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Utility of Retention Prediction Model for Investigation of Peptide Separation Selectivity in Reversed-Phase Liquid Chromatography: Impact of Concentration of Trifluoroacetic Acid, Column Temperature, Gradient Slope and Type of Stationary Phase

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Peptide separation selectivity in reversed-phase liquid chromatography was investigated using a training set of 165 peptides with a total of 1698 amino acid residues. Gradient separation was performed at selected chromatographic conditions, varying column temperature, gradient slope, ion-pairing reagent concentration, and type of stationary phase. The retention times for each set of experiments were utilized to calculate the amino acid retention coefficients using a published prediction model (*Rapid Commun. Mass Spectrom.* 2007, *21*, 2813–2821). The calculated retention coefficients reflect the contribution of each type of amino acid residue to peptide retention at a given chromatographic condition. For example, the concentration of ion-pairing reagent (trifluoroacetic acid) had the strongest impact on the retention of peptides containing an increasing number of basic (charged) amino acids, such as arginine, lysine, and histidine, while the retention coefficients of other amino acids are minimally affected. Increasing the separation temperature resulted in a moderate decrease of amino acid retention coefficients with the exception of isoleucine, leucine, valine, and proline. This finding suggests that these residues enhance peptide retention at elevated temperature. Amino acid residue retention coefficients were also helpful to understand the impact of sorbent pore size (130 vs 300 Å) on peptide retention selectivity. In addition we investigated the selectivity differences of various reversed-phase chromatographic sorbents. The trends suggested by calculated retention coefficients were confirmed using a set of synthetic peptides with specifically designed sequences.

Reversed-phase liquid chromatography (RPLC) is a powerful method for analysis of peptides. RPLC is often used for peptide mapping applications,[1−3] characterization of therapeutic peptides, and proteomic research.[4−6] A number of studies have investigated the impact of separation conditions on peptide peak shape, retention, and separation selectivity[7−15] with the goal to rationalize method development. While the effect of ion-pairing reagent on retention and selectivity is well understood,[7,8] the impact of other separation parameters on peptide selectivity is less obvious.[12,14]

When separating complex peptide mixtures, such as in peptide mapping applications, separation optimization is not trivial. The ion-pairing reagent type and concentration, gradient slope, and column temperature have different impacts on different types of peptides. This makes method development of complex mixtures rather difficult. Optimization is usually performed by trial and error or using computer simulation software.[15,16]

Several laboratories have developed approaches for a robust prediction of peptide retention behavior using peptide training sets.[17−21] Majority of the published algorithms are based on the

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Table 1. Synthetic Peptides Used for the Experiment

	sequence	peptide length	charge[a]	MW	PVIL[b]	WY[b]	PVIL-WY-HKR[c]
set 1, constant length, various charges							
1a	MAVTGNGILAG	11	1	1002.52	3	0	3
1b	MGGGVNGILAR	11	2	1043.55	3	0	2
1c	MARTINGILAR	11	3	1214.69	3	0	1
1d	MVRTLHGILAR	11	4	1265.74	4	0	1
set 2, constant length, various PIVL-WY factors							
2a	MPIVLGILAG	10	1	982.59	6	0	6
2b	MIPVLGILAT	10	1	1026.61	6	0	6
2c	MWYWYGYYAG	10	1	1358.54	0	6	−6
2d	MYWYWGYYAT	10	1	1402.57	0	6	−6
set 3,various lengths, constant charge							
3a	ILAF	4	1	462.28	2	0	2
3b	YGGFL	5	1	555.27	1	1	0
3c	AGTINGIL	8	1	757.43	3	0	3
3d	MGTWGYAG	8	1	841.34	0	2	−2
3e	TAGAGATINGILAGAG	16	1	1313.69	3	0	3
3f	AGLTATGTGAANGWIG	16	1	1416.70	2	1	1

[a] Charge was calculated as sum of H, K, R residues + 1 for amino terminal group. [b] Sum of P, V, I, L or W, Y amino acid residues in the peptide sequence. [c] Calculated from eq 2.

assumption that the retention contributions of amino acid residues comprising the peptide sequence are additive and neglect the nearest neighbor effect and positions of amino acid residues in the sequence. After calculation of the amino-acid retention coefficient from a training peptide set, retention of any additional peptide can be predicted. The prediction models have been successfully used for data filtering and removal of false positive peptide hits in proteomic applications.[22−25]

While published algorithms allow for robust retention prediction ($R^2 \approx 0.96-0.99$), the prediction accuracy is not sufficient to be directly applicable for peptide mapping method development (required prediction accuracy is comparable to peak width, that is, several seconds, while the current algorithm's accuracy is on order of minutes for ∼1 h long gradient; see Supporting Information Figure 1).

In a recent publication,[24] we investigated the impact of pH on peptide retention in RP LC and developed retention prediction models for low (2.6) and high pH (10.0) conditions. Basic peptides were better retained at pH 10 conditions, while the acidic peptides exhibit more retention when acidic mobile phases are used.[5,23,24] This trend was clearly reflected in the values of amino acid retention coefficients. Specifically, retention coefficients of histidine, arginine, and lysine residues were negative at acidic pH (residues were charged, hence hydrophilic and poorly retained on RP sorbent), while at pH 10.0, the contribution of these residues to peptide retention became highly positive (amino acids were uncharged, peptides became more hydrophobic).[24] For acidic amino acid residues, aspartic and glutamic acid, we observed the opposite trends. Retention coefficients of neutral (not-ionizable) residues did not significantly change with the mobile phase pH.[24] These observations suggest that the retention coefficients closely reflect the nature of peptide interaction in RPLC and potentially can be utilized to explore the impact of separation conditions on peptide retention and selectivity.

In this work, we propose a novel method for investigation of the peptide separation selectivity in RPLC. The goal was to use amino acid retention coefficients calculated for various peptide data sets to visualize the impact of chromatographic conditions on separation selectivity. We aim to illustrate the impact of trifluoroacetic acid ion-pairing reagent, and investigate the unknown or underappreciated effects of column temperature, gradient slope, and type of stationary phase.

EXPERIMENTAL SECTION

Materials and Reagents. Formic acid (FA) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade acetonitrile (MeCN) and ammonium hydroxide were purchased from J.T. Baker (Phillipsburg, NJ). A Milli-Q system (Millipore, Bedford, MA) was used to prepare deionized water (18 MΩ cm) for HPLC mobile phases. MassPREP protein tryptic digestion standards of enolase, alcohor dehydrogenase (ADH), phosphorylase b, hemoglobin, and bovine serum albumin (BSA) were obtained from Waters (Milford, MA).

Synthetic Peptide Samples. Selected peptides of desirable sequence were obtained from Biomatik Corporation (Cambridge, Ontario, Canada). The sequences and other details are listed in Table 1. Set number 1 was designed to have similar retention but different overall charge (different number of basic amino acids R, K, and H). Peptides in set 2 have the same length and charge, similar retention in RPLC, and different content of PIVL and WY amino acid residues. Set number 3 included peptides with similar retention but significantly different lengths (molecular weight, MW). The explanation of one and three letter amino acid convention is provided in Supporting Information Table 1.

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LC-UV-MS Instrumentation, Columns, and Conditions. LC-MS experiments were carried out using the following instruments: ACQUITY UPLC system with a tunable ultraviolet detector connected online to a time-of-flight (LCT) MS instrument (all Waters, Milford, MA). The instrument was equipped with a 425 μL mobile phase mixer and 15 μL PEEKsil nondeactivated sample needle. The 100 × 2.1 mm RP columns packed with 1.7 or 1.8 μm sorbent were obtained from Waters. The ion-pairing reagent concentration, temperature, and gradient slope studies were performed using BEH130, C18 Peptide Separation Technology column. Additional columns used to investigate the impact of the type of RP sorbent were as follows: BEH300 C18, BEH Shield RP 18, BEH phenyl (based on bridged-ethyl-hybrid sorbent, BEH), and silica-based C18 columns, packed with 1.8 μm HSS T3, HSS C18 SB, and HSS C18 sorbents. Column dimensions were 100 mm × 2.1 mm.

The standard gradient was 0−50% MeCN in 50 min, unless stated otherwise. Mobile phases used for gradient slope, temperature, and sorbent type studies were 0.02% TFA in water as mobile phase A and 0.018% TFA in MeCN as mobile phase B. Column temperature was 40 °C, and flow rate was 0.2 mL/min.

The ion-pairing reagent concentration study was carried out with the following mobile phases: A 0, 0.02, 0.05, and 0.1% TFA in water; B 0, 0.018, 0.045 and 0.09% TFA in MeCN, respectively. For 0% TFA conditions, both mobile phases contained 0.1% FA to maintain the pH.

The gradient slope study was performed with standard conditions (0.02% TFA, 40 °C, 0−50% B) by varying gradient time from 12.5, 25, 50, to 100 min. Similarly, the temperature study used standard conditions (0.02% TFA, 0−50% B), and the column temperature was set to 20, 30, 40, and 50 °C. BiopharmaLynx, version 1.1, software (Waters) was used to process the peptide maps and extract peptide retention data from LC-MS chromatograms.

Peptide Retention Prediction. Development of a peptide retention prediction algorithm shown in eq 1 was described earlier.[24] Retention time (RT) of a peptide is calculated from the contribution of the retention coefficients b_i of all amino acid residues AA_i in the peptide sequence. The retention model is linearized by natural logarithm ln function using an interactively optimized coefficient a ($a = 0.21$). L denotes peptide length (number of amino acids residues; $L = \text{sum}(AA_i)$), b_0 is the intercept in the model.

$$RT = (1 - a \cdot \ln L) \cdot \left(\sum b_i \cdot AA_i + b_0\right) \quad (1)$$

The basic assumption of the model is that the retention coefficient values b_i calculated from the retention times of the peptide training set (165 peptides, 1698 amino acid residues) accurately reflect the relative contribution of different amino acid residues to overall peptide retention at given chromatographic conditions. Therefore, it is possible to visualize the impact of ion-pairing reagent concentration, separation temperature, and stationary phase on peptide retention using b_i coefficients. The take-one-out cross-validation experiment indicated that values of amino acid retention coefficients b_i change on average by 1% when sequentially removing a single peptide from the training set.

Supporting Information Table 1 lists the calculated retention coefficients b_i. The retention times of 165 peptides at investigated chromatographic conditions are provided in Supporting Information Table 2. Supporting Information Table 3 summarizes the numbers of amino acid residues used for calculation and RSD values for the take-one-out experiment. The Excel spreadsheet peptide retention time calculator is provided as Supporting Information. Example of correlation between experimental and predicted retention times for 165 peptides for selected data set is shown in Supporting Information Figure 1. If Supporting Information Tables 1 and 2 and the spreadsheet calculator are combined, it is possible to investigate correlations for all experiments and columns used in this study.

RESULTS AND DISCUSSION

Effect of Ion-Pairing Reagent. Thanks to the excellent studies from Shibue et al.[7,8,26] the impact of ion-pairing reagent concentration and type on peptide retention behavior is well understood. Therefore, we chose to evaluate the validity of the proposed approach by studying the effect of ion-pairing reagent (TFA) on retention coefficients. Results are summarized in Figure 1. The retention behavior of 165 peptides chromatographed with 0% (0.1% FA), 0.02%, 0.05%, and 0.1% TFA in the mobile phase is plotted either as retention times (Figure 1A) or as retention coefficient of 20 amino acid residues (Figure 1B). Because we are correlating results for 0% TFA (0.1% FA) concentration against all other experiments including the same data set), the plots are diagonally divided with a straight line and with slope 1 and intersect at 0,0 coordinates. We assume here that 0% TFA (0.1% FA) mobile phase represent a system with negligible ion-pairing properties compared to TFA.[15,27]

From the offset between series in Figure 1A, one can appreciate that peptide retention generally increases with TFA concentration. However, the retention time plots are not parallel. The visible data scatter is because retention of some peptides is more affected by ion-pairing reagent concentration than others.

Figure 1B shows the same data presented as retention coefficients of amino acid residues, which reflect how much each of the residues contributes to peptide retention. As expected, the aromatic amino acid residues tryptophan (W) and phenylalanine (F) contribute more significantly, while the contribution of charged amino acids histidine (H), lysine (K), and arginine (R) is negative. Although the retention coefficients were calculated from retention times of peptides, their values are not directly proportional to time units, since they are dynamically linearized by $(1 - a \ln L)$ coefficient in eq 1.

The most revealing part of the graph in Figure 1B is the region of H, K, R residues. These charged amino acid residues are clearly affected by ion-pairing; their retention coefficients, which have negative values at 0% TFA (0.1% FA) conditions, increase significantly at elevated TFA concentration and K, and R residues ultimately become positive contributors to the peptide retention when TFA concentration exceeds 0.05%. As one would expect, the uncharged amino acid residue values are not affected by the ion-pairing interactions. Some minor changes (I, L, F, W) are believed to be caused by the slight change of the plot's slope at elevated TFA concentrations (presumably caused by the contribu-

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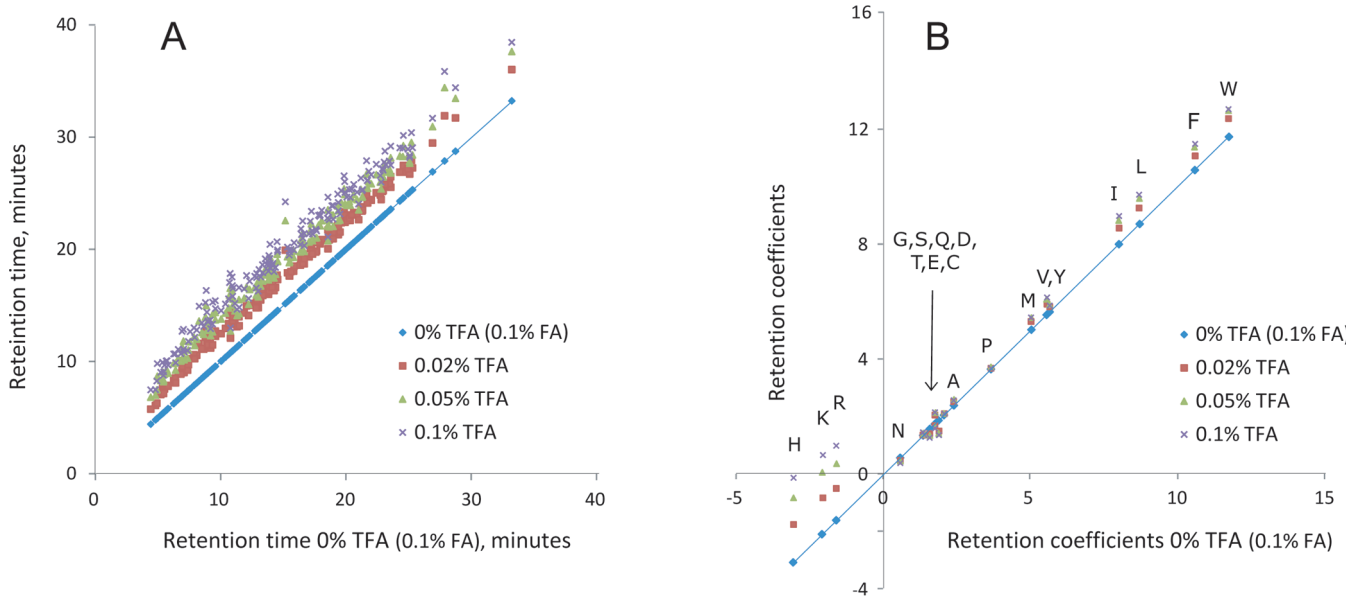

Figure 1. Effect of ion-pairing reagent concentration on peptide retention times (A) and amino acid residue retention coefficients (B).

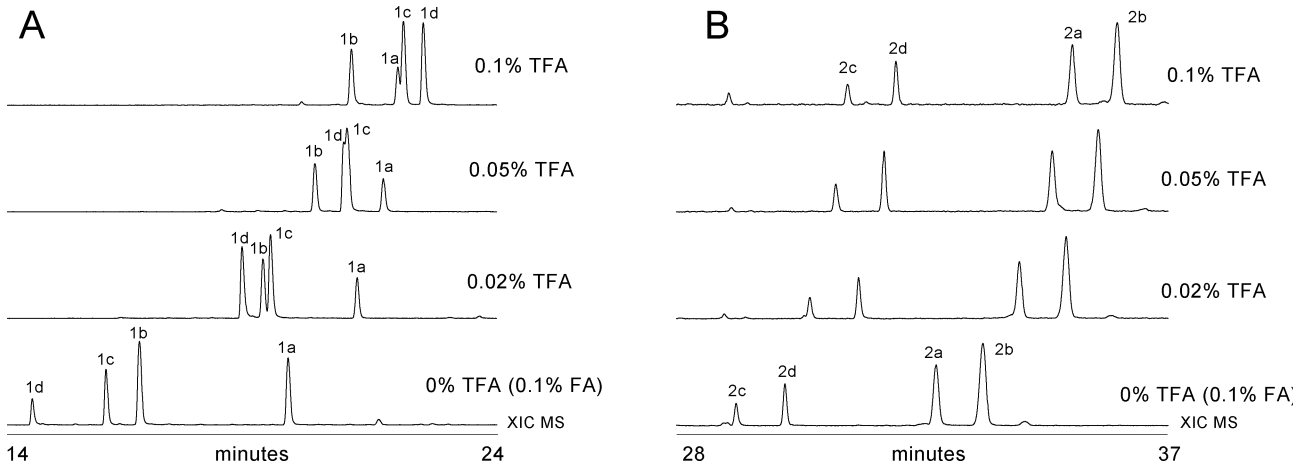

Figure 2. Effect of ion-pairing reagent concentration on peptide retention. Separation selectivity is strongly affected for set 1 (Table 1) with different peptides charges (A). Peptides **1a**, **1b**, **1c**, and **1d** carry 1+, 2+, 3+, and 4+ charges, respectively. The separation selectivity of peptides in set 2 (carrying the same 1+ charge) does not change appreciably (B). Peak relative areas in LC-MS XIC chromatogram are affected by ionization efficiency of peptides at different concentrations of TFA.

tion of the charged N-terminal amino group, which also ion-pairs with TFA, in addition to H, K, and R residues).

The results in Figure 1 correlate well with the literature data[7,8,26] suggesting that ion-pairing reagents affect peptide retention via a charge-to-charge interaction with basic H, K, and R residues. To confirm this hypothesis experimentally, we utilized two sets of synthetic peptides listed in Table 1. Peptides **1a**−**1d** differ in the number of basic residues, while peptides **2a**−**2d** have constant charge (1+ because of the terminal amino group).

Figure 2A illustrates changes of retention behavior of peptides with different charge (**1a**−**1d**). For example, the least retained peptide in 0% TFA (0.1% FA) becomes the most retained one in 0.1% TFA conditions (**1d**, 4+ charge). The magnitude of retention change correlates well with peptide charge.[8]

In contrast to Figure 2A, Figure 2B demonstrates that the relative retention and separation selectivity of peptide set **2a**−**2d** carrying the same 1+ charge is not altered by ion-pairing reagent concentration. This has direct implications on peptide separation method development. If one aims to resolve coeluting peptides, altering ion-pairing reagent concentration is a rational optimization strategy only if the peptides have different charge.

The ion-pairing study suggests that the calculated retention coefficients are useful for elucidating the principles of peptide retention. The agreement with the literature[7,8,26] and experiment data validates the chosen approach.

Effect of Separation Temperature. Column temperature directly affects peptide retention and separation selectivity.[13,14] Several studies have investigated the impact of temperature on dimerization and unfolding of a special class of analytes, the amphipathic helical peptides.[11,12] To the best of our knowledge, there has been no study rationalizing peptide separation optimization using temperature for generic peptides (without strong secondary structure).

Figure 3 shows how the retention coefficients for amino acid residues change with column temperature. Since retention in RPLC usually decreases with temperature, one may expect an

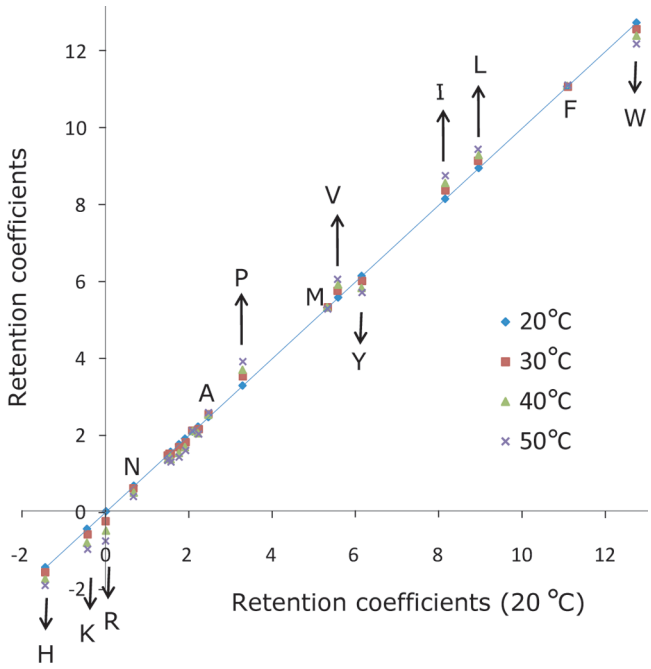

Figure 3. Impact of separation temperature on the amino acid retention coefficients. P, V, I, and L residue contributions to peptide retention are enhanced at elevated temperature.

overall decrease of all retention coefficients. Instead, some of the coefficients show a greater descending trend than others and some amino acid residue values appear to be increasing. These trends will be examined separately in the following discussion.

Since charge-to-charge interaction strength increases at elevated temperatures, one may expect that ion-pairing interaction follows the same trend. However, H, K, and R residue values show a descending trend. This could be explained by poorer adsorption of the hydrophobic TFA on the C18 surface at elevated temperatures, which consequently leads to less efficient ion-paring.

The aromatic amino acid residues Y and W show a noticeable decrease at elevated temperature. Interestingly, phenylalanine (F), another member of the aromatic amino acid group, does not show the same trend.

Perhaps the most interesting are the trends for P, V, I, and L amino acid residues with aliphatic alkyl side chains (alicyclic in case of proline). These residues show increasing retention with temperature. Alanine with a short $-CH_3$ side chain also shows an increase, although minor. To experimentally validate the retention coefficient trends observed in Figure 3, we designed a set of synthetic peptides **3a**−**3d** (Table 1). While peptides **3a** and **3b** are P, V, I, and L rich, peptides **3c** and **3d** contain instead multiple W and Y residues. The peptide lengths and charges are the same.

Figure 4 illustrates that the P, V, I and L rich peptides indeed increase their retention with temperature, while the W and Y rich peptides decrease retention significantly. Above 50−60 °C we observed a gradual decrease in retention of **2a** and **2b** peptides; however, the retention time difference between the two sets of peptides (**2a**, **2b** versus **2c**, **2d**) continues to increase.

Figures 3 and 4 allow one to propose a simple rule for retention of peptides at elevated temperature shown in eq 2.

$$\mathrm{TF} = \sum \mathrm{P,V,I,L} - \sum \mathrm{W,Y} - \sum \mathrm{H,K,R} \qquad (2)$$

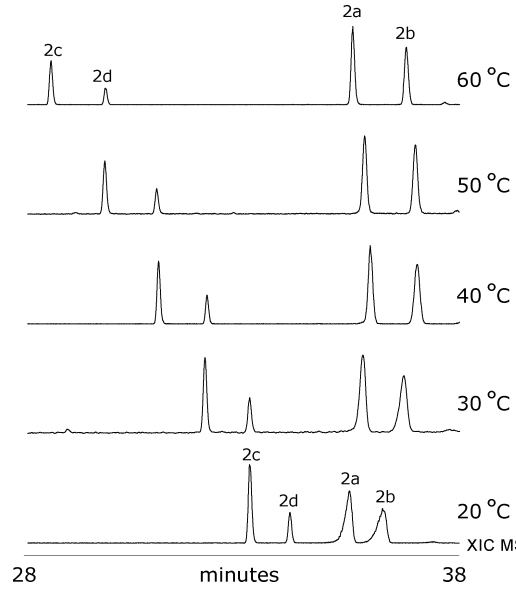

Figure 4. Impact of temperature on retention and separation selectivity of synthetic peptides **2a**−**2d** (Table 1). The retention of peptides with high P, V, I, and L content (**2a**, **2b**) increases with temperature. The retention of peptides rich in W and Y (**2c**, **2d**) significantly decreases with temperature.

When plotting the "temperature factor" (TF) against the change in retention (ΔTR) at elevated temperature, we observed a clear trend (Supporting Information Figure 2). The greater the calculated TF value is, the lower was the decrease in retention for a given peptide. Several peptides with very high TF values (4−6) exhibited an absolute increase in retention at elevated temperature (Suppoting Information Figure 2).

Equation 2 provides guidelines for the optimization of peptide separations as a function of temperature. If the separation of a critical pair of peptides (of similar length) is required, the change of column temperature will be helpful in cases where the peptides have significantly different TF.

The rationale for a positive impact of P, V, I, and L residues on retention is not known. We do not believe this behavior is the result of an impact of secondary structure (potentially "induced" by the presence of these residues). Some evidence for the positive impact of P, V, I, and L on retention at elevated temperature can be found in Chen et al. for short random coil peptides.[12] We speculate that peptides composed of P, V, I, and L residues (with the alkyl side chains) behave as thermo-responsive polymers,[28] effectively increasing the side chain's hydrophobicity at elevated temperatures.

Effect of Gradient Slope. It is well-known that gradient slope affects the relative retention in RPLC,[29] including peptides.[14,16] Similar to that of temperature, the impact of gradient slope on peptide retention is complex. Although gradient slope is often used for peptide separation optimization, method development is often performed by trial-and-error with limited understanding of the impact of the gradient slope on the selectivity of the separation.

We have calculated the retention coefficients for a series of experiments using different gradient slopes. Figure 5 shows the results for a 0−50% MeCN gradient executed at 12.5, 25, 50, and

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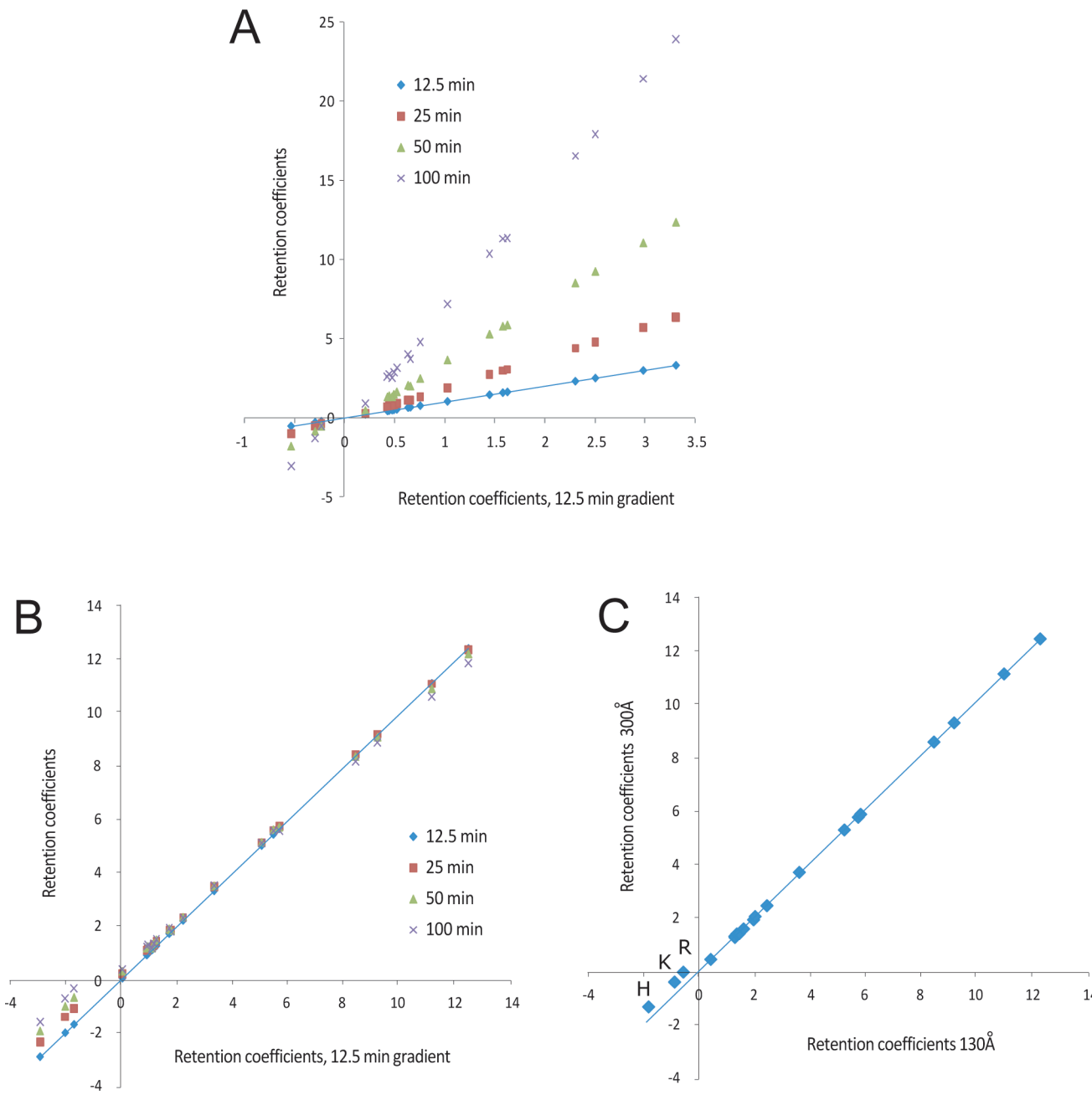

Figure 5. Effect of gradient slope on amino acid residue retention coefficients (A) calculated using peptide retention times, (B) calculated using % of MeCN at the point of peptide elution, and (C) calculated using peptide retention times for columns packed with 130 and 300 Å C18 sorbents.

100 min. Because the values of retention coefficients are calculated from absolute peptide retention times, the coefficients in Figure 5A have different values for different gradient slopes, which complicates interpretation of the results. Therefore, we have recalculated the peptide retention as percentage of MeCN at the point of elution. With this data normalization, the plots in Figure 5A lined up in Figure 5B and retention coefficients can be readily compared. As one may conclude, gradient slope does not affect the value of retention coefficients to any significant extent, with the exception of H, K and R residues. Apparently, gradient slope has some impact on the efficiency of ion-pairing. The ion-pairing interaction appears to be more pronounced at shallower gradients. The reasons for this effect are not obvious. Because of the differences in retention behavior of peptides and TFA the change of gradient slope may alter the ion-pairing reagent adsorption on the stationary phase to a different degree than peptides. Effectively the peptides may be eluted at slightly different TFA concentration. This effect can potentially produce the pattern observed in Figure 5B.

Impact of Sorbent Pore Size. Practitioners analyzing peptide maps are aware that columns packed with chemically identical sorbents with different pore sizes (e.g., 130 versus 300 Å) have noticeably different separation selectivity. This knowledge is often utilized as one of the separation optimization strategies.

To understand the sources of different selectivity of 130 and 300 Å C18 sorbents, we have calculated the corresponding retention coefficients for two sets of experimental data. The experiments were performed using identical column dimensions

(100 mm × 2.1 mm) with the same gradients and chemically identical C18 BEH sorbents; the only difference was the sorbent pore size (130 and 300 Å). The correlation between two sets of retention coefficients is shown in Figure 5C.

When excluding H, K and R residues, the correlation between the sets is absolute with slope 1 and $R^2 = 1$. In other words, retention coefficients are identical for both 130 and 300 Å columns. The small difference in values of H, K, and R retention coefficients may mean that ion-pairing interaction is stronger for 300 Å compared to 130 Å sorbent, which results in different separation selectivity. Virtually the same trends were observed by Krokhin,[21] who compared retention coefficients for 300 Å and 100 Å C18 columns (Table 2 in reference[21]). However, as shown in Figure 5B, a similar offset in H, K, and R values was observed for a 130 Å column upon changing the gradient slope. The correlation between retention coefficients in Figure 5B (any of the series, excluding the H, K, and R data points) is also high ($R^2 > 0.999$). Therefore, we need to consider whether the observed offset in H, K, and R values in Figure 5C between 300 Å and 130 Å columns can potentially be caused by a gradient slope effect. This question will be discussed in the following paragraphs.

It is known that the column retentivity depends on the available surface of the sorbent packed in the column as defined by eq 3, where k is the retention coefficient, c_s, c_m the concentrations of analyte in stationary or mobile phase, and V_s and V_m are the volumes of stationary or mobile phases. In adsorption chromatography, V_s could be substituted by A_s, the surface area of the sorbent packed in column, and V_m by the column void volume V_0. The ratio of c_s/c_m is equal to the equilibrium constant K, while the ratios V_s/V_m or A_s/V_0 constitute the phase ratio β.

$$k = \frac{c_s}{c_m} \cdot \frac{V_s}{V_m} = K \cdot \frac{A_s}{V_0} = K\beta \tag{3}$$

It is also known that sorbents with larger pore size have less available surface area (in the present study 130 Å and 300 Å sorbents had surface areas 194 and 85 m^2/g, respectively). Because the surface areas of both columns are different, while the difference in V_0 is rather small and nearly negligible, the phase ratio changes significantly. This in turn strongly impacts the gradient slope. By using the same gradient volume V_g in both experiments, we have effectively performed a steeper gradient for the 130 Å column that has the greater available surface area.

Method transfer between columns of different dimensions follows simple rules. For example, the gradient 0−50% MeCN in 50 min at 0.2 mL/min for 2.1 × 100 mm long column translates to 25 min gradient for 2.1 × 50 mm column at the same flow rate, or alternatively the flow rate can be lowered to 0.1 mL/min while maintaining 50 min long gradient for the shorter column. In fact, the accurate the method transfer should utilize the actual measured V_0 of both columns to compensate for possible differences in the column packing density. When transferring the gradient correctly, the ratio of V_g/V_0 is constant regardless of column dimensions. However, it is often overlooked that the gradient conversion is more complicated in cases when using chromatographic sorbents with significantly different surface area.

To design the experiment with the same gradient slope for columns with different A_s, the gradient volume needs to be adjusted in proportion to the phase ratio, calculated from eq 4, where ε_i is interstitial porosity of packed bed, V_{sp} is specific pore volume and ρ_{sk} is the skeletal density of the packing.[29]

$$\beta = (1 - \varepsilon_i) \cdot \frac{A_s}{V_{sp} + \varepsilon_i/\rho_{sk}} \tag{4}$$

To evaluate the hypothesis that the differences in apparent selectivity of 300 Å and 130 Å columns are caused by gradient slope differences, we performed the experiment shown in Figure 6. The first two chromatograms, Figure 6A and B, were acquired using a flow rate of 0.2 mL/min. The chromatograms appear to be distinctly different (see the highlighted sections). This is why practitioners believe that 130 and 300 Å sorbents have different separation selectivity.

To equalize the gradient slope, we adjusted the gradient volume by adjusting the flow rate (eq 5). The gradient time and span were kept constant (50 min, 0−50% MeCN). Because the values for calculating phase ratio β cannot be easily obtained, we approximated the calculation using the sorbent's specific surface areas ${}^1A$, ${}^2A$ and void volumes ${}^1V_0$, ${}^2V_0$ measured for both columns. The smaller specific surface of the 300 Å column suggests a lower flow rate calculated from eq 5.

$$FR_2 = FR_1 \cdot \frac{\beta_2}{\beta_1} \sim FR_1 \cdot \frac{{}^2V_0}{{}^1V_0} \cdot \frac{{}^2A}{{}^1A} \tag{5}$$

When the analysis was run at a flow rate of 0.11 mL/min for the 300 Å column (Figure 6C), the original separation selectivity was clearly altered and differs from the separation previously obtained at 0.2 mL/min (Figure 6A). The peak order and selectivity starts to closely resemble the chromatogram in Figure 7B obtained with the 130 Å column. Comparison of chromatograms B and C in Figure 6 suggests that the selectivity of columns packed with 130 and 300 Å C18 sorbents is essentially identical. We have highlighted three sections that specifically demonstrate the similarity between the separation of the 130 Å column at 0.2 mL/min and the 300 Å column at 0.11 mL/min.

When scaling the flow rate using eq 5 for the 130 Å column from 0.2 to 0.365 mL/min, we obtained a chromatogram Figure 6D closely resembling Figure 6A obtained with 300 Å column. This experiment further confirms that selectivity of both columns is similar.

Impact of Gradient Slope on Peptides with Different Molecular Weight. Previous sections discussed the impact of gradient slope on separation selectivity. While it is understood that relative peak retention changes at different gradient slopes, it is less obvious how to exploit this knowledge for rational method development.

In principle one may chose to vary the gradient slope by changing the gradient time while keeping the gradient span constant, or as discussed previously, by adjusting the flow rate, while keeping both gradient time and span constant. The selectivity changes are similar in both scenarios, depending primarily on

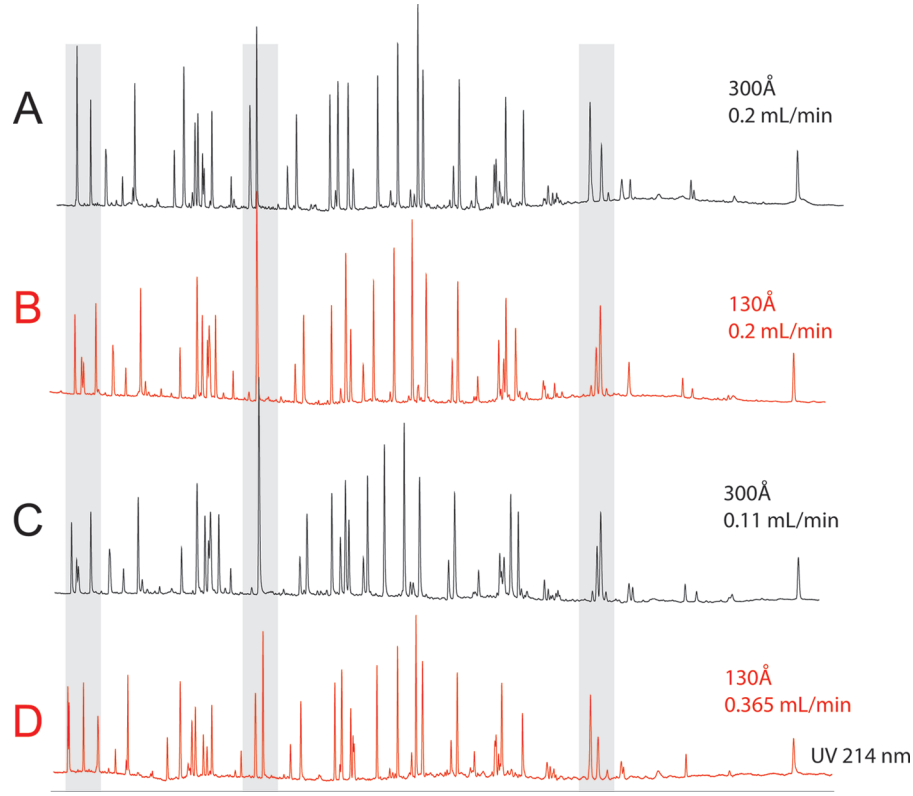

Figure 6. Separation of yeast enolase tryptic map using 300 Å BEH C18 column at flow rate 0.2 mL/min (A), 130 Å BEH C18 column at 0.2 mL/min (B), 300 Å BEH C18 column at flow rate 0.11 mL/min (C), and 130 Å BEH C18 column at flow rate 0.365 mL/min (D). The 300 and 130 Å columns appear to have different selectivity (compare chromatograms A and B). When adjusting the flow rate to maintain the gradient volume proportional to available sorbent surface packed in the column (eq 5), the selectivity of 300 and 130 Å columns appears to be similar (compare chromatograms A and D, or B and C). Other conditions: 100 × 2.1 mm columns were packed with 1.7 μm sorbent. Mobile phase A was 0.02% TFA in water, mobile phase B was 0.018% TFA in MeCN. Gradient was 0−50% B in 50 min, column temperature 40 °C, flow rates are indicated in figure.

gradient volume V_g (or more generically on ratio V_0/V_g when considering different column dimensions; we assume constant A_s).

Figure 7 illustrates the impact of gradient slope on two sets of peptides. Set 3 (Table 1), comprised of analytes with significantly different molecular weight, shows strong selectivity changes, while the relative retention of set 2 peptides (Table 1) with similar MW does not change appreciably (see Figure 7B). The explanation of this behavior lies in eq 6,[30] which defines the change in retention factor k of the analyte with the change of isocratic mobile phase strength ϕ (portion of organic modifier in the mobile phase). The speed of the change is defined by the slope S, which increases with the MW of the analyte.[31,32]

$$\log k = \log k_0 - S\phi \qquad (6)$$

Consequently, when analyzing peptides with different size (MW), the linear functions of *log k* have different slopes S and therefore cross as shown in the inset of Figure 7A. In such case, the selectivity of separation is altered both by a change of isocratic elution conditions or by gradient slope. For such scenario Snyder and Dolan[30] coined the term *irregular samples*, while for *regular samples*, the selectivity is not affected by gradient slope. Regular samples are, for example, homopolymers,[31,32] but similar retention behavior is also observed for peptides of similar MW as shown in Figure 7B. Because the log k functions of peptides of the same length (and charge) do not cross (inset in Figure 7B), their relative retention is not appreciably affected by gradient slope.

Closer inspection of Figure 7A reveals that retention times of peptides of similar size are affected by gradient slope to a similar degree. The relative retention (separation selectivity, resolution) of 4 and 5 amino acid (AA) peptides does not change significantly. Similarly, retention differences for pairs of 8 AA (or 16 AA) peptides remains approximately the same at all conditions. In contrast, the relative differences in retention of 4, 5, and 16 AA peptides are apparent. This observation represents a rationale for peptide separation optimization. If two coeluting peptides have significantly different MW, gradient slope is a suitable optimization parameter.

Selectivity of RP Sorbents for Peptide Analysis. Different types of C18 or other RP sorbent chemistries have presumably different selectivity for peptide separation (and other types of molecules). We have utilized calculated retention coefficients to investigate how the selectivity differs between silica based sorbents (1.8 μm high strength silica, HSS, 101 Å, surface area ~240

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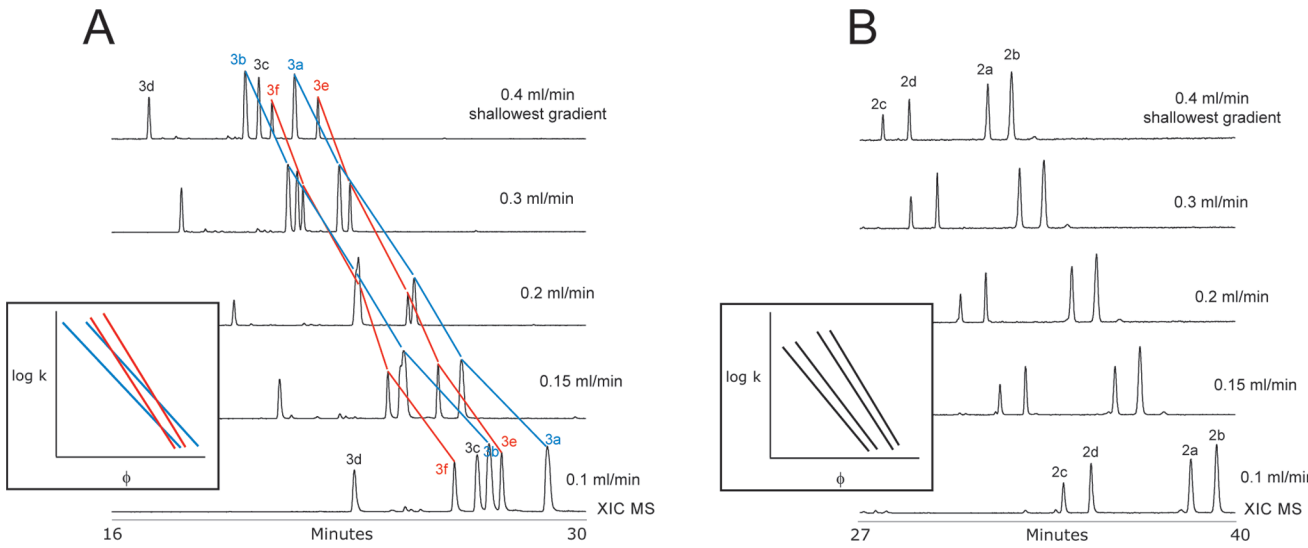

Figure 7. Effect of gradient slope on relative retention of peptides. Separation selectivity of peptides with different MW is strongly affected by gradient slope (A). Peptides with similar MW are little affected by gradient slope (B). The insets in figures are for illustration of trends between log k and ϕ, rather than experimentally measured data for actual peptides.

m^2/g, and 1.7 μm hybrid silica BEH, 130 Å with surface area ~194 m^2/g). We also used various types of surface chemistry as follows: HSS C18 (3.25 $\mu mol/m^2$, end-capped), HSS C18 T3 (low density C18, 1.7 $\mu mol/m^2$, end-capped), HSS C18 SB, selectivity for bases (1.74 $\mu mol/m^2$, non-end-capped), BEH C18 (3.0 $\mu mol/m^2$, end-capped), BEH Shield RP18 (3.38 $\mu mol/m^2$, polar embedded group with C12 ligand, end-capped), BEH Phenyl (phenyl ligand, 2.8 $\mu mol/m^2$, end-capped).

As pointed out in the previous section, selectivity depends on the gradient slope, which is related to the available surface of the column. To perform the column comparison at constant gradient slope we adjusted the gradient flow rate as shown in eq 5 (0.2 mL/min for BEH and 0.25 mL/min for HSS columns, 50 min gradient).

Retention coefficients calculated for selected column chemistries were compared to BEH C18 130 Å data in Figure 8. Straight lines in the graphs represent correlation between the same BEH C18 and therefore divide the graph into two parts. Offset of retention coefficients from this line reflects the differences in overall retentivity of sorbents compared against BEH C18, while the scatter indicates the differences in selectivity. Figure 8 A suggests that BEH C18, HSS C18, and HSS C18 T3 sorbents have similar selectivity, but the retentivity of HSS C18 and in particular HSS T3 sorbents is greater. Figure 8B illustrates that HSS C18 SB sorbent provides for stronger retention with basic H, K, and R residues. An overall greater retentivity of this sorbent is reflected as elevated slope versus the straight line. Interestingly, Y residue (thyrosine) appears to have stronger interaction with HSS C18 SB sorbent.

As one expects, the sorbents that are chemically distinct from C18 such as BEH Phenyl, and BEH Shield RP18 (polar embedded group) exhibit noticeably different selectivity compared to BEH C18. The retention coefficients of residues with planar aromatic (F, W, in part Y) moieties were elevated at BEH Phenyl (Figure 8C). We suspect that $\pi-\pi$ interaction is responsible for the relative differences of these residues. Interestingly, proline residue value (P, cyclic structure) is also relatively elevated on BEH Phenyl sorbent. The retention contribution of basic residues K and R decreased compared to BEH C18, suggesting less efficient ion-pairing interaction on BEH Phenyl (adsorption of TFA on phenyl sorbent is less efficient compared to BEH C18). We speculate that in case of histidine (H) the contributions for of elevated retention for planar cyclic moiety and less efficient ion-pairing cancel each other.

Figure 8D illustrates overall similar retentivity of BEH C18 and embedded polar group BEH Shield RP18 sorbents. However, clear differences were observed for Y and W residue values. It is known that polar embedded sorbent (carbamide group) provides for H-bonding interactions and have increased affinity for phenol-like structures (such as thyrosine residue). The improved retentivity on tryptophan may be explained similarly by H-bonding interaction.[33]

Limitations of the Retention Coefficients Method. The proposed method of utilizing peptide retention coefficients for investigation of peptide separation selectivity under various chromatographic conditions is useful. However, one needs to be aware of method limitations. (1) The retention coefficient values are only as valid as the prediction model used for their calculation and depend on the quality of experimental data used for model training. One have to be aware that retention data acquired on one LC system may differ between laboratories because of the contribution of gradient delay and other instrument factors. (2) The retention behavior of certain peptides does not follow the retention models. This is probably because of the impact of secondary structure.[12] In fact, a bias from the prediction model could be utilized to discover peptides with strong secondary structure. In the present study, we have excluded ~10 peptides as outliers to avoid undesirable bias in calculated retention coefficient values. (3) We included 165 peptides and 1698 amino acid residues in this study. While this appears to be sufficient number, several residues with a low frequency occurrence are poorly represented in the data set (methionine, 17; tryptophan,

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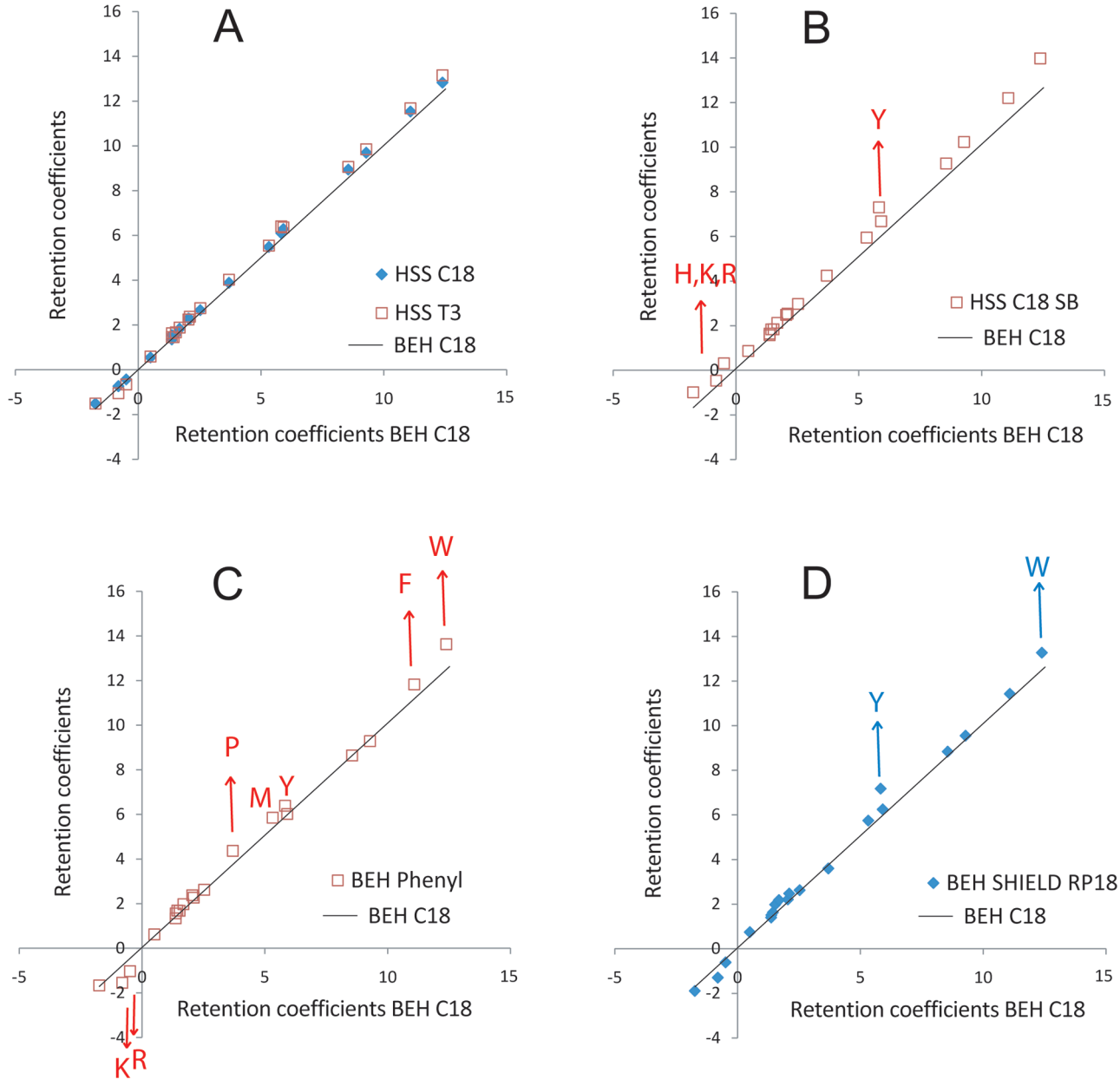

Figure 8. Correlations between amino acid residues retention coefficients calculated for selected stationary phases. BEH C18 versus HSS C18 and HSS T3 columns (A); BEH C18 versus HSS C18 SB (B); BEH C18 versus BEH Phenyl (C); and BEH C18 versus BEH Shield RP18 (D). The solid line represents correlation between two identical BEH C18 data sets; the line has slope 1 and intersect at 0.

17; cysteine, 32 residues; see Supporthing Information Table 3). However, the RSD values for the take-one-out experiment for these residues do not show an elevated level of variability (Supporting Information Table 3).

CONCLUSIONS

The following conclusions can be made from the presented results: (1) Calculated retention coefficients are useful for elucidating the rationale of peptide retention behavior. (2) The concentration of the ion-pairing reagent (TFA) predominantly affects charged amino acid residues (H, K, R). Co-eluting peptides differing in charge can be resolved by adjusting the ion-pairing concentration. (3) The presence of P, V, I, and L residues cumulatively tend to enhance peptide retention at elevated temperatures, while Y and W aromatic residues have the opposite effect. The remaining residues are less responsive to temperature changes. The retention contribution of H, K, and R residues also decreases with temperature, probably because of a decrease in ion-pairing efficiency (lower TFA adsorption at the C18 surface). (4) The gradient slope has a strong impact on peptide separation selectivity for peptides of different length (MW). This is caused by the relationship between S (see eq 6) and the molecular weight of peptides. The relative retention of peptides that have the same length does not change significantly with gradient slope. (5) When comparing the selectivity of columns, one needs to consider not only the column dimensions, but also the sorbent surface area, especially for columns packed with sorbents with greatly different available surface areas (300 Å versus ~100 Å sorbents, superficially porous sorbents, etc.). The 300 and 130 Å C18 sorbents of

identical surface chemistry appear to have similar selectivity when the gradient slope is scaled in proportion to the available sorbent area.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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