based on repeated experiments at nominal conditions. The expected variation would be 50% of the bars shown in Figure 3 since ±1 levels were used corresponding to twice the expected variation. Consequently, the expected random variation in pH of +/-0.05 units would result in a variation of $\alpha_{4\text{-OBSA/Tol}}$ of +/-0.10 *etc*. $\alpha_{4\text{-OBSA/Tol}}$ values obtained in the current study for columns having a positive charge

ranged from 0.8 to 30 units with an average of 7.7, hence, based on this it can be concluded that the robustness of the protocol should be acceptable.

While it is understandable that %MeCN, C and T would have a noteworthy influence, it is surprising that pH has a significant effect since the pK_a of 4-OBSA was calculated to be -0.4 in water. Despite the large difference between the estimated pK_a of 4-OBSA and the pH of the eluent pH 2.4 – 2.6, it is possible that the pH effect observed on retention could be due to a change in pK_as of the analyte and the buffer due to the addition of MeCN. In the literature there have been both reports of either minor [28] or substantial [29, 30] effects on pK_a, depending on the nature of the analyte, due to the addition of organic modifiers to aqueous solutions.

Exemplar chromatograms for stationary phases believed to possess no and low levels of positive characters are shown in Supplementary Material 3.

3.3 Evaluation of a range of RP columns possessing varying degrees of positive character

Using the old and new methodologies for assessing varying degrees of positive character, a range of five commercially available RP columns (no positive character) were compared to a range of ten commercially available RP columns purported to possess low (*i.e.*, CSH C18 [26], the Sym C8 phase containing residual basic catalyst [5,6]) and moderate to high positive character (*i.e.*, residual amino functionality as a result of incomplete acylation reaction in the production of a polar embedded phase [18, 31], proprietary bonding and a BEH C18 AX mixed mode phase [26]).

The Tanaka parameters and the new $\alpha_{4\text{-OBSA/Tol}}$ values for the fifteen RP columns can be seen in Table 2. As expected, there is a poor correlation between the old $\alpha_{BSA/Tol}$ and new $\alpha_{4\text{-OBSA/Tol}}$ values for positive charge on the column. The RP columns reported to possess no positive character yielded $\alpha_{4\text{-OBSA/Tol}} < 0.62$ whereas the columns possessing differing levels of positive character ranged from 0.76 to >30.

Section 3.1 highlights that certain claimed neutral RP columns, based on pure silica, may in fact demonstrate small but discernible positive character when employed with mobile phases possessing low buffer concentrations. It has been shown that the Express C18 and BEH C18 display this phenomenon and have $\alpha_{4\text{-OBSA/Tol}}$ values of 0.54 and 0.57 respectively, hence we conclude that phases with $\alpha_{4\text{-OBSA/Tol}} > 0.5$ possess a positive character, while those exhibiting a $\alpha_{4\text{-OBSA/Tol}} < 0.5$ do not possess demonstrable positive character (i.e., HPH C18 and Kinetex C18) under these chromatographic conditions.

The columns possessing high positive character such as the BEH C18 AX mixed mode, polar embedded phases and the proprietary amino bonding generated the largest $\alpha_{4\text{-OBSA/Tol}}$ values (i.e., > 12.5). The popular CSH type of phases possessing low positive character generated intermediate $\alpha_{4\text{-OBSA/Tol}}$ values ranging between 0.8 – 4.1 depending on the bonding technology and manufacturer. The $\alpha_{4\text{-OBSA/Tol}}$ value of 1.22 for the Symmetry C8 indicated a reasonable degree of positive character confirming the conclusions previously reported [5,6].

3.4 Assessment of the electrostatic repulsion of the fifteen commercially available columns

A range of eight basic and three neutral analytes of structural diversity and differing hydrophobicity (log *D* at pH 2.5 ranged from -2.9 to 2.7, see Supplementary Material 2) were chromatographed using practically relevant RP conditions (i.e., low buffer concentration and gradient conditions at pH 2.5) as a way of assessing the phenomena of electrostatic repulsion with real-life chromatographic conditions. Comparison of the CSH C18 ($\alpha_{4\text{-OBSA/Tol}} = 0.78$) to a range of phases containing no discernible positive charge ($\alpha_{4\text{-OBSA/Tol}} = 0.37 – 0.57$) can be seen in Figure 4. The HPH C18 and Kinetex C18 phases possess very similar Euclidean distances [18,20] for all of the Tanaka parameters shown in Table 2 in addition to possessing very similar $\alpha_{4\text{-OBSA/Tol}}$ values. Both phases demonstrate very similar chromatography for the basic / neutral mixture and as the differences in the Euclidean distances and $\alpha_{4\text{-OBSA/Tol}}$ values increased, so did the differences in the chromatographic selectivity for the basic / neutral mixture. In comparison to the neutral phases, the hydrophilic bases in the CSH C18 chromatogram all elute early with respect to the neutral analyte 1,4-benzenedimethanol (BDM) indicating that they are experiencing an electrostatic repulsion with the positively charged pyridyl functionality on the CSH C18 phase [26,27]. The degree of electrostatic repulsion could be assessed by the selectivity factor between the hydrophilic bases / 1,4-benzenedimethanol (*i.e.*, for benzylamine {B} the selectivity factor $\alpha_{B/BDM}$ ranged from 0.46 – 0.52 for the five neutral columns and 0.33 – 0.17 for the columns with positive character) see Table 3.

Poor correlation was observed between the relative effects of electrostatic attraction and repulsion for the columns evaluated. However, it was feasible to group the alkyl columns run under these RP mobile phase conditions into three subsets: significant positive character (BEH C18 AX, Bonus RP and Arata C18), medium to low positive character (Sym C8, CSH C18, AdvanceBio Plus, PS C18 and Cortecs C18+) and insignificant positive character (EVO C18, Express C18, HPH C18, Kinetex C18 and BEH C18). The non-alkyl phases (i.e., CSH Fluoro-Phenyl and CSH Phenyl-hexyl) did not follow the same trend. The results highlight that Euclidean distances [20] are a better measure of determining the chromatographic similarity / dissimilarity of RP-LC columns compared to Principal Component Analysis (PCA) because the former methodology does not suffer from the loss of information associated with PCA when the ten Tanaka parameters are reduced to two principal components. The usefulness of Euclidean distances in assessing similar and dissimilar columns can be seen in Figures 4 and 5 respectively. As the Euclidean distance increases, so does the chromatographic dissimilarity between the columns, this is especially true for the CSH Fluoro-phenyl and BEH C18 AX phases (see Figure 5) and highlights the usefulness of the inclusion of CSH C18 and CSH Fluoro-phenyl type phases in method development strategies.

3.5 Real-Life Biopharmaceutical Application

An equivalent "*back-up*" column to the CSH C18 was required which would separate a basic impurity from the neutral Active Pharmaceutical Ingredient (API). Both the impurity and the API were large (MW ~ 1000 Daltons) and bulky in nature and a TFA/MeCN gradient was necessary. The positively charged amino impurity eluted before the API on the positively charged CSH C18 phase as a result of electrostatic repulsion (see Figure 6). In contrast, on "*neutral*" columns such as the BEH C18, Kinetex C18, EVO C18, and HPH C18 the impurity eluted after the API due to the lack of the repulsive phenomena (see Figure 6). The new

parameter ($\alpha_{4\text{-OBSA/Tol}}$) suggested that a range of positive character columns should be evaluated ($\alpha_{4\text{-OBSA/Tol}}$ ranged from 0.8 – 29.2). As observed with the positively charged basic analytes, there was little correlation between the relative retention of the positive charged impurity to the neutral API. However, in most cases the impurity eluted before the API indicative of it experiencing a repulsive effect. In contrast, two columns (i.e., PS C18 and Cortecs C18+) failed to elute the impurity before the API despite their positive character (see Figure 6). However, the new anionic exchange retention parameter ($\alpha_{4\text{-OBSA/Tol}}$) does provide a practically relevant measure of the potential of a RP column to undergo electrostatic attraction or repulsion with negatively or positively charged analytes respectively but the degree of the interaction that the analyte experiences depend on its accessibility to the positively charged site within the stationary phase [35,36].

Conclusions

A simple and convenient protocol for the characterization of RP-LC stationary phases containing low to medium amounts of positive charge has been developed. To date, column characterization databases do not report this measurement, or they fail to discriminate between columns of purportedly low level positive and neutral characters. The paper reports that the relative retention between the negatively charged 4-OBSA and the neutral analyte Tol at pH 2.5, with a low buffer strength MeCN/water mobile phase, is a good measure of the positive character of the stationary phases. A two-level reduced factorial design experiment proved that the methodology was robust with respect to pH, %MeCN, temperature, buffer concentration and injection volume. The new methodology was found to be appropriate for stationary phases possessing low positive character (i.e., $\alpha_{4\text{-OBSA/Tol}}$ ranging between 0.37 – 4.13) but for phases possessing significant amounts of positive character (i.e., $\alpha_{4\text{-OBSA/Tol}}$ ranging between 12.53 – >30), the original $\alpha_{BSA/Tol}$ determination is more appropriate. Hence, for column classification purposes it is recommended that if the original $\alpha_{BSA/Tol}$ value >1, then this should be used whereas if the value is <1, then the new $\alpha_{4\text{-OBSA/Tol}}$ value should be employed. The $\alpha_{4\text{-OBSA/Tol}}$ results and the behaviour of 4-OBSA retention to buffer concentration suggested that, in accordance with previous reports, even the new generation neutral alkyl phases, based on pure silica, may exhibit a positive character under certain chromatographic conditions (i.e., low buffer concentration) due to the presence of residual basic catalyst from the alkyl silane bonding process. Under LC-UV conditions using typical buffer concentrations between 10-20 mM, chromatographers would practically observe no positive character with these columns. In contrast, when low buffer concentrations are employed (i.e., typically employed for LC-MS analysis) then this is where effects of the positive charge associated with these neutral columns may be observed. Mobile phases containing low %MeCN were demonstrated to promote mixed mode (anionic exchange / hydrophobic) retention whereas at high %MeCN, the effects associated with the positive charge on the column (i.e., ion exchange retention) dominated.

When the $\alpha_{4\text{-OBSA/Tol}}$ value was combined with the existing Modified Tanaka parameters, it was possible to identify columns possessing a positive charge and reduce the number of columns with complementary chromatographic selectivity required for method development screening strategies. Evaluation of the results (see Table 2) suggests that the chemometric tool of PCA is an extremely useful and visual tool in identifying columns for method development. However, the use of Euclidean distances between the ten Tanaka parameters is a superior approach for the identification of "*backup / equivalent*" columns as, contrary to PCA, no information is lost. It should be noted that under certain chromatographic conditions the retention mechanism(s) may be too complex to allow direct location of a "*backup /*

equivalent" column(s). The use of column characterization will, however, allow identification of a suitable set of potential backup columns to screen.

The measure of electrostatic repulsion between positively charged columns and positively charged analytes was assessed by evaluating the relative retention of a range of bases and neutral analytes of differing structural diversity and hydrophobicity. The greatest repulsion was observed with hydrophilic bases. In contrast, the hydrophobic bases were not influenced by the positive charge of the column. While there was no absolute correlation between the positive character of the RP column as measured by electrostatic attraction or repulsion, the columns could be broadly divided into three subsets – those with significant positive character (BEH C18 AX, Bonus RP and Arata C18), medium to low positive character (Sym C8, CSH C18, AdvanceBio Plus, PS C18 and Cortecs C18+) and insignificant positive character (EVO C18, Express C18, HPH C18, Kinetex C18 and BEH C18) under the investigated mobile phase conditions. Finally, the results were used to highlight the usefulness of the column characterization database containing the new positive charge character parameter ($\alpha_{4\text{-OBSA/Tol}}$), for the selection of an equivalent column(s) possessing a low amount of positive character in the analysis of a real-life biopharmaceutical application.

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Table legends

Table 1. List of columns used with manufacturer description.[19,26,27].

Table 2. Chromatographic parameters for the 15 commercially available RP columns ranked by their increasing positive character (i.e., increasing $\alpha_{4\text{-OBSA/Tol}}$ value) see references [15-18,20] for a description and explanation of the significance of each parameter.

Table 3. Selectivity factors for the basic analytes / neutral hydrophobic ranked by their increasing positive character (i.e., decreasing $\alpha_{B/BDM}$ value). See section 2.2 for chromatographic conditions. B = benzylamine, S = salbutamol, A = atenolol, Prop = propranolol, D = diphenhydramine, C = carvedilol, N = nortriptyline, Am = amitriptyline, BDM = 1,4-benzenedimethanol and Tol = Toluene.

Figure legends

Figure 1. Dependence of 4-OBSA and Tol retention on the concentration of KH_2PO_4 pH 2.5 in MeCN/water (35:65% v/v) on the neutral column (Express C18) and one containing a low level of positive character (PS C18). Experimental conditions as described in section 2.2.1.

Figure 2A - D. Dependence of 4-OBSA and Tol on the proportion of MeCN in the mobile phase containing 5 mM KH_2PO_4 pH 2.5 in MeCN/water (v/v) on the neutral columns (Express C18 and BEH C18) and those containing a low level of positive character (PS C18 and CSH C18). Experimental conditions as described in section 2.2.1.

Figure 3. Coefficients for coded factors and 95% confidence intervals. For example, changing the pH by +0.1 unit will decrease the $\alpha_{4\text{-OBSA/Tol}}$ value by 0.2 units.

Figure 4. Chromatography of the basic / neutral test mixture on the A) CSH C18, B) BEH C18, C) Kinetex C18 and D) HPH C18 columns plus the $\alpha_{4\text{-OBSA/Tol}}$ parameter and Euclidean distances relative to the CSH C18 phase. Chromatographic conditions are as described in section 2.2. 1. Benzylamine, 2. Salbutamol, 3. Atenolol, 4. 1,4-Benzenedimethanol, 5. Benzyl alcohol, 6. Propranolol, 7. Diphenhydramine, 8. Carvedilol, 9. Nortriptyline, 10. Amitriptyline, 11. Toluene.

Figure 5. Chromatography of the basic / neutral test mixture on the A) CSH C18, B) AdvanceBio Plus, C) BEH C18 AX and D) CSH Fluoro-phenyl columns plus the columns $\alpha_{4\text{-OBSA/Tol}}$ parameter and Euclidean distances relative to the CSH C18 phase. Chromatographic conditions are as described in section 2.2. Peak identity as shown in Figure 4.

Figure 6. Schematic representation of the separation of a large bulky neutral API and its structurally similar positively charged impurity on a range of C18 columns possessing varying degrees of positive character. The structure and chromatographic conditions are of a proprietary nature and cannot be divulged. The black arrow indicates the elution of the positively charged impurity.

Table 1

Column Name	Abbreviated Name	Manufacturer's Description	Manufacturer
AdvanceBio Peptide Plus	AdvanceBio Plus	Superficially porous, hybrid silica modified to have a charged surface, endcapped, C18, 100 Å pore size, 2.7 µm particle	Agilent
InfinityLab Poroshell Bonus RP	Bonus RP	Superficially porous, triple endcapped, polar embedded amide functionality in the alkyl ligand, 120 Å pore size, 2.7 µm particle	Agilent
Infinity Poroshell HPH C18	HPH C18	Superficially porous, double endcapped, C18 ligand, 120 Å pore size, 2.7 µm particle	Agilent
Ascentis Express C18	Express C18	Superficially porous, endcapped, monofunctional C18 alkyl ligand, 90 Å pore size, 2.7 µm particle	Merck
Kinetex C18	Kinetex C18	Superficially porous, endcapped, C18 alkyl ligand, 100 Å pore size, 2.6 µm particle	Phenomenex
Kinetex EVO C18	EVO C18	Superficially porous, endcapped, organo-silica ethyl cross-linking, C18 alkyl ligand, 100 Å pore size, 2.6 µm particle	Phenomenex
Luna Omega PS C18	PS C18	Totally porous, endcapped, C18 alkyl phase with a positve charge on the surface of the particle, 100 Å pore size, 1.6 µm particle	Phenomenex
Shim-pack Arata Peptide C18	Arata C18	Proprietary endcapping, C18 bonding, 120 Å pore size, 2.2 µm particle. Manufacturer describes properties that are suggestive of a charged surface material	Shimadzu
Acquity UHPLC Peptide BEH C18	BEH C18	Totally porous, endcapped, trifunctional C18 alkyl ligand bonded to ethyl bridged silica hybrid material, 130 Å pore size, 1.7 µm particle	Waters
Acquity UHPLC Peptide CSH C18	CSH C18	Totally porous, endcapped, trifunctional C18 alkyl ligand bonded to ethyl bridged silica hybrid material possessing a low level positive surface charge (pyridyl group positively charged below pH 5), 130 Å pore size, 1.7 µm particle	Waters
Acquity UHPLC Peptide CSH Fluoro-Phenyl	CSH Fluoro-Phenyl	Totally porous, non-endcapped, trifunctional pentafluorophenyl ligand bonded to ethyl bridged silica hybrid material possessing a low level positive surface charge (pyridyl group positively charged below pH 5), 130 Å pore size, 1.7 µm particle	Waters
Acquity UHPLC Peptide CSH Phenyl-hexyl	CSH Phenyl-hexyl	Totally porous, endcapped, trifunctional C6 alkyl ligand with a terminal phenyl functionality bonded to ethyl bridged silica hybrid material possessing a low level positive surface charge (pyridyl group positiovely charged below pH 5), 130 Å pore size, 1.7 µm particle	Waters
Atlantis Premier BEH C18 AX	BEH C18 AX	Totally porous, endcapped, trifunctional C18 alkyl ligand bonded to ethyl bridged silica hybrid material with an anion exchange functionality (tertiary alkylamine positively charged below pH 8), 95 Å pore size, 2.5 µm particle	Waters
Cortecs C18 Plus	Cortecs C18+	Superficially porous, endcapped, trifunctional, 100% aqueous compatible C18 alkyl phase with a positive surface with reduced ligand density, 90 Å pore size, 2.7 µm particle	Waters
Symmetry C8	Sym C8	Totally porous, C8 column with monofunctional bodning on a high purity base deactivated silica, 100 Å pore size, 3.5 µm particle	Waters

Table 2

	Chromatographic parameter										
Column	k_{PB}	α_{CH2}	$\alpha_{T/O}$	$\alpha_{C/P}$	$\alpha_{B/P\ pH\ 7.6}$	$\alpha_{B/P\ pH\ 2.7}$	$\alpha_{TNB/Tol}$	$\alpha_{BSA/Tol}$	$\alpha_{P/BA}$	$\alpha_{1,2/1,4-DNB}$	$\alpha_{4-OBSA/Tol}$
Kinetex C18	3.25	1.46	1.25	0.45	0.43	0.11	0.18	0.01	1.00	1.05	0.37
HPH C18	3.89	1.49	1.26	0.45	0.38	0.10	0.18	0.01	1.00	0.92	0.41
Express C18	4.83	1.50	1.42	0.42	0.70	0.10	0.14	0.01	1.00	0.89	0.54
BEH C18	4.18	1.47	1.38	0.37	0.23	0.09	0.17	0.00	1.00	1.00	0.57
EVO C18	2.82	1.44	1.12	0.42	0.37	0.09	0.19	0.03	1.00	1.09	0.61
Cortecs C18+	5.40	1.51	1.37	0.51	1.85	0.10	0.12	0.02	0.89	0.94	0.76
CSH C18	4.04	1.44	1.35	0.40	0.38	0.09	0.21	0.03	0.98	1.00	0.78
Sym C8	3.62	1.38	0.95	0.39	0.40	0.02	0.29	0.16	1.01	1.14	1.22
CSH Phenyl-Hexyl	1.55	1.31	1.13	0.70	0.42	0.07	0.85	0.16	0.94	1.22	1.30
AdvanceBio Plus	2.82	1.46	1.25	0.37	0.32	0.03	0.20	0.04	1.09	1.00	1.71
PS C18	3.83	1.43	1.11	0.53	0.51	0.08	0.22	0.14	1.00	1.12	2.32
CSH Fluoro-Phenyl	0.37	1.00	3.10	1.72	1.24	-0.06	0.48	0.74	0.96	1.48	4.13
Arata C18	4.31	1.42	2.02	0.37	0.35	0.01	0.22	2.09	1.40	0.95	12.52
Bonus RP	1.73	1.34	1.57	0.39	0.31	0.01	0.28	1.60	1.27	1.31	29.15
BEH C18 AX	5.62	1.47	1.25	0.49	0.36	-0.03	0.21	3.09	1.00	1.00	>30

Table 3

	Selectivity factors							
Column	$\alpha_{B/BDM}$	$\alpha_{S/BDM}$	$\alpha_{A/BDM}$	$\alpha_{Prop/Tol}$	$\alpha_{D/Tol}$	$\alpha_{C/Tol}$	$\alpha_{N/Tol}$	$\alpha_{Am/Tol}$
BEH C18	0.52	0.76	0.89	0.68	0.73	0.81	0.86	0.87
Kinetex C18	0.51	0.70	0.84	0.66	0.72	0.83	0.85	0.87
HPH C18	0.49	0.67	0.79	0.65	0.69	0.79	0.83	0.84
Express C18	0.48	0.63	0.73	0.60	0.65	0.74	0.77	0.78
EVO C18	0.46	0.58	0.67	0.62	0.67	0.80	0.81	0.82
CSH Fluoro-Phenyl	0.43	0.52	0.60	0.72	0.61	1.22	0.97	1.02
CSH Phenyl-Hexyl	0.34	0.39	0.41	0.62	0.68	0.85	0.84	0.86
Cortecs C18+	0.33	0.42	0.50	0.57	0.62	0.73	0.75	0.76
PS C18	0.32	0.44	0.53	0.59	0.63	0.74	0.75	0.77
AdvanceBio Plus	0.32	0.36	0.36	0.53	0.57	0.73	0.74	0.74
CSH C18	0.30	0.44	0.49	0.61	0.66	0.77	0.80	0.81
Sym C8	0.30	0.33	0.34	0.49	0.54	0.61	0.65	0.66
Bonus RP	0.30	0.34	0.34	0.46	0.48	0.75	0.68	0.68
Arata C18	0.27	0.33	0.33	0.52	0.53	0.72	0.68	0.68
BEH C18 AX	0.17	0.20	0.22	0.42	0.45	0.56	0.57	0.58

Figure 1

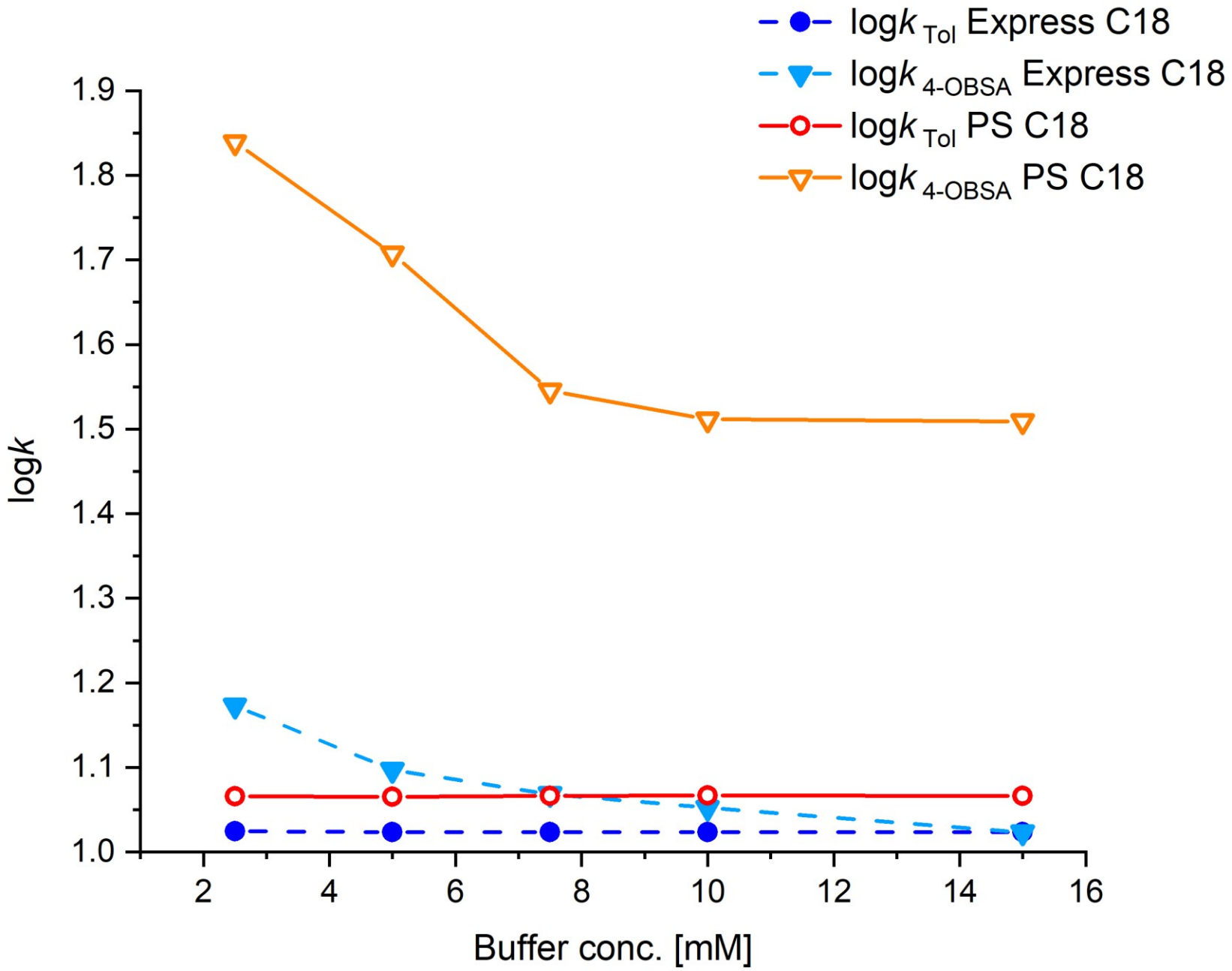

logk_{Tol} Express C18
log$k_{4\text{-OBSA}}$ Express C18
logk_{Tol} PS C18
log$k_{4\text{-OBSA}}$ PS C18
log$k$
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
2
4
6
8
10
12
14
16
Buffer conc. [mM]

Figure 2

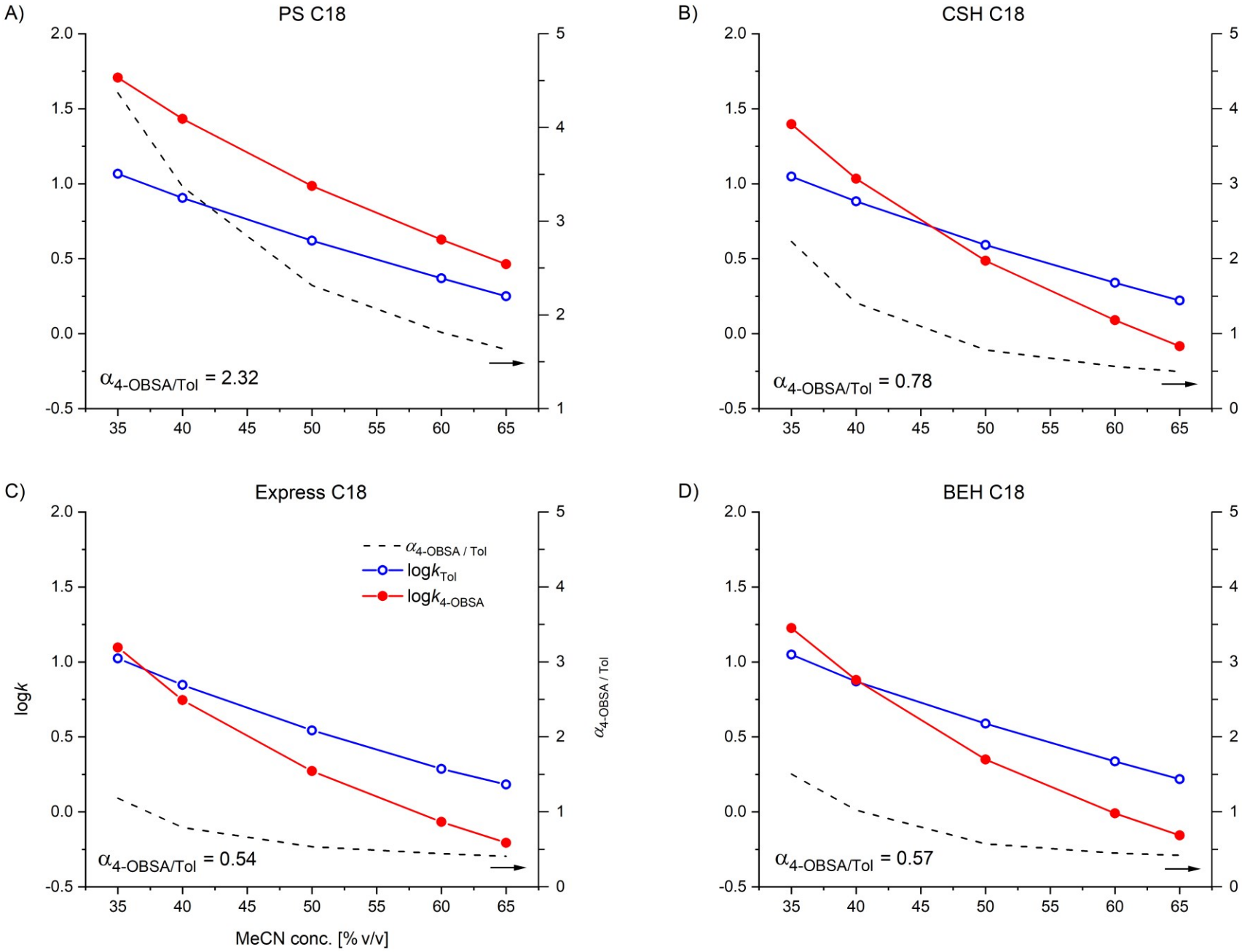
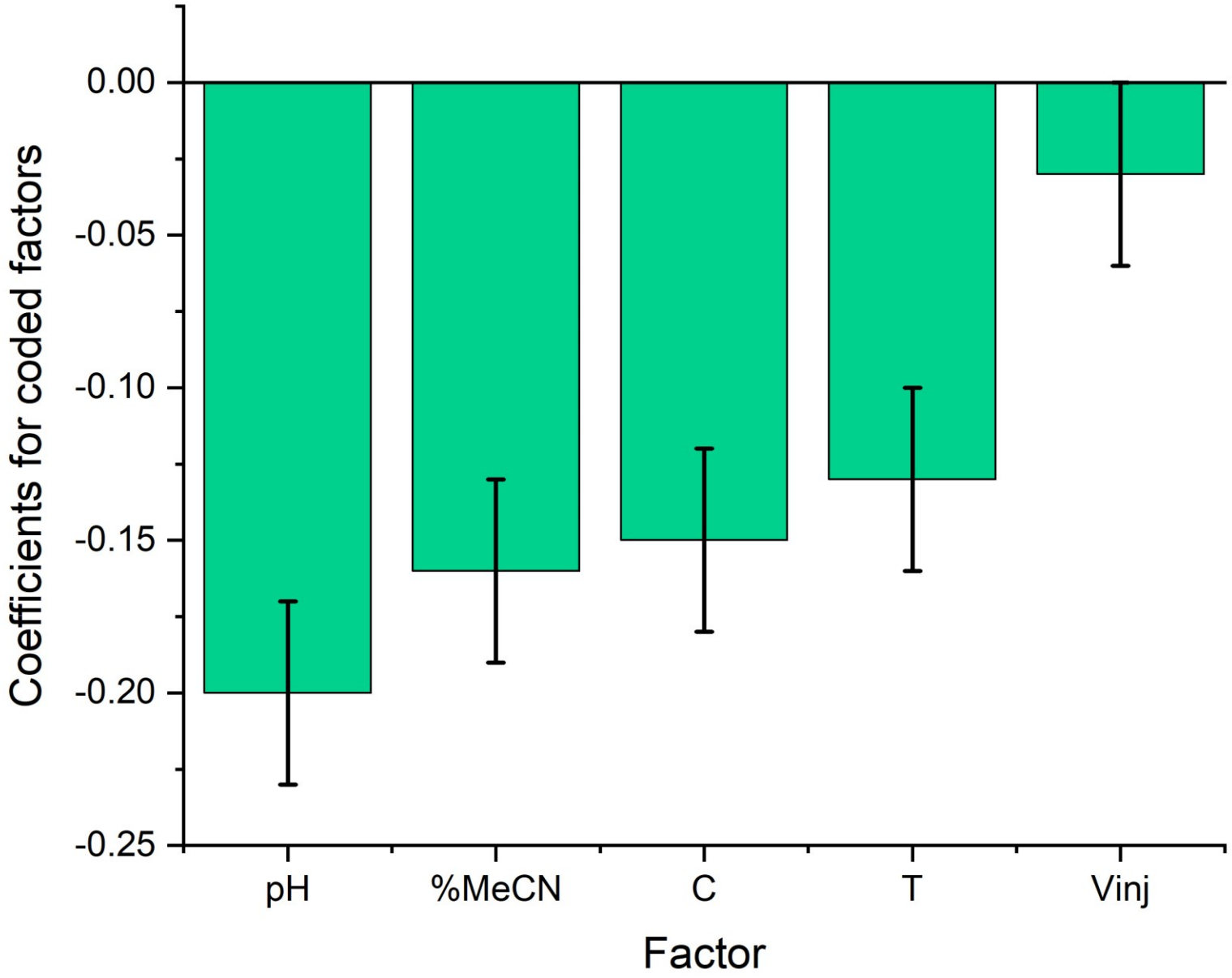

A)
PS C18
$\alpha_{4\text{-OBSA/Tol}}$ = 2.32
B)
CSH C18
$\alpha_{4\text{-OBSA/Tol}}$ = 0.78
C)
Express C18
$\alpha_{4\text{-OBSA / Tol}}$
logk_{Tol}
log$k_{4\text{-OBSA}}$
$\alpha_{4\text{-OBSA/Tol}}$ = 0.54
log$k$
$\alpha_{4\text{-OBSA / Tol}}$
MeCN conc. [% v/v]
D)
BEH C18
$\alpha_{4\text{-OBSA/Tol}}$ = 0.57

Figure 3


0.00
-0.05
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Coefficients for coded factors
pH
%MeCN
C
T
Vinj
Factor

Figure 4

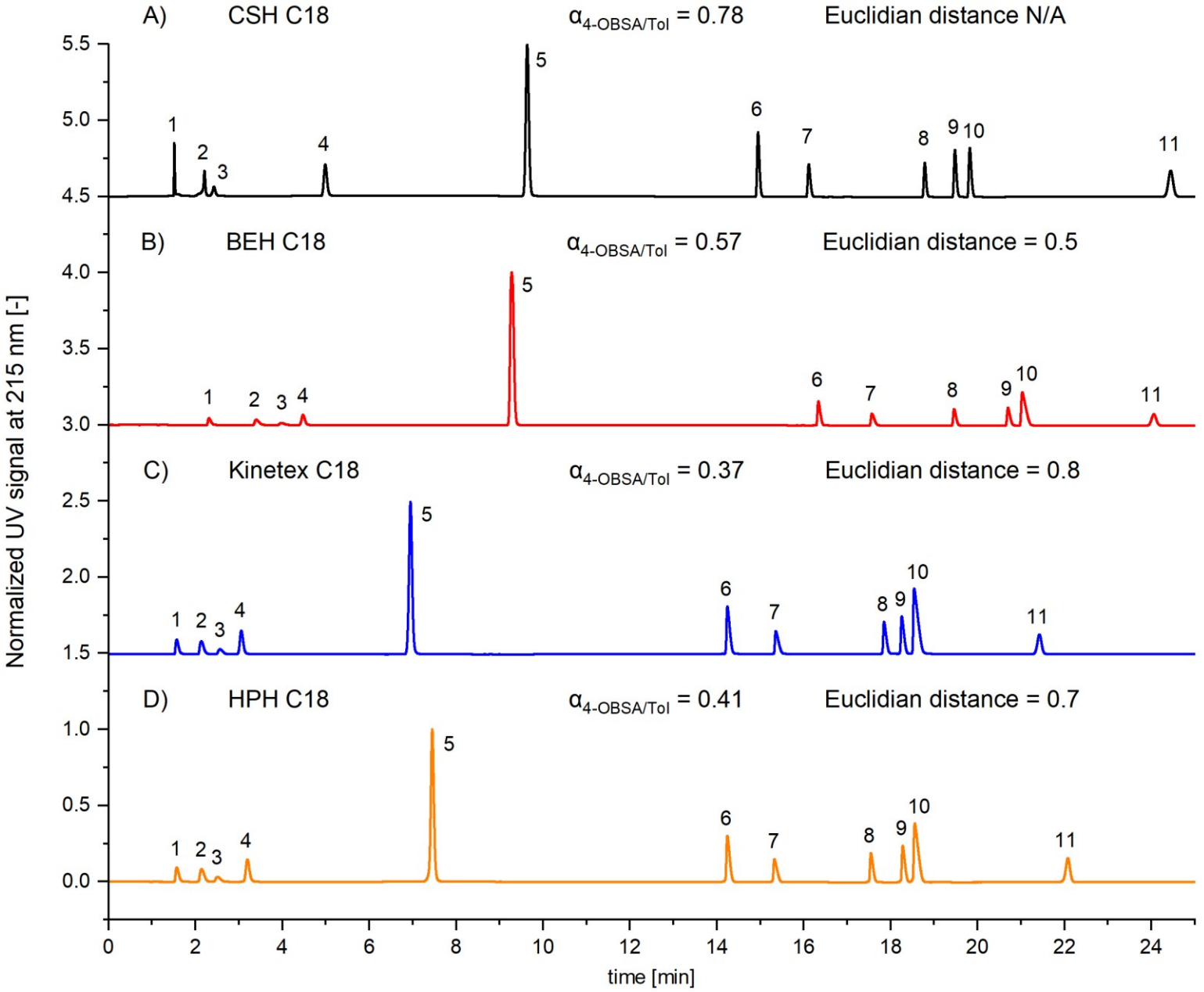
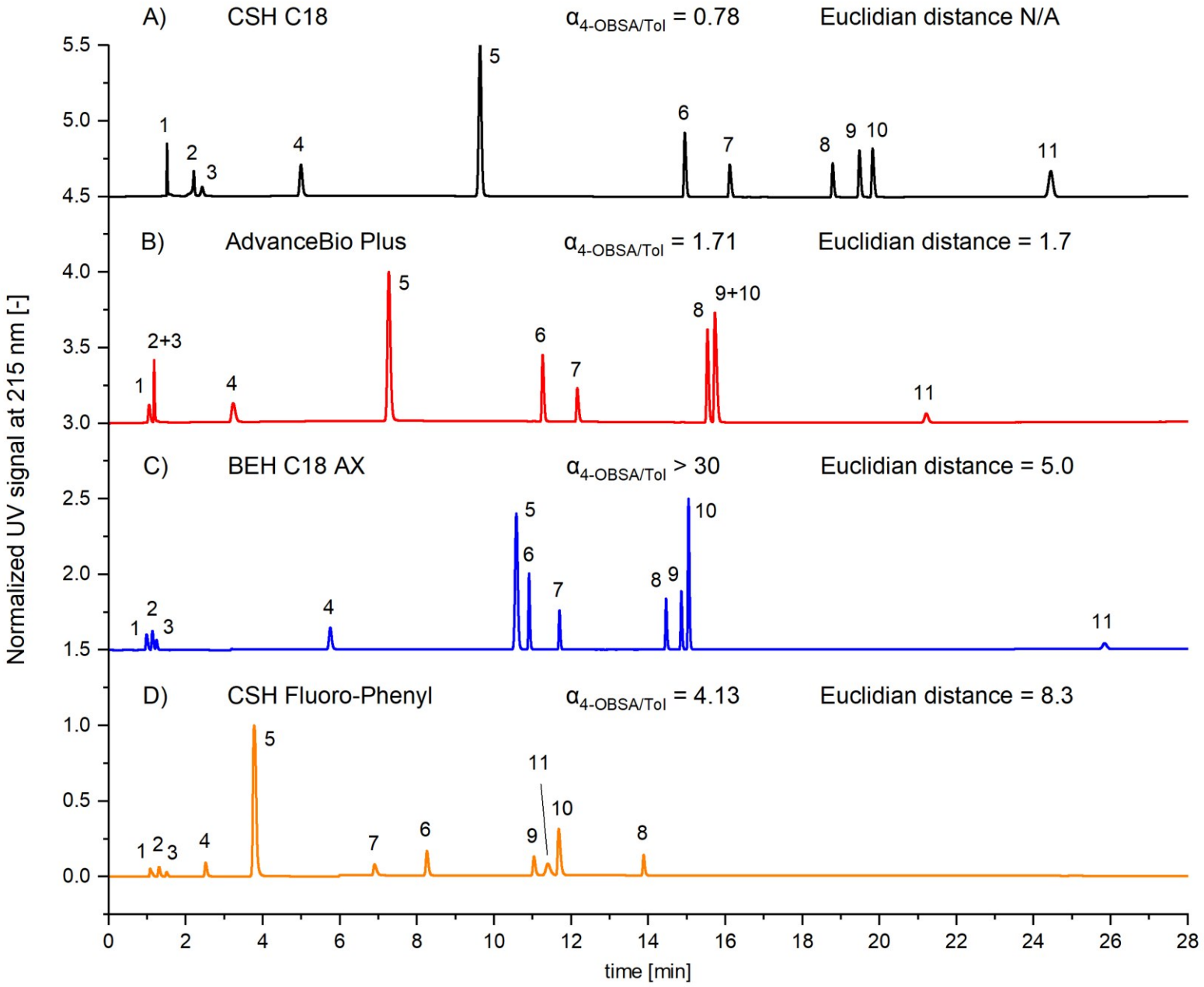

A) CSH C18
$\alpha_{4\text{-OBSA/Tol}}$ = 0.78
Euclidian distance N/A
B) BEH C18
$\alpha_{4\text{-OBSA/Tol}}$ = 0.57
Euclidian distance = 0.5
C) Kinetex C18
$\alpha_{4\text{-OBSA/Tol}}$ = 0.37
Euclidian distance = 0.8
D) HPH C18
$\alpha_{4\text{-OBSA/Tol}}$ = 0.41
Euclidian distance = 0.7
Normalized UV signal at 215 nm [-]
time [min]

Figure 5


A) CSH C18
$\alpha_{4\text{-OBSA/Tol}}$ = 0.78
Euclidian distance N/A
B) AdvanceBio Plus
$\alpha_{4\text{-OBSA/Tol}}$ = 1.71
Euclidian distance = 1.7
C) BEH C18 AX
$\alpha_{4\text{-OBSA/Tol}}$ > 30
Euclidian distance = 5.0
D) CSH Fluoro-Phenyl
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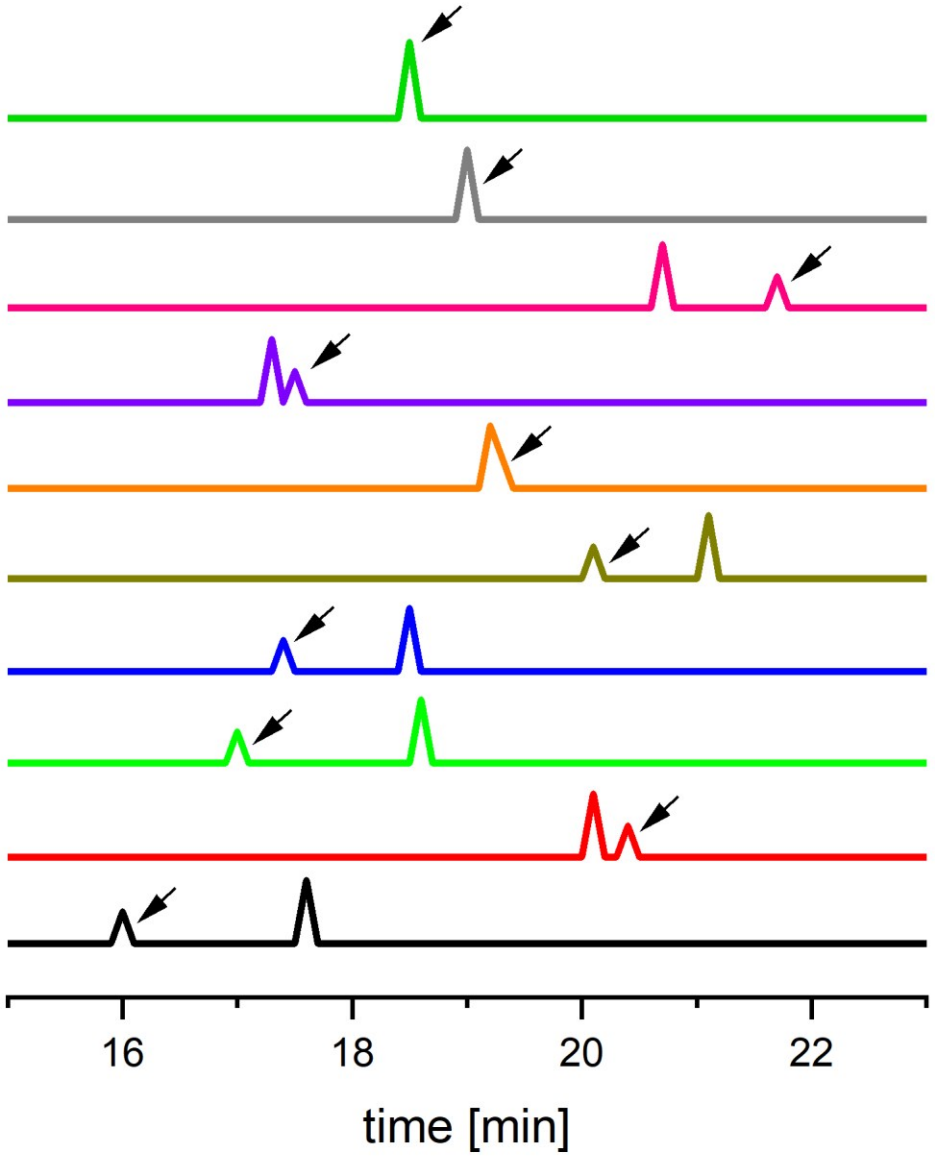
Euclidian distance = 8.3
Normalized UV signal at 215 nm [-]
time [min]

Figure 6



Column	$\alpha_{4\text{-OBSA / Tol}}$	Euclidian distance
Kinetex C18	0.37	0.83
HPH C18	0.41	0.74
BEH C18	0.57	0.52
EVO C18	0.61	1.10
Cortecs C18+	0.76	3.70
CSH C18	0.78	N/A
Sym C8	1.22	1.91
AdvanceBio Plus C18	1.71	1.66
PS C18	2.32	1.06
Bonus RP	29.15	4.99

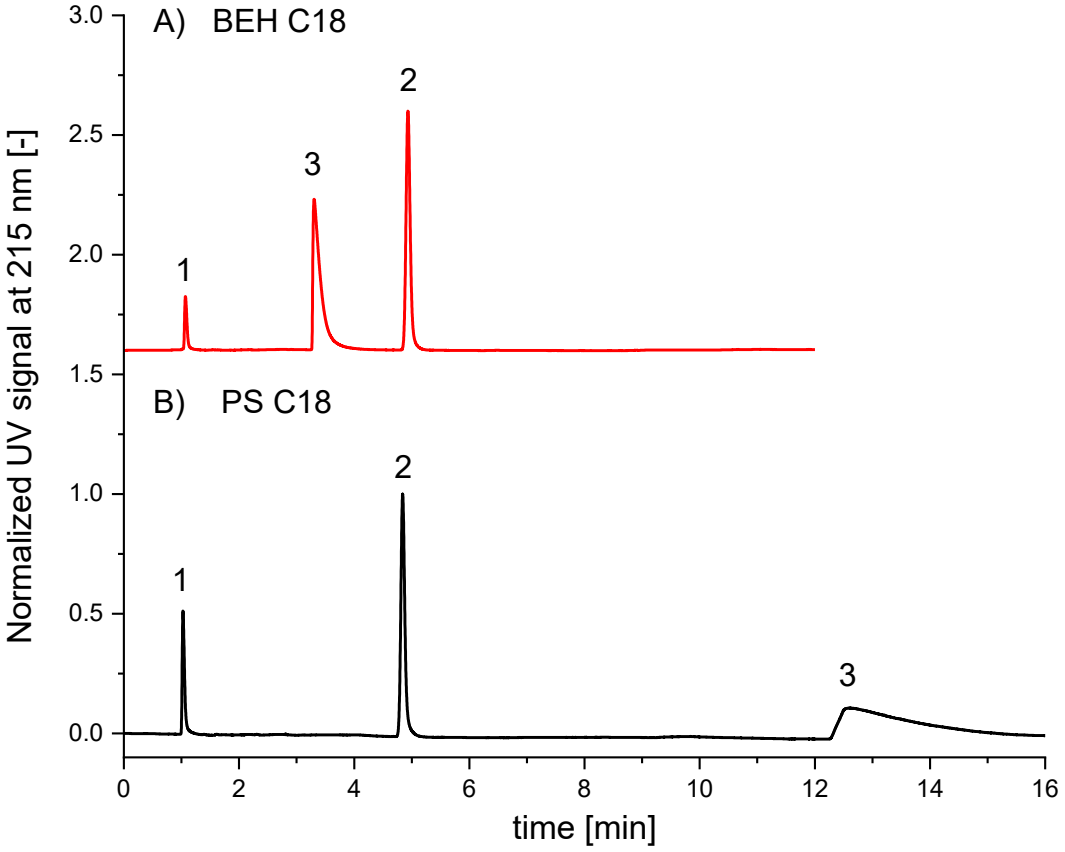
Supplementary Materials

Chemical	log *D* at pH 2.5	MeCN/Water (v/v)
Benzenesulfonic acid	-3.1	0 / 100
2-Napthalenesulfonic acid	-1.4	0 / 100
2-Bromo-1-napthalenesulfonic acid	-2.2	0 / 100
p-xylene-2-sulfonic acid	-2.3	0 / 100
4-*n* -Octylbenzenesulfonic acid	0.9	10 / 90
4-Methoxy-1-Napthalenesulfonic acid	-0.9	5/ 95
1,4-Benzenedimethanol (hydrophobic probe)	0.0	10 / 90
Benzyl alcohol (hydrophobic probe)	1.2	25 / 75
Toluene (hydrophobic probe)	2.6	50 / 50
Uracil (t_o marker)	-0.4	50 / 50

Supplementary Material 1. Aryl sulfonic acid and hydrophobic probes (all at 0.5 mg/mL concentration) for the development of the new anionic exchange parameter.

Chemical	Log *D* at pH 2.5	pK_a of most basic moiety	Amino functionality	MeCN/Water (v/v)
Atenolol	-2.9	9.4	2°	5 / 95
Salbutamol	-2.5	9.6	3°	5 / 95
Benzylamine	-2.0	9.1	1°	5 / 95
1,4-Benzenedimethanol (hydrophobic probe)	0.0	N/A	N/A	10 / 90
Propranolol	0.2	9.5	2°	50 / 50
Diphenydramine	0.6	8.8	3°	25 / 75
Carvedilol	2.0	8.2	2°	50 / 50
Benzyl alcohol (hydrophobic probe)	2.2	N/A	N/A	25 / 75
Amitriptyline	2.6	9.2	3°	50 / 50
Toluene (hydrophobic probe)	2.6	N/A	N/A	50 / 50
Nortriptyline	2.7	10	2°	50 / 50

Supplementary Material 2. Basic analytes and hydrophobic probes (all at 0.5 mg/mL concentration) for the evaluation of the repulsive nature of the columns. Analytes with Log *D* values <0 are considered hydrophilic and those and >0 hydrophobic in nature.



Supplementary Material 3. New Anionic Exchange Test protocol chromatogram on the A) BEH C18 column, $\alpha_{4\text{-OBSA/Tol}} = 0.57$ and B) PS C18, $\alpha_{4\text{-OBSA/Tol}} = 2.32$. 1. Uracil (unretained t_0 marker), 2. Toluene (neutral hydrophobic probe) and 3. 4-n-Octylbenzenesulfonic acid (Anionic probe). Chromatographic conditions as described in section 2.2.1.