Solvent selectivity and strength in reversed-phase liquid chromatography separation of peptides

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ABSTRACT

A set of tryptic peptides was analyzed in reversed-phase liquid chromatography using gradient elution with acetonitrile, methanol, or isopropanol. We used these retention data as training sets to develop retention prediction models of peptides for the three organic eluents used. The coefficients of determination, R^2, between predicted and observed data were approximately 0.95 for all systems. Retention coefficient values of twenty amino acids calculated from a model were utilized to investigate differences in separation selectivity between acetonitrile, methanol, or isopropanol eluents. The experimentally observed difference in separation selectivity appears to be a complex interplay of multiple amino acids, each contributing to a different degree to overall peptide retention. While retention contribution of hydrophilic amino acids was higher in methanol than acetonitrile, peptides containing aromatic amino acids (tyrosine, phenylalanine, tryptophan) exhibit relatively lower retention in methanol compared to acetonitrile. The differences between acetonitrile and isopropanol eluents were less pronounced. We also compared the relative elution strength of the three organic eluents for peptides. The relationship between the elution strength of two solvents is not linear, rather it was best fitted by a cubic polynomial function. Three solvents can be arranged in the order of increasing elution power as methanol<acetonitrile<isopropanol. The equations for relative solvent strength conversion were proposed.

Keywords: Peptides; separation selectivity; eluotropic strength; reversed-phase; method transfer; isoeluotropic conditions

1. Introduction

The development and commercialization of protein based pharmaceuticals requires reliable analytical methods. One of the tools for protein characterization and quality control is peptide mapping. Peptide mapping is typically performed using reversed-phase liquid chromatography (RP LC) with UV spectroscopic [1-5] or mass spectrometric detection [6-10], often a combination of both. Since the enzymatic digestion of proteins can generate between tens and hundreds of peptides, highly efficient columns [7] and proper method development [5] are utilized to achieve desirable peptide resolution. To this end, the impact of sorbent, mobile phase, and mobile phase additives on peptide separation selectivity has been thoroughly studied over the past thirty years [11-14].

The general interest in understanding the retentivity and selectivity in RP LC dates back to the commercial introduction of C18-functionalized sorbents [15,16]. It was recognized that both the stationary phase and the mobile phases employed will affect the separation selectivity [17-19]. Altering elution solvents, e.g. switching from acetonitrile (MeCN) to methanol (MeOH) or tetrahydrofuran (THF) was seen as a tool to alter not only solute retention (due to the differing elution strength of each solvent), but also the relative retention of a mixture of solutes, and thus altering their resolution [20-22]. Recent acetonitrile shortages revived the interest in alternative eluents in RP LC [23]. Although the impact of different solvents on separation selectivity is complex, alternative solvent options present an attractive method development tool for both small and large molecule analyses [24].

We have studied the impact of a few key chromatographic conditions on the separation of peptides in several publications, under both RPLC conditions [14,25,26] and HILIC conditions [27]. In these, we applied the peptide retention prediction model, which assumes that the retention of peptides depends largely on the contribution of the peptide building blocks - its amino acid residues. Several alternative peptide retention models have been proposed, with the authors of each using a peptide teaching set to calculate the retention coefficient (RC) values for 20 amino acids [28-31]. While the absolute values of retention coefficients differ between reports (due to different LC conditions), their relative values correlate rather well (typically $R^2>0.9$), despite the differences in LC conditions and peptides used for retention data acquisition [32-34].

More recently we illustrated that amino acid retention coefficient values reflect the strength of ion-pairing interactions, as well as the impact of temperature on peptide retention [14,25]. The retention coefficients also serve as tools for the investigation of differences between types of RP LC columns or HILIC sorbents [27]. This suggests that retention coefficients can be employed to investigate the chromatographic interactions in a manner similar to that done in both the linear free energy relationship model (LFER) [35-38] and the hydrophobic-subtraction model (HSM) [17,39].

The aim of the present work is to utilize the retention coefficient method to study the impact of organic eluent choice on the separation selectivity of peptides. We selected acetonitrile, methanol, and isopropanol as they are most commonly used for peptides separations. The obtained data were also useful for comparison of the eluotropic strengths [20,21] of these solvents.

2. Theory and background

Many semi-empirical models have been proposed for the prediction of peptide retention time in RP LC. Early models assumed simple additivity of retention contributions of the amino acid residues in the peptide sequence [28,29]. Later models recognized that the contribution of the summed retention coefficient must be corrected for the peptide length [40]. These later models typically included a logarithm function of peptide length to linearize the predicted versus experimental retention relationship [30,41-43]. We proposed a similar model, as shown in equation 1 [25].

$$t_r = \left(1 - const \cdot \ln L\right) \cdot \left(b_1 \cdot \#AA_1 + b_2 \cdot \#AA_2 + b_3 \cdot \#AA_3 + ...b_{20} \cdot \#AA_{20} + b_0\right) \quad (1)$$

The retention time t_r of a given peptide is predicted from the sum of the retention coefficient of each amino acid (of the twenty used in the model) b_i multiplied by the number of occurrences of each amino acid $\#AA_i$ in the sequence. This sum is then linearized by peptide length L ($L=\Sigma\#AA_i$ in the sequence). The values of b_i, b_0, and *const* are obtained from multi-linear regression of the measured retention time of each peptide in the training set against their known sequence. The retention coefficients b_i represent the interaction strength of each type of amino acid residue with the stationary phase for a given set of chromatographic conditions.

While the model derived here has no direct connection to the linear-free-energy relationship model [35,44] (equation 2) or its modified form, the hydrophobic subtraction model [17,45] (equation 3), the manner in which they are both derived (multi-linear regression of measured data) and implemented in a predictive fashion are strikingly similar. In LFER and HSM the analyte retention log k (k = retention factor) is calculated as a sum of the retention contributions of "retention coefficients". Although the coefficients are termed differently in LFER and HSM, they loosely represent hydrophobicity, hydrogen bonding, ionic interactions, and steric analyte-stationary phase interactions.

$$\log k = c + rQ_2 + s\pi_2^{*H} + a\sum \alpha_2^H + z\sum \beta_2^H + vV_x \quad (2)$$

$$\log k = \log k_{ref} + \eta'\mathbf{H} + \sigma'\mathbf{S} + \beta'\mathbf{B} + \alpha'\mathbf{A} + \kappa'\mathbf{K} \quad (3)$$

The detailed description of symbols in equations 2 and 3 is beyond the scope of this paper; the subscribed parameters Q, π, α, β, V (LFER) and η', σ', β', α', κ' (HSM) are solute related, while r, s, a, z, v (LFER) and **H**, **S**, **B**, **A**, **K** (HSM) are column related parameters. Please note that certain symbols in equation 2 were changed from the convention found in literature [35,44], since the originals are used in different meaning throughout this publication. The solute parameters must be known (or estimated) [45,46], while column parameters are fitted using multi-regression analysis using the experimental log k values of a training set of solutes. The solute and column parameters together represent an interaction strength of five different forces, which cumulatively govern solute retention on an LC column.

Similarity between LFER/HSM (equation 2 and 3) and retention coefficient methods (equation 1) becomes more apparent when considering that all methods utilize retention data for a training set of solutes, multi-linear regression, and all can serve as retention prediction models.

Besides similarities between equations 1-3, there are also important differences. The primary goal of LFER/HSM experiments is to understand retention forces in chromatography, and their methods are often utilized for describing column similarities or dissimilarities [17]. In contrast, the peptide retention prediction method described in equation 1 is solely used to predict peptide retention. This simple endpoint can make it quite useful in proteomic research, where it can rapidly eliminate false positive identifications [25,26,31,42]. The method of retention coefficients (equation 1) is also simpler than LFER/HSM in that it does not require upfront knowledge of solute descriptors and considers only the number of amino acid residues present in the sequence (equation 1). In this sense the method is somewhat limited in the information that it provides. Retention coefficients b_i represent the interaction strength with which the amino acid residues interact with the stationary phase. It is not possible to distinguish between hydrophobic, hydrophilic, ionic and other interactions because all amino acid residues contribute to those interactions to some extent. Luckily, the amino acids represent a diverse range of physiochemical properties. For example: arginine, lysine, and histidine residues in peptide sequence are basic; aspartate and glutamate are acidic; valine, leucine, isoleucine, and proline are hydrophobic with an aliphatic side chain; phenylalanine, tyrosine, and tryptophan are hydrophobic with an aromatic side chain; asparagine, glutamine, and glycine are strongly hydrophilic; while remaining amino acid residues are moderately hydrophilic/hydrophobic. These properties help to interpret the value of retention coefficients b_i in meaningful ways, as we have shown in previous studies [14,27], in spite of the fact that the contribution of amino acid residues to retention is to a certain extent affected by nearest neighbor effects [47,48], residue position in the sequence [31], or terminal group [49].

3. Experimental

3.1. Materials and reagents

Formic acid (FA), >99%, was purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol (MeOH), acetonitrile (MeCN) and isopropanol (IPrOH) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). A Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare deionized water (18 MΩ cm) for HPLC mobile phases. MassPREP™ protein tryptic digestion standards of Enolase, alcohol dehydrogenase (ADH), Phosphorylase b, Hemoglobin, and bovine serum albumin (BSA) were obtained from Waters (Milford, MA, USA). Non-tryptic peptides in the study were either purchased from Sigma; custom peptides were synthesized by Biomatic Corporation (Cambridge, ON, Canada).

3.2. LC-MS instrumentation, columns, and conditions

Chromatographic experiments were carried out using an ACQUITY H-class UPLC system. Mobile phases were mixed by quaternary pump where A was water, B acetonitrile, C methanol, and D isopropanol. All water and organic solvent contained 0.1% (v/v) FA. Gradients started from 0% of organic mobile phase and reached the final percentage of organic in 25 minutes; the final percentage for each experiment is listed in Table 1. The chromatographic column and conditions were as follows: 50 × 2.1 column packed with 1.7 µm BEH C_{18} sorbent. Flow rate was 0.2 mL per minute, temperature 40 °C.

Synapt MS Q-Tof instrument (Waters, Milford, MA, USA) was utilized to record retention time and verify the identity of tryptic peptides by accurate mass. Instrument was tuned to a mass resolution of 20,000 with an average mass accuracy below 5 ppm. Capillary voltage was set to 3.5 kV, cone to 35 V, and extraction cone to 2V. Source temperature was 100 °C, desolvation temperature 250 °C, cone gas flow was 10 L/Hr, and desolvation gas flow was 550 L/Hr. Scan time was 0.3 seconds with an interscan time of 0.02 seconds. [Glu1]-Fibrinopeptide B was used for LockMass mass correction, 1 second scan was acquired every 30 seconds.

BioPharmalynx v 1.3 software (Waters) was used to process the peptide maps and extract peptide retention data from LC-MS chromatograms.

3.3. Peptide retention prediction and amino acids retention coefficients

The peptide retention prediction algorithm in equation 1 was described in earlier publications [14,25]. Peptides used as training set originated by tryptic digestion of the following five proteins: Enolase (Swiss prot accession number P00924), alcohol dehydrogenase (ADH, P00330), Phosphorylase b (P00924) Hemoglobin (alpha-P01966, beta-P02070), and bovine serum albumin (BSA, P02769). All proteins with the exception of Enolase were reduced and alkylated prior to digestion; samples were HPLC purified after digestion. The selected training set contained 214 peptides.

Retention times obtained in LC experiments were recorded and processed in MatLab (MathWorks, Natick, MA, USA) using a multi-regression fit to calculate the retention coefficients b_i which provide the best linear correlation between experimental and calculated retention times. The value of fitted regression constant *const.*(equation 1) was 0.21; a list of optimized retention coefficients b_i is given as Supplemental data S1 along with experimental retention times, calculated retention times, and retention time calculator spreadsheet.

Table 1
Gradient conditions used in experiments.

Experiment description	A % [a] Water	B % [a] MeCN	C % [a] MeOH	D % [a] IPrOH
MeCN	50	50	0	0
MeOH	25	0	75	0
IPrOH	60	0	0	40
MeCN:MeOH, 1:1	37.5	25	37.5	0
MeCN: IPrOH, 1:1	55	25	0	20
MeOH: IPrOH, 1:1	42.5	0	37.5	20
MeCN:MeOH, 3:1	43.75	37.5	18.75	0
MeCN:MeOH, 1:3	31.25	12.5	56.25	0

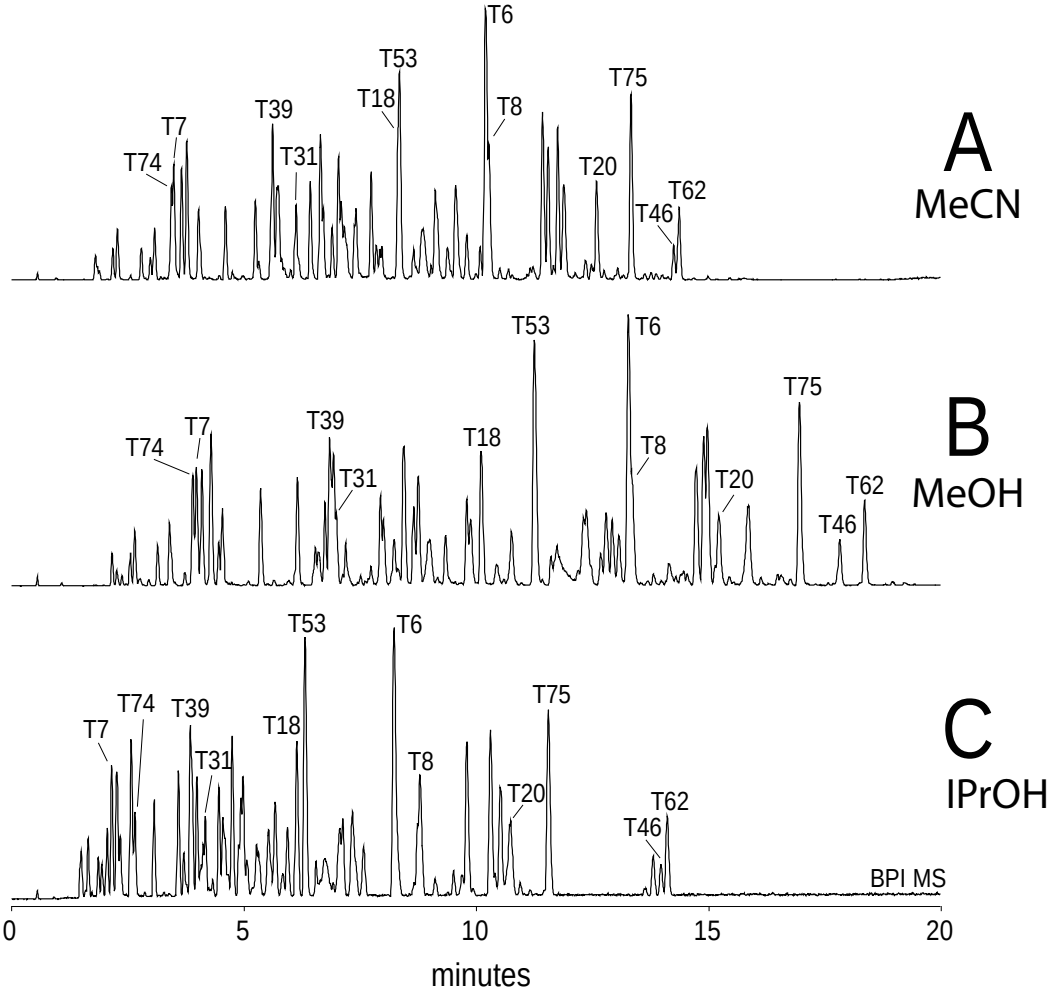
[a] Final percentage at the end of gradient. Gradient starts at 0 % organic eluent (100% aqueous). The final percentage of organic solvents is reached at 25 minutes after the start of the gradient. For other conditions see Experimental section.

4. Results and discussion

4.1. Peptide separation selectivity.

Chromatograms in **Fig. 1** show the LC-MS analysis of BSA tryptic digest with three different mobile phase eluents – acetonitrile, methanol and isopropanol. Selected peptides were annotated to illustrate the selectivity differences between three different mobile phases. For example, peptides T18 and T53 co-eluted in the acetonitrile gradient, while they are completely resolved under methanol conditions, and are just barely resolved in the isopropanol gradient. Another pair, peptides T6 and T8, is well resolved only under isopropanol conditions, and unresolved in both the acetonitrile and methanol gradients. Selectivity changes appear to be rather complex, and cannot be meaningfully interpreted by analyzing the retention data in chromatograms. Therefore we employed the method of retention coefficients.

Fig. 1. RP LC separation of tryptic digest of bovine serum albumin using acetonitrile (A), methanol (B), or isopropanol (C) as organic eluent. For chromatographic conditions see experimental. MS signal intensity was similar for all three solvents.



4.2. Organic eluent strength and retention data linearization.

Because the value of retention coefficients depends on the retention times of the training set, it is important to maintain similar elution windows for the training set across all three organic solvent experiments. This is complicated by the varying eluotropic strength of the three solvents employed: methanol is a weaker eluent than acetonitrile, and isopropanol is generally stronger than both. To compensate for this, a few preliminary separations were performed, allowing for the appropriate adjustment of the final organic eluent strength, as shown in Table 1. However, **Fig. 2a** shows that this solution was only partially effective. Retention data of peptides in the acetonitrile experiment were chosen as the reference set (data for x-axis) and all three experiments were plotted on the y-axis of **Fig. 2a**. By doing so, the MeCN data represent a straight line with slope of 1 (correlation of MeCN data with identical data set) against which the other two experiments are compared. It is clear that weakly retained peptides in the MeOH gradient have similar retention to those in the MeCN system (appear close to the line in **Fig. 2a**), but after ~5 minutes the peptides are eluted increasingly later than in acetonitrile. The isopropanol experiment shows the opposite trend – weakly retained peptides are eluted earlier than in acetonitrile, and only for later eluting peptides are the retention times comparable between the IPrOH and MeCN gradients. Apparently, the relationship between elution strength of acetonitrile, methanol and isopropanol is non-linear. It is not possible to simply adjust the final concentration of the organic solvent in the linear gradients employed (Table 1) and achieve elution of all peptides in the same retention window.

To highlight the differences in elution strengths of the three organic eluents, we converted the time scale in **Fig. 2a** into a percentage of organic eluent at the point of peptide elution. Given the sensitivity of peptide retention behavior to mobile phase strength (a feature common to large molecules) it is reasonable to assume that below a certain "critical" mobile phase strength, the peptides are strongly retained, while above this "critical" mobile phase strength they become unretained. We assumed that this "critical" mobile phase strength was equal to the percentage of organic eluent at the point of elution. For steep gradients used in the experiments here, this is an acceptable assumption [50].

Fig. 2b compares the elution strength using acetonitrile as the reference experiment (MeCN data are shown as straight line with slope 1). **Fig. 2b** clearly shows that isopropanol is a stronger eluent and methanol a weaker eluent than acetonitrile. The mutual relationship with MeCN was fitted with cubic polynomial function. Similar relationships for other experiments described by Table 1 are provided as Supplemental data S2.

As stated above, our inability to maintain a uniform range of peptide elution for all experiments complicates the calculation of retention coefficients [14,27]. Because the retention times of peptides do not adhere to the same elution time window for each experiment (**Fig. 2a**), we resorted to further normalization of each data set. The retention time window was arbitrarily broken into four "linear" portions; 0-5, 5-10, 10-15, and 15-20 minutes. Linear slope and intercept in those sections were adjusted such that the entire 0-20 minute time span was linearized (**Fig. 2c**). The details for linearization are provided as Supplemental data S3.

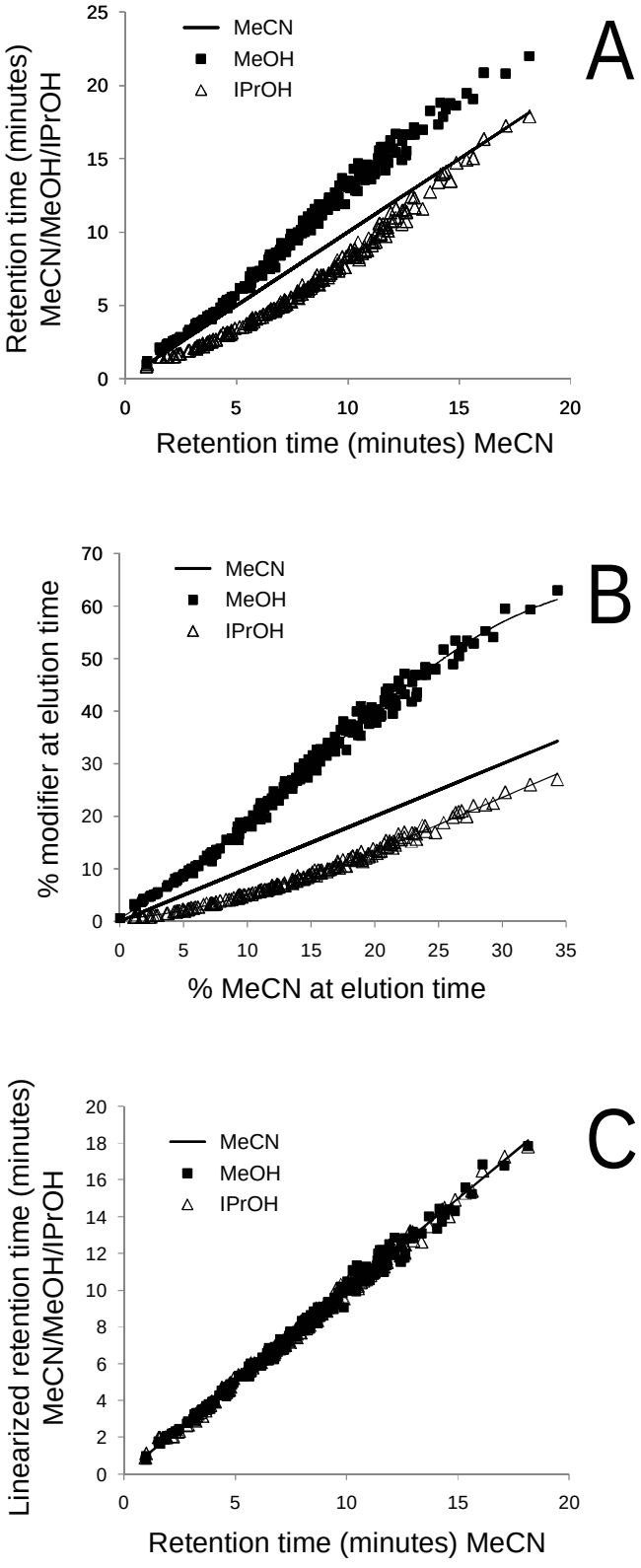


Fig. 2. Comparison of the retention of 214 tryptic peptides in RP LC using acetonitrile, methanol, and isopropanol as eluents. (A) Correlation between retention times in acetonitrile based and methanol/isopropanol based gradient systems (gradient conditions in Table 1), (B) similar correlation represented in percentage of organic mobile phase at point of elution, (C) retention time data after linearization.

It is important to state that linearization does not alter the relative retention of peptides. Peptides with a certain amino acid composition will remain to be retained relatively stronger than others. Therefore the retention coefficients will reflect the contribution of each amino acid to peptide retention regardless of normalization. However, the absolute value of each amino acid's contribution to retention will be altered. The retention coefficients calculated for linearized retention time data sets are provided as Supplemental data S4. They can be compared with non-linearized RCs given in Supplemental data S1.

4.3. Selectivity differences of organic solvents reflected in retention coefficients.

Retention coefficients reflect the retention contribution of amino acid residues present in peptide sequences. We have previously shown that this could be used to illustrate the peptide retention mechanism and lend insight into method development options. For example changing the concentration of acidic ion-pairing agents affected mainly the RC values of basic amino acids like arginine, lysine and histidine. Increases in the separation temperature increased RC values of isoleucine, leucine, proline, and valine [14].

We compare the retention coefficients for each amino acid (calculated via linearized data sets) for all three solvents in **Fig. 3a**. Similarly, acetonitrile data were used as a reference set (line with slope 1and intercept 0). Retention coefficients for methanol or isopropanol positioned above or below the line have greater or lower retention

contribution for these amino acid residues, respectively. The differences in RCs for methanol and isopropanol compared to their acetonitrile analogues are subtle. To better visualize the impact an alternative organic eluent had on each individual amino acid's RC, we calculated the difference between them and their acetonitrile RC and plotted them in **Fig. 3b**. Interestingly, even though methanol and isopropanol are both alcohols, they don't always show similar trends in RC changes.

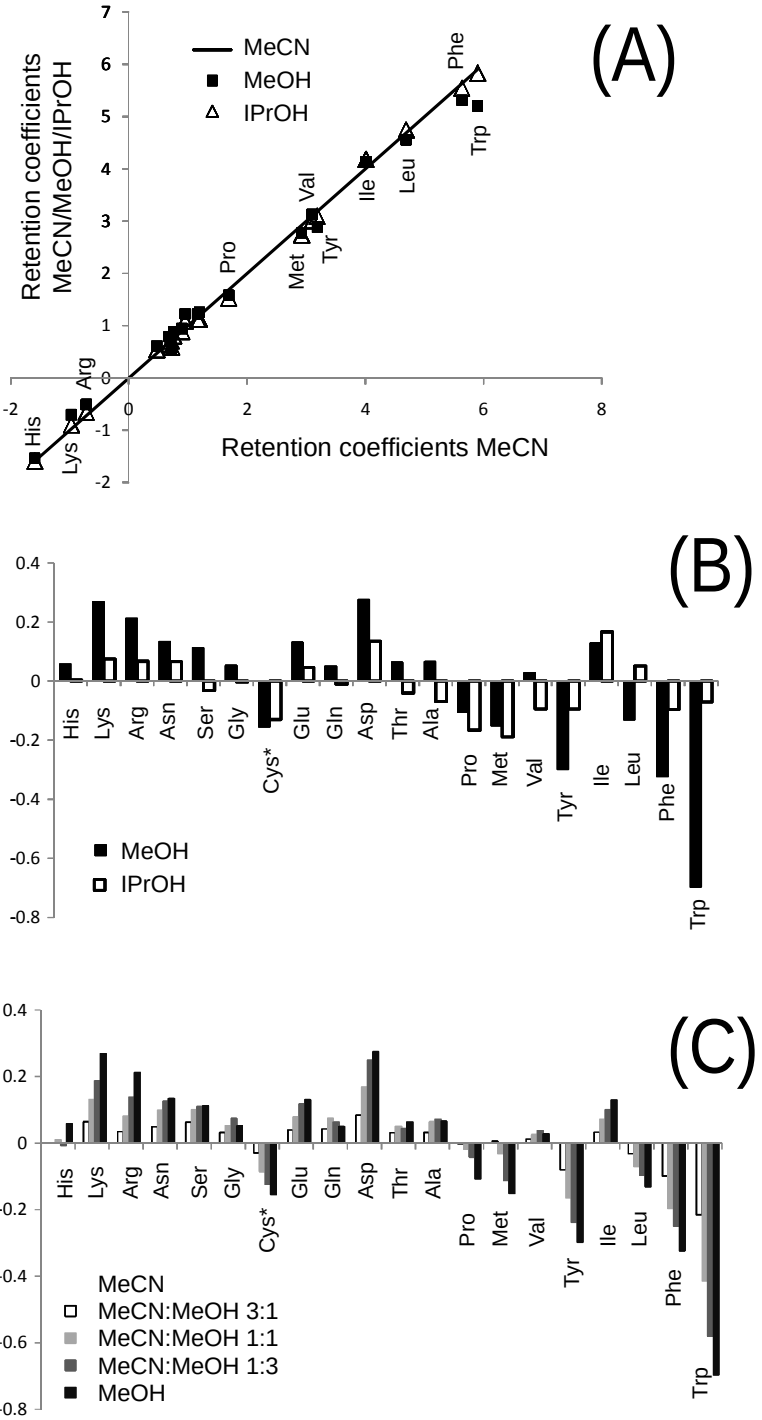


Fig. 3. (A) Comparison of amino acid retention coefficients optimized using a training set of 214 tryptic peptides analyzed using acetonitrile, methanol, and isopropanol as eluents. Retention coefficients represent relative contributions of 20 amino acids to RP LC peptide retention. (B) The differences between retention coefficients in acetonitrile and pure methanol/isopropanol experiments. (C) The difference between retention coefficient values in acetonitrile versus mobile phases containing mixtures of MeOH and MeCN or pure MeOH.

We needed to determine if the source of these minor changes in retention coefficients are tied to organic modifier type, or just artifacts of regression calculation. Therefore we performed an additional series of experiments, wherein the acetonitrile was gradually replaced with methanol in the organic mobile phase (see Table 1) until the mobile phase consisted of methanol only. Retention coefficients from those experiments were compared in **Fig. 3c**. Acetonitrile conditions in this plot are represented by the x-axis (y = zero). As the mobile phase content increases in methanol, values of retention coefficients show clear trends suggesting that the RC differences shown in **Fig. 3b** cannot be attributed to noise alone.

Fig. 3c shows two distinct trends. First, all three aromatic amino acid residues

(tyrosine, phenylalanine and especially for tryptophan) have relatively lower retention contribution in methanol compared to acetonitrile. This becomes more obvious as the methanol content in mobile phase increases. Second, basic residues (lysine, arginine), and acidic amino acid residues (aspartate, glutamate) show relatively greater contribution to retention in MeOH conditions. While remaining RCs show smaller differences between MeCN and MeOH, overall it appears that hydrophilic amino acid residues contribute relatively more to retention in MeOH, while hydrophobic residues relatively less. Intriguingly, isoleucine and leucine, positional isomers, behave very differently in MeOH and MeCN conditions.

It was of interest to confirm whether the differences in RCs shown in **Fig. 3** translate into observable chromatographic behavior of peptides. **Fig. 4** shows the correlation between acetonitrile retention times (x-axis) and the difference between retention in methanol and acetonitrile (y-axis) for the tryptic peptides of phosphorylase B (peptides not in the training set). Since the retention times were longer under MeOH conditions than MeCN (**Fig. 2a**), the trend of data points in **Fig. 4** resembles the graph in **Fig. 2a**. However, **Fig. 4** also captures the selectivity difference in peptide retention – in other words it illustrates which peptides are retained more strongly when methanol is used as the organic eluent. It is evident that peptides containing tryptophan, phenylalanine, and tyrosine are retained relatively more weakly in MeOH than MeCN; in contrast the peptides containing multiple acidic and/or basic amino acid residues are retained relatively more strongly in MeOH. **Fig. 4** confirms that calculated retention coefficients capture the reality of the very complex chromatographic behavior inherent to peptides. The observed differences in RCs are truly manifested in peptide retention behavior. Selectivity variations for peptides with on single amino acid substitution in MeOH and MeCN based mobile phases were studied by Mant and colleagues [51,52]. The effect of aromatic amino acids on peptide retention was similar as in work presented here.

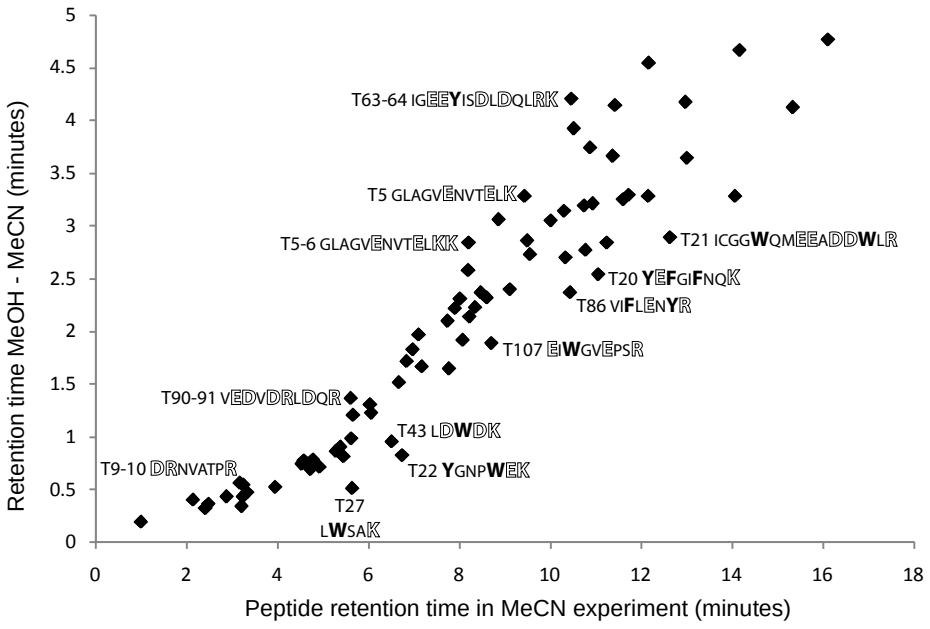


Fig. 4. Differences in peptide retentivity when using acetonitrile or methanol as the organic eluent for the RP LC analysis of the tryptic peptides of phosphorylase B. Y-axis values represent difference in peptide retention time between MeOH and MeCN mobile phases. Retention is enhanced in MeOH compared to MeCN for peptides rich in aspartic acid D, glutamic acid E, arginine R, and lysine K. Peptides containing tryptophan W, phenylalanine F, and tyrosine Y are retained relatively less in MeOH experiment. Sequences of selected peptides are indicated in the plot.

While the retention coefficient trends for isopropanol (**Fig. 3a** and **3b**) are more subtle compared to methanol, we believe they illustrate the true contribution of amino acid residues to peptide retention and highlight the differences in selectivity between IPrOH, MeOH and MeCN. Apparently, the changes in selectivity for peptides are complex; all amino acids to a certain degree contribute to the differences in peptide separation selectivity observed in **Fig. 1**. Comparison of linearized retention coefficients to those of MeCN for all experiments shown in Table 1 are provided in additional tables and plots as Supplemental data S4.

4.4. Organic eluent eluotropic strength for peptides.

Information about elution strength of organic mobile phases has practical implications in RP LC. When transferring isocratic chromatographic methods between acetonitrile, methanol or other eluents, it is useful to know their relative strength. Unfortunately, the method transfer is complicated by non linear relationship of solvent strengths, similar to those observed in **Fig. 2b**.

In the early days of reversed-phase chromatography, several authors estimated the eluotropic strengths of solvents [15,20,53-55]. Schoenmakers et al. proposed equations for conversion of solvent strengths between methanol, acetonitrile, and tetrahydrofuran, allowing for the estimation of isoeluotropic conditions (percentage of organic eluents giving approximately the same retention of analytes) [21].

Fig. 2b defines the relationship between "critical" (isoeluotropic) strength of acetonitrile, methanol, and isopropanol. The relationships in **Fig. 2b** were fitted with a cubic polynomial function, yielding equations 4 and 5, which can be used for isoeluotropic strength conversion from acetonitrile to methanol or isopropanol.

$$\%MeOH = -0.0011 \times \%MeCN^3 + 0.0454 \times \%MeCN^2 + 1.5025 \times \%MeCN + 0.4893 \quad (4)$$

$$\%IPA = -0.0003 \times \%MeCN^3 + 0.0286 \times \%MeCN^2 + 0.2063 \times \%MeCN + 0.391 \quad (5)$$

These equations are valid for peptides within the range of organic eluent concentrations used in this work (see **Fig. 2b**, x-axis range).

Supplemental data S5 spreadshet calculator is provided for strength conversion of one type of solvent to another. For example 20 % MeCN has the same elution strength as 40.1 % of MeOH and 13.5 % IPrOH. In this range (20% MeCN) the relative elution strength of acetonitrile is approximately 2-times higher than methanol, and 1.5-times lower than isopropanol.

While it is tempting to use the proposed equations 4 and 5 for prediction of isoeluotropic conditions for small molecules, it should be noted that the predicted isoeluotropic values differ significantly from the methanol-acetonitrile conversion proposed by Schoenmakers et al. [21]. According to our evaluation (data not shown) Schoenmaker's method developed for small molecules within retention range $1<k<10$ [21]) gives more fitting predictions than equations 4 and 5 presented here.

We tested the success of isoeluotropic conversion between acetonitrile and methanol (equation 4) as well as acetonitrile and isopropanol (equation 5) using 14 peptides not included in the original training set. The match between the observed percentage of organic solvent measured at isocratic experiment for k=2 and percentage calculated from equations 4 and 5 was estimated by external validation according to Martens and Næs, chapter 4 [56] (for details see Supplemental data S5). The relative prediction error was 8.2 % for methanol and 6.6 % for isopropanol for the studied 14 peptides. The Supplemental data S5 provides a calculator for isoeluotropic conversion between the solvent systems used in this study.

5. Conclusions

The retention coefficients method was applied to the investigation of peptide separation selectivity in RP LC for three different solvents - MeCN, MeOH and IPrOH. The results show that the differences in peptide selectivity when each of these solvents are used is complex and multi-faceted. Optimized retention coefficients indicate greater similarity between IPrOH and MeCN, and some difference in peptide behavior for MeOH and MeCN. Methanol containing mobile phase shows two noticeable trends: (i) Aromatic amino acid residues in peptide sequences contribute relatively less to retention than in acetonitrile conditions. This is especially significant for tryptophan. (ii) Hydrophilic amino acid residues, most notably basic (lysine, arginine) and acidic (aspartate, glutamate) ones, contribute relatively more to retention than in acetonitrile gradients. These observations could be useful for peptides LC method development. For example the resolution of co-eluted critical peptide pairs may be achieved by adding methanol into mobile phase if the unresolved peptides have different content of aromatic or hydrophilic amino acid residues.

The experimental retention data for 214 tryptic peptides were useful for the comparison of the eluotropic strength of each of the investigated solvents. The relationship between elution strength was not linear; rather, it was best described by a cubic polynomial function. While the isocratic method transfer between different mobile phases is difficult, the proposed equations for acetonitrile percentage conversion to methanol and isopropanol mobile phases could be of practical value. The elution strength of methanol is approximately 2-times weaker than acetonitrile, which in turn is 1.5-times weaker than isopropanol. More accurate prediction of mobile phase relative strength can be obtained from calculator provided as supplemental material S5.

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