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Accurate modelling of the retention behaviour of peptides in gradient-elution hydrophilic interaction liquid chromatography

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ABSTRACT

The applicability of models to describe peptide retention in hydrophilic interaction liquid chromatography (HILIC) was investigated. A tryptic digest of bovine-serum-albumin (BSA) was used as a test sample. Several different models were considered, including adsorption, mixed-mode, exponential, quadratic and Neue-Kuss models. Gradient separations were performed on three different HILIC stationary-phases under three different mobile-phase conditions to obtain model parameters. Methods to track peaks for specific peptides across different chromatograms are shown to be essential. The optimal mobile-phase additive for the separation of BSA digest on each of the three columns was selected by considering the retention window, peak width and peak intensity with mass-spectrometric detection. The performance of the models was investigated using the Akaike information criterion (AIC) to measure the goodness-of-fit and evaluated using prediction errors. The *F*-test for regression was applied to support model selection. RPLC separations of the same sample were used to test the models. The adsorption model showed the best performance for all the HILIC columns investigated and the lowest prediction errors for two of the three columns. In most cases prediction errors were within 1%.

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1. Introduction

Proteomics is a field comprising of different techniques used to identify and quantify the proteins present in cells, tissues and organisms [1]. A distinction can be made between top-down proteomics [2], where intact proteins are analysed, and bottom-up proteomics [3], where proteins are first digested to yield peptides, prior to analysis and interpretation. The identification and quantification is challenging, due to the high complexity of the sample, especially in bottom-up proteomics, and the great differences in the relative abundance of proteins in a cell proteome [4]. An indispensable analytical technique in this field is mass spectrometry (MS). However, data quality can be detrimentally impacted if many species are infused at the same time. Therefore, MS alone cannot be used to analyse complex samples, such as whole-cell lysates. For this reason, separation techniques are typically coupled to MS analysis, providing the much needed simplification of the sample prior to its introduction into the MS.

Liquid chromatography (LC) is one of the most frequently employed separation techniques, since it can be directly coupled to MS. Moreover, for common LC modes employed, little or no additional sample preparation is needed. The most commonly used LC separation mode for bottom-up proteomics is reversed-phase liquid chromatography (RPLC). In RPLC, analytes are separated based on differences in partitioning between the hydrophilic (aqueous) mobile phase and the hydrophobic stationary phase. To facilitate timely elution of strongly retained analytes from the stationary phase, the fraction of organic modifier can be gradually increased using a gradient program. However, one limitation of RPLC is the lack of separation based on the polar functional groups which are abundantly present in peptides. Therefore, a complementary technique that would be able to retain polar compounds is needed to extend the analysis of a proteomic sample. This is especially relevant for multi-dimensional separations, in which two (or three) vastly different (“orthogonal”) retention mechanisms are employed to greatly improve the separation of complex mixtures [5,6].

One method with a retention mechanism and selectivity that is very different from that of RPLC is hydrophilic-interaction liquid chromatography (HILIC). HILIC was introduced as a separation mode for polar compounds [7], but it is also used as a fractionation

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technique for bottom-up proteomics prior to a RPLC separation to decrease sample complexity [8]. Whereas hydrophobic alkyl-based stationary-phase chemistries are used in RPLC, HILIC employs a polar stationary-phase, such as bare silica, or silica modified with amide, amino or diol groups [9]. Charged stationary-phases can also be used such as silica modified with cationic groups (e.g. poly aspartamide) or zwitterionic groups (e.g. ZIC HILIC). The mobile phases in HILIC mainly comprise of non-polar organic solvents, with small percentages (e.g. 3%) of water or aqueous buffer. The exact retention mechanism is still being investigated. However, there is a general consensus that retention is based on partitioning between an aqueous layer formed on the surface of the stationary phase and the mostly organic bulk mobile phase, with electrostatic interactions (ionic interactions and hydrogen bonding) also influencing the retention [7,10,11]. The exact magnitude of the different interactions highly depends on the employed stationary and mobile phases, but also on the properties of the analyte.

The large influence on retention of the selected stationary phase, mobile-phase solvent and additives, dramatically complicates method development for HILIC separations. In order to stimulate the proliferation of HILIC, computational tools for method development are needed. Such tools generally rely on prediction of retention times with respect to the combination of stationary phase and mobile phase. Several models have been proposed for predicting the retention times of peptides, based on their amino-acid composition, sequence and conformation [12–15], assessing the chemical structure of the analyte to predict retention. However, the development of such models depends heavily on large numbers of experiments using various mobile and stationary phases.

An alternative approach is based on establishing retention parameters of (unknown) analytes using the concept of so-called gradient-scanning techniques [16]. Here, the retention times are recorded for each analyte in a few experiments under pre-set conditions and the resulting data are fed into the underlying retention model. Entirely theoretical models require a thorough understanding of the underlying retention mechanism, which is challenging for HILIC. Alternatively, (semi-) empirical models can be used to describe the data.

Computer-aided method development for HILIC has been extensively studied by several groups [17,18]. Recently, the feasibility of accurate prediction of retention times of peaks eluting before, during or after a gradient was demonstrated, using only a small number of scouting measurements [19]. Several retention models were investigated and the prediction performance was shown to depend on the type of stationary-phase chemistry and the mobile-phase components. In addition, while the method was found to have great potential for smaller molecules, such as metabolites, dyes and tea components, its application for predicting retention times of peptides proved fruitless. However, in the above study only a small number of peptide standards were included, which were not representative of the peptides typically encountered in bottom-up proteomics.

In this study, we investigate the prediction of retention times of peptides for a larger number of combinations of stationary-phase chemistries and mobile-phase additives. A more-complex sample (Bovine serum albumin digest), is used that is much-more representative of a bottom-up-proteomics sample than is a set of standard peptides. Also mass-spectrometric detection is employed. Bovine serum albumin is attractive as a bench mark sample because it is easily available and it includes a sufficient number of diverse peptides (>40). Moreover, we rigorously evaluate the contemporary tools used to assess prediction performance. Computer aided method development for HILIC has been massively restricted by shortcomings in retention modelling on certain types of columns (particularly amide) and for certain types of analytes, especially peptides. The results of the present work remove these

restrictions. In addition, the results help understand the retention behaviour in HILIC and they provide means to reduce the uncertainty in peptide identification. Finally, a number of general recommendations for HILIC separations of peptides are proposed.

2. Experimental

2.1. Materials

Milli-Q water (18.2 mΩ) was obtained from a purification system (Millipore, Bedford, MA, USA). Acetonitrile (ACN, MS grade), 2-propanol (IPA, HPLC grade) and toluene were purchased from Biosolve Chimie (Dieuze, France). Ammonium formate (AF, BioUltra; ≥ 99%) and ammonium bicarbonate (Bioultra; ≥ 99.5%) were purchased from Fluka Analytical (Buchs, Switzerland). Acetic acid (glacial) was obtained from ACROS organics (Geel, Belgium).

The following chemicals were purchased from Sigma-Aldrich (Darmstadt, Germany), bovine serum albumin (BSA, ≥96%), urea (bioreagent, ≥ 98%), dithiothreitol (DTT, ≥ 99%), iodoacetamide (IAA, ≥ 99%), trypsin (BRP), uracyl (≥ 99%), ammonium acetate (AA, for molecular biology, ≥98%) trifluoroacetic acid (TFA, ≥99%), Formic acid (FA, Analytical grade; 98%), SPE cartridges (3 mL, C18), thiourea (GR for analysis ACS) and sodium hydroxide (for analysis).

2.2. Sample preparation

The peptide samples were obtained by trypsin digestion. Denatured protein (100 μL, 10 μg/μL) in urea (6M) was reduced with DTT (5 μL, 30 mg/mL in 25 mM ammonium bicarbonate) for an hour at 37 °C. The protein was alkylated with IAA (20 μL, 36 mg/mL in 25 mM ammonium bicarbonate) for one hour in the dark at room temperature. Then 20 μL of DTT and 900 μL of 25-mM ammonium-bicarbonate solution and finally trypsin (1:30 weight ratio trypsin:protein) were added. The protein was digested overnight at 37 °C. The next day TFA (10%, 40 μL) was added to acidify the sample to pH 2–3 before desalting the peptides using SPE cartridges (C18). The peptide solution was freeze-dried and reconstituted in 80% ACN, 20% buffer (1 mg/mL) before use.

2.3. Instrumentation

The LC-MS measurements were performed on an Agilent 1100 Series LC system with a quaternary pump (G1311A), an auto-sampler (G1313A) (Agilent, Waldbronn, Germany) in combination with a Micro-QTOF from Bruker (Bremen, Germany). The electrospray ionization (ESI) parameters used were end-plate offset –500 V, capillary voltage 4.4 kV, nebuliser 1 bar, dry gas 8 L/min, dry temperature 220 °C. Compass Data analysis from Bruker was used to extract the *m/z* and retention time information. The dwell volume of the LC system was experimentally determined to be 0.81 mL and the dead time for the HILIC columns was 0.33 mL, measured using toluene and an Agilent DAD detector (1-μL flow cell, 1290 Infinity diode-array detector (G4212A)).

A system comprised of an Eksigent Ekspert nanoLC 425 (Sciex, Singapore) coupled to a TripleTOF 5600+ mass spectrometer (Sciex, Singapore) was used for MS/MS measurements for sample identification. The columns used during this investigation are listed in Table 1.

2.4. Methods

2.4.1. HILIC separation of peptides

Three different columns were chosen for the HILIC separations, W-silica (Waters), Z-silica (Zorbax) and amide. The effect of mobile phase additives on the retention and selectivity of the HILIC column was investigated using formic acid or two buffers, 10 mM

Table 1
Columns used for the separation of BSA digest.

Column	Brand and type of stationary phase	Selectivity	Designation	Dimensions (mm)	Particle size (μm)	Pore size (\AA)
1	Waters, Acquity, BEH	Amide	Amide	2.1 \times 150	1.7	130
2	Waters, Atlantis	Silica	W-silica	2.1 \times 150	3	100
3	Agilent, Zorbax, HILIC Plus	Silica	Z-silica	2.1 \times 150	1.8	95
4	Phenomenex [*] , Kinetex	RP	XB-C18	4.6 \times 150	3.5	100
5	In house packed, Magic C18 ^{**}	RP	M-C18	0.075 \times 100	5	100

^{*} Phenomenex (Torrance, CA, USA).

^{**} NanoLCMS Solutions (Oroville, CA, USA).

ammonium formate, pH 3, and 10 mM ammonium acetate, pH 6. These conditions were selected based on the MS compatibility of the volatile additives and their useful pH range (within the working pH range of the columns), and to observe the effect of using a buffer compared to only an acidic environment. At acidic pH the silanol groups present in the stationary phase will be protonated, thus minimizing electrostatic interactions. All the HILIC columns were chosen to have the same dimensions, but the particle size varied (see Table 1). Bovine serum albumin (BSA) digested with trypsin was used to provide a good range of peptides with varying properties and concentrations.

For each combination of mobile and stationary phase, six gradients were measured. Mobile-phase A was always 97% ACN with 3% water or buffer and B was 100% water or buffer. In the case of formic acid 0.3% (volume) was added to both A and B. The initial condition, isocratic 100% A was held for 0.25 min. This was followed by a linear gradient from 0% B to 40% B (amide and Z-silica column) or 50% (W-silica) in 10, 17, 30, 52, 70 or 80 min. The final condition was maintained for 1 min (amide and Z-silica) or 5 min (W-silica), after which the system was switched back to the initial conditions in 1 min. The equilibration time was set to 30 min (amide) or 50 min (Z-silica and W-silica). The flow rate was 0.2 mL/min. The sample was dissolved in 80% ACN 20% buffer with a concentration of 1 mg/mL. The injection volume was 5 μL for the three shortest gradients and 10 μL for the three longest gradients to overcome the problem of dilution.

In order to identify the peptides in the gradient runs, the same sample was measured on C18 column 75 μm ID 10 cm length (M-C18) coupled to a high-resolution mass spectrometer. The peptides identified using MS/MS were compared to peptides measured on the microQTOF and were considered a match if the m/z value was within 0.02 of the MS/MS identified peptides. A list of 15 peptides was constructed by comparing measurements with all stationary-phases and seven of these were selected to show the influence of mobile-phase additives due to their similar intensity.

The separation method was developed initially for the amide column and then adapted for the silica columns. A scouting gradient from 97% ACN to 40% ACN was used and the final solvent composition was adjusted to improve the peak spreading. The equilibration time was initially set to 20 min and then increased to 30 min. With this later duration significant variations were observed in the retention times for triplicate measurements. Therefore the column was considered to be well equilibrated. Changes had to be made during measurements for the other columns. In the case of the Z-silica column, a peak shift was noticed between triplicate measurements. Therefore, the equilibration time after each run was increased. For the W-silica column, carry-over and peak shifting were observed, and therefore the final percentage of aqueous eluent was increased and the equilibration time was chosen the same as for the Z-silica column. The equilibration time has previously [20] been correlated to the water uptake capability of the stationary phase, with faster equilibration corresponding to higher water uptake. The amide stationary phases were reported to have the highest water uptake followed by bare silica, which was in line with our observations.

2.4.2. RPLC separation of peptides

BSA digest was separated on an RPLC column using the same linear gradient lengths as for HILIC, with 0.1% FA in water and with 10 mM ammonium formate pH 3 buffer as mobile phase A and 80% ACN mobile-phase B. The flow-rate used was 0.4 mL/min since the internal diameter was larger than that of the HILIC columns (4.6 mm). The gradient ran from 5% to 60% B, followed by a 10 min equilibration. We observed a slight decrease in retention when using buffer. However, the resolution between some peptides was increased.

2.5. Data processing and retention modelling

The data were processed using Compass Data Analysis from Bruker and PIOTR [21]. A longer gradient (52 min or 70 min) was chosen from each data set and the dissect option was used to obtain the m/z and retention-time list. The m/z values were assigned to a peptide sequence using MS/MS measurements with the same sample on the Sciex TripleTOF 5600+ MS. The MS confidence of identification was chosen to be 95% or above and no modifications were considered. The observed ions in the HILIC measurements were matched to a peptide sequence if the value was within 0.02 m/z . Once the longer gradient was assigned, the same peptide list was searched in the other gradients using extracted-ion chromatograms (EIC). A unique list for all the columns of 15 peptides was obtained after processing all the data sets. Peak lists consisting of the retention time of each peptide for each gradient experiment were prepared for each column. These data were supplied to the PIOTR program to fit the different retention models. The computational approach has been explained previously [19,21]. Briefly, the retention models were used to calculate the model coefficients and the goodness-of-fit values, to compute the F-test of regression, and to predict retention. For the Z-silica and W-silica columns the 10-min gradient gave rise to a high degree of co-elution, which hindered peak detection and diminished the accuracy of the extracted retention times. Therefore, only five gradients were used in the analysis for these columns.

3. Results and discussion

3.1. Effect of additives in HILIC separation of peptides

Among the conditions explored – three different columns (amide and two B type silica stationary phases) and three mobile-phase additives (0.3% formic acid, 10 mM ammonium acetate pH 6, 10 mM ammonium formate pH3) – not all chromatograms showed good chromatography, in terms of retention and peak shape. Therefore, we first set out to establish the optimal combinations of columns and additives (Fig. 1). For this purpose, we compared the peak width, peak intensity and elution window for each of the conditions (see Table 2). The performance of the amide column was good with all three mobile-phase additives. When using a buffer (ammonium acetate and formate), slightly sharper peaks were obtained. However, the intensity decreased by one order of magnitude. Retention was also affected by the use of buffers.

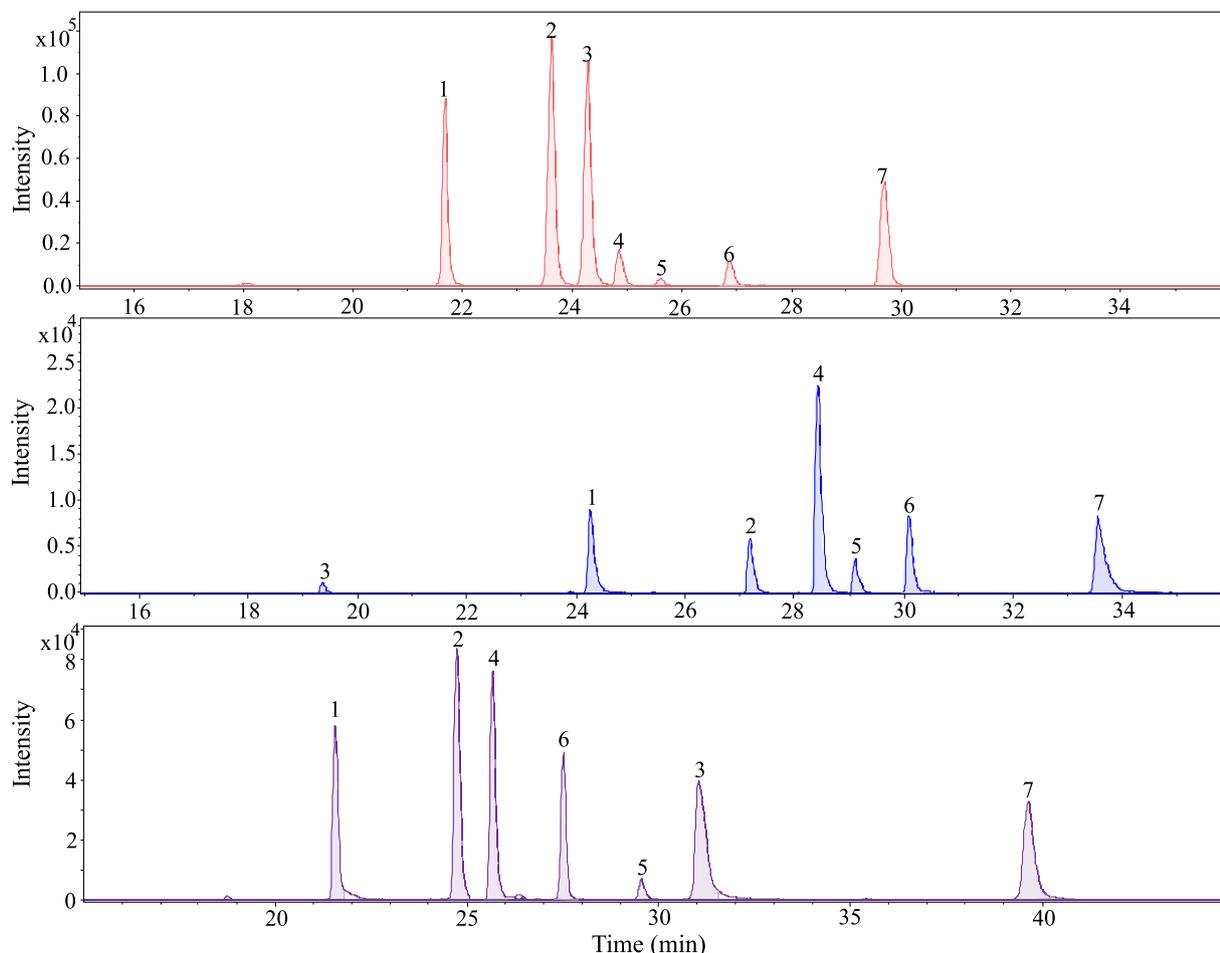


Fig. 1. Optimal conditions for the separation of BSA digest on the amide column (red, top), Z-silica column (blue, middle), W-silica column (purple, bottom). For details see text. Analyte peptides: 1. $m/z=1002.5830$, 2. $m/z=740.4014$, 3. $m/z=509.2956$, 4. $m/z=789.4716$, 5. $m/z=689.3729$, 6. $m/z=922.4880$, 7. $m/z=571.8608$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

Seven peptides that were used to assess the optimal mobile-phase additive for the HILIC separation. The 30 min gradient duration measurements were used. FA=formic acid, AF=ammonium formate, AA=ammonium acetate.

Column/additive	Max t_R (min)	Min t_R (min)	Retention window (min)	Average peak height (counts) $\times 10^3$	Average peak width (min)
Amide FA	29.70	21.71	7.99	56.3	0.133
Amide AF	31.54	24.49	7.04	6.74	0.114
Amide AA	32.60	15.86	16.74	20.2	0.124
W-silica FA	23.18	17.27	5.90	83.7	0.185
W-silica AF	28.89	21.64	7.25	24.2	0.157
W-silica AA	39.64	21.60	18.04	49.5	0.201
Z-silica FA	28.41	19.86	8.55	9.91	0.225
Z-silica AF	34.97	25.03	9.93	39.2	0.125
Z-silica AA	37.21	24.33	12.88	27.9	0.223

Formic acid gave rise to the lowest retention, followed by ammonium acetate and then ammonium formate (Fig. S1). This could be explained by an expansion of the water layer when using buffers. Dinh et al. [20] showed that when ammonium acetate (5–50 mM) was added to the ACN/water mobile phase, the ions were adsorbed on the surface of the stationary phase. The authors observed an increase in the water layer of up to 50% for bare silica phases. The elution order was also found to vary with varying conditions. Due to the higher signal intensity and adequate resolution, formic acid was chosen as the optimal additive for the amide stationary phase.

The Z-silica column required a buffer for the elution and separation of the peptides (Fig. S2). Therefore, the separations using formic acid as additive were not considered for modelling. The elution order was the same with the two buffers. However, with

ammonium acetate the peaks were tailing and the resolution was decreased. At pH=6 a significant fraction of the silanol groups will be dissociated, whereas some groups (arginines, lysines and histidines) on the peptides may still be positively charged. This creates a strong ion-exchange contribution to a mixed retention mechanism, which may explain the tailing. Therefore, ammonium formate was chosen as the optimal additive for the Z-silica column.

Finally, also the separations using the W-silica column required a buffer (Fig. S3) [22]. Good peak shapes were obtained with both buffers. The elution order was also the same, with the exception of two peptides (3 and 5), which showed a decreased retention with ammonium formate. Both peptides had a theoretical pI of about 9.7 (basic). McCalley showed previously that for this silica column the retention of basic solutes increased when increasing the pH

from 3 to 6 [23]. The number of negatively charged silanol groups at the surface increases at higher pH, providing stronger interaction with the positively charged solutes. Ammonium acetate (pH 6) gave higher retention and a better resolution. Hence, it was chosen as the optimal buffer.

3.2. Retention modelling

The models used to fit the data were the exponential, mixed-mode, adsorption, quadratic and Neue-Kuss models.

The exponential model has been shown to fit RPLC data [24] and has the following form

$$\ln k = \ln k_0 - S \phi \quad (1)$$

where k_0 represents the extrapolated retention of an analyte at $\phi = 0$ (100% water in case of RPLC) and S the so-called "solvent-strength parameter", describing the change in retention with increasing concentration (volume fraction) of strong solvent (ϕ).

The adsorption model is typically used to describe normal-phase separations [25].

$$\ln k = \ln k_0 - n \ln \phi \quad (2)$$

Here, n is meant to represent the ratio between the surface occupied by the analyte molecules and the molecules of strong solvent.

The mixed-mode model is a combination of the previous two models and is thought to take into account both partitioning and adsorption [26].

$$\ln k = \ln k_0 + S_1 \phi + S_2 \ln \phi \quad (3)$$

The quadratic model was developed to characterize retention over a larger range of mobile-phase compositions [27].

$$\ln k = \ln k_0 + S_1 \phi + S_2 \phi^2 \quad (4)$$

The Neue-Kuss model is an empirical model that can easily be integrated to predict retention under gradient conditions [28].

$$\ln k = \ln k_0 + 2 \ln(1 + S_2 \phi) - \frac{S_1 \phi}{1 + S_2 \phi} \quad (5)$$

This study was conducted using retention times obtained from gradient-elution runs. Thus, the retention models were applied for gradient separations as described previously [19]. For the mixed-mode and quadratic model the gradient equation cannot be solved. Therefore, a numerical approach based on the Simpsons' approximation was applied.

The PIOTR program was used to fit these different retention models to the experimental data for each analyte. We have previously described this approach to establish the retention parameters [19,21]. Briefly, PIOTR utilizes a non-linear programming solver which searches for the minimum residuals. In essence, the constants (e.g. $\ln k_0$ and S for the exponential model) are varied until the simulated result matches the experimental retention times with a minimum of residual error. This is carried out within the constraints of the applied gradient to record the experimental data.

The goodness of fit of the five models was determined using the Akaike information criterion (AIC) [29]. The minimum number of scouting gradients needed was three to fit all the models since the quadratic, mixed-mode and Neue-Kuss contain three parameter model coefficients. The retention time of the peptides under different gradient conditions were used as the input data. The data sets contained 15 peptides, analysed with the three HILIC columns run at optimal conditions as described in the previous section and one RPLC column. The 15 peptides featured different properties with regard to length, amino-acid composition, net charge, pI, and the grand average of hydrophobicity index (GRAVY). The properties of the peptides can be found in Table 3. Peptides KVPQVSTPTLVEVSR

and KQTALVELLK were removed from the final results due to large variations in the AIC values and prediction errors. The AIC values were calculated and predictions were performed using the in-house-developed Matlab program PIOTR [21].

The AIC parameter is calculated as follows.

$$AIC = 2p_m + n \left(\ln \left(\frac{2\pi * SSQ}{n} \right) + 1 \right) \quad (6)$$

where n is the number of input data points, p_m is the number of parameters of the model and SSQ is the sum of squared errors. By using this value, we can compare models that have different numbers of parameters. A good fit is indicated by a small, often negative, AIC value. Each peptide considered gives an AIC value for each model. Therefore, we considered the average values and the standard deviations across all peptides. The AIC value itself does not provide any qualitative information about the fit. AIC values can only be used to relatively compare a series of values. Even then, as can also be seen in Fig. 3, the AIC values are not always conclusive, especially not when a large standard deviation is observed. Therefore, we also considered the average error of prediction and the F-test of regression to draw clear conclusions.

3.3. RPLC retention modelling

Separation of BSA digest with reversed-phase liquid chromatography was performed to facilitate the identification of the peptides using existing libraries on the Triple TOF instrument. RPLC data were also used to verify the functionality of the models and to compare the selectivity with the HILIC separations. RPLC has been extensively characterized [30] and the retention of the analyses can be accurately described by an exponential model (Eq. (1)).

Using the same procedures for the data treatment as outlined in Section 2.6 we calculated the goodness of fit and prediction errors with the five models. We observed that only the exponential, mixed-mode and quadratic models performed well, showing low prediction errors ($\leq 0.5\%$) and negative AIC values (Fig. 2). The adsorption and Neue-Kuss models did not perform well. When inspecting the models (Section 3.2), we observed that the three equations that provided a good fit shared the terms of the exponential model, with one extra parameter in the case of the mixed-mode and quadratic models. The mixed-mode and the quadratic models can be viewed as the exponential model when considering only the first two parameters. This could be an indication that the third parameter does not contribute significantly to the performance of the model. To test this hypothesis, we looked into the influence of the third parameter by using the statistical F-test for regression [31]. In contrast to the AIC value, this statistical F-test does not assess the fit in general. Instead, it allows a comparison of a model with a reduced version. For example, the exponential model (Eq. (1)) can be seen as a reduced version of the quadratic model (Eq. (4)), differing by one term. The F-test can be used to compare the residual sum-of-squares of the full model ($SS_{res,full}$) with that of the reduced model ($SS_{res,red}$) and consequently determine the significance of the additional parameter. This is shown in Eq. (7)

$$F = \frac{MS_{res,diff}}{MS_{res,full}} = \frac{(SS_{res,full} - SS_{res,red}) / (df_{red} - df_{full})}{SS_{res,full} / df_{full}} \quad (7)$$

where MS denotes the mean squares and df_{red} and df_{full} are the degrees of freedom of the reduced and full model, respectively. Using PIOTR, the cumulative distribution function of the F-distribution is assessed to yield a p value. If the p value is statistically significant (< 0.05), then this indicates that the additional term (and thus the full model) is statistically significant. It is good to emphasize that this specific F-test provides no information on the goodness-of-fit.

Table 3

Peptides used for the retention modelling; Properties were obtained from [32].

Sequence	<i>m/z</i>	Measured charge	MW	pI	GRAVY index
LGEYGFQ	407.193	2+	812.369	4.00	-0.35
GFQNALIVR	509.296	2+	1016.575	9.75	0.57
FWGK	537.282	1+	536.274	8.75	-
KQTALVELLK	571.861	2+	1141.705	8.59	0.19
TDLTK	577.319	1+	576.310	5.50	-1
LVNELTEFAK	582.319	2+	1162.621	4.53	0.13
AWSVAR	689.373	1+	688.364	9.79	0.26
STVFDK	696.356	1+	695.347	5.55	-0.31
GLVLIAF	732.465	1+	731.456	5.52	2.92
LGEYGFQNALIVR	740.401	2+	1478.786	6.00	0.29
LVTDLTK	789.472	1+	788.462	5.84	0.42
KVPQVSTPTLVEVSR	820.473	2+	1638.928	8.75	-0.06
AEFVEVTK	922.488	1+	921.479	4.53	0.17
LVVSTQTALA	1002.583	1+	1001.574	5.52	1.39
Q TALVELLK	1014.619	1+	1013.61	6.00	0.64

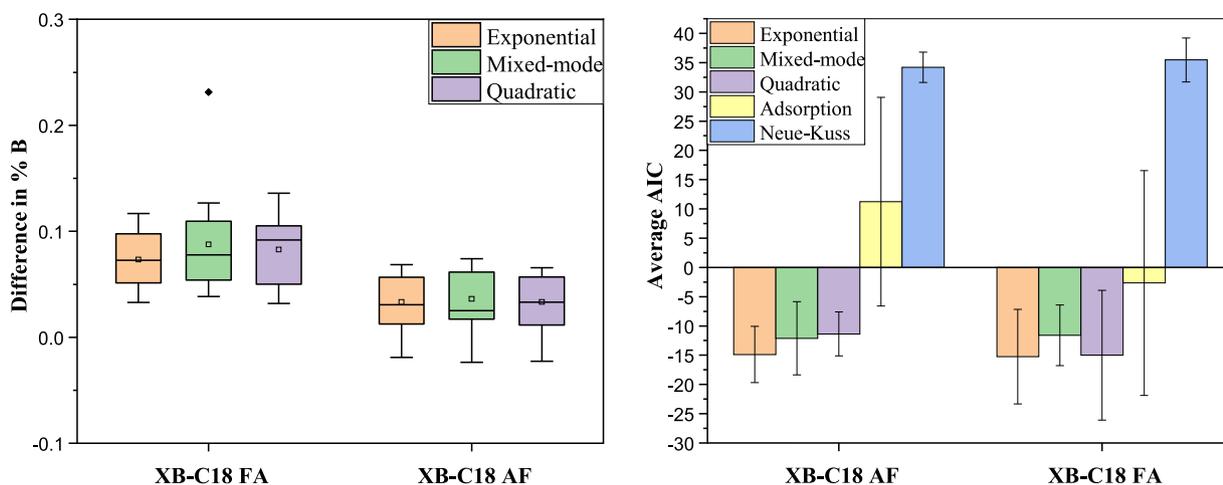


Fig. 2. BSA digest separation of XB-C18; left: average AIC values and right: errors in prediction expressed in % of mobile-phase B; 3 input gradients were used 17, 52 and 80 min duration and 30 min gradient was predicted.

All the values obtained were added in the supplementary information (Table S1). The minimum *p* values obtained were 0.26 for the mixed-mode and 0.51 for the quadratic model. From this it can be concluded that the added contribution of the third parameter in the mixed-mode and quadratic models was not statistically significant.

3.4. HILIC – goodness of fit

Firstly, we investigated how the number of input gradients affect the AIC values. We observed that the standard deviation decreased significantly when four gradients were used as input instead of three (Fig. S5), whereas only a slight additional decrease was observed when five input gradients were used (Fig. S6). The differences were more noticeable for the quadratic and Neue-Kuss models. Based on these observations, we used four input gradients to decide on the best model(s) to describe our data (Fig. 3).

Secondly, we investigated which model yielded the lowest AIC average for each column. For the amide and Z-silica columns, the lowest AIC values were obtained with the adsorption model with relatively low standard deviations (2.15 and 1.18 respectively). For the W-silica the lowest values were for the quadratic model. However, it showed a large standard deviation (11.04). The second lowest AIC average value was obtained with the adsorption model, with a much lower standard deviation (3.88). Therefore, we concluded that for all columns the adsorption model could best be used to accurately fit the data.

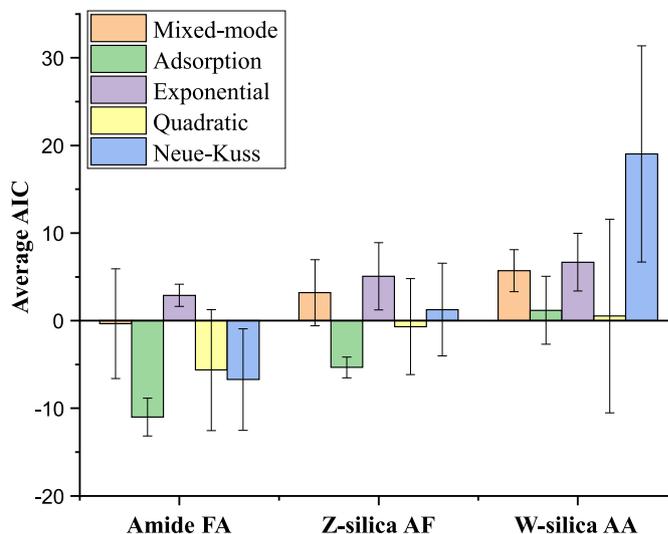


Fig. 3. AIC values and standard deviations for five models on three different columns, obtained using gradients of 17, 52, 70 and 80 min duration.

3.5. HILIC – retention-time prediction

Prediction of retention times is an important tool in method development. An accurate model and a small number of scouting

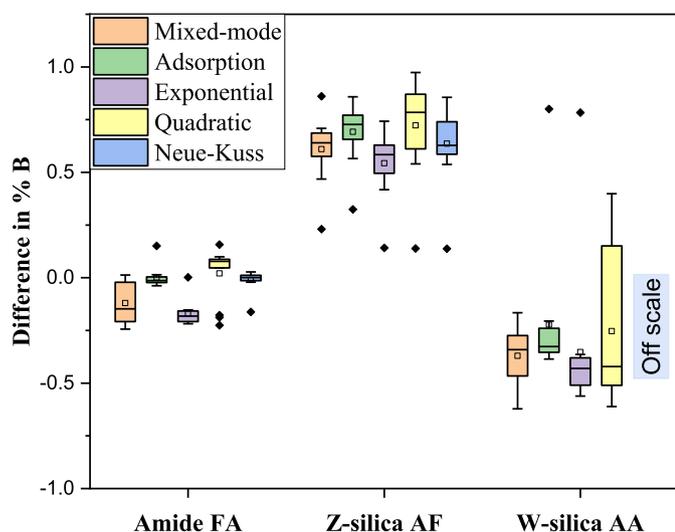


Fig. 4. The error in prediction of a 30 min gradient for the separation of BSA digest expressed in mobile-phase B composition in the three HILIC columns. The input gradients used were 17, 52, 80 min duration.

gradients may suffice to optimize a separation. We used prediction of retention times for the three HILIC columns to validate the results obtained from the goodness-of-fit for the five tested models. As previously, when investigating AIC values, we explored three or four gradients as inputs and we attempted to predict one of the measured gradients that were not used as an input. In Fig. 4 the results for the three-gradient-input are shown. The results obtained with four-gradient-input data are shown in supplementary material (Fig. S7). We observed that there is no significant gain in accuracy from adding a fourth input gradient for prediction. Therefore, only three measurements suffice for prediction. The column with the lowest error of prediction was the amide column, followed by W-silica and then Z-silica.

The amide column showed average prediction errors close to 0 for the adsorption (0.08%), quadratic (0.35%) and Neue-Kuss (0.2%) models. However, the standard deviations for the latter two models were larger. The exponential model showed standard deviations similar to the adsorption model. However, the average error was larger (0.36%). The mixed-mode model showed errors in prediction up to 0.8%. The significance of the third parameter to the model performance was calculated for the quadratic compared to exponential model and mixed-mode compared to adsorption model. There was no significant gain from adding a third parameter for the adsorption model (lowest p value was 0.31). However, for six of the thirteen peptides, the third factor in the quadratic model did prove to be significant (p values ≤ 0.01). Ultimately, the adsorption model was found to be the most suitable for retention-time prediction of peptides on the amide column. This model was previously also found suitable for predicting the retention of small molecules [19].

The Z-silica column was found to give rise to a systematic error, with all models showing an average prediction error close to 0.5 min. The exponential model showed an average prediction error closer to zero (1.36%). We evaluated the significance of the third parameter in the quadratic model compared to the log-linear model. The p values for all the peptides were above 0.05, with 0.1 being the minimal value, thus indicating no significant contribution. When comparing the adsorption model with the mixed-mode model, no significance of the third parameter was observed either (lowest p value was 0.44). The exponential model performed reasonably well. However, the adsorption model may still be preferred since the difference in prediction error was just 0.5%.

The W-silica column showed a very high error of prediction for the Neue-Kuss model and a large standard deviation for the quadratic model. Therefore, these models were not further considered. When inspecting the other three models, the mixed-mode model showed a larger standard deviation, whereas the exponential and adsorption models exhibited a relatively narrow range of errors. The contribution of the third parameter in the mixed-mode compared to the adsorption model was found to be insignificant, with a lowest p value of 0.3. Among the exponential and adsorption models, the latter showed lower prediction errors (i.e. $\leq 0.36\%$). Hence, it was considered the best model for prediction.

4. Concluding remarks

In this work, we have investigated the retention of peptides in HILIC and we have explored five models to fit the data. The performance of the models was characterized by the Akaike information criterion (AIC) to determine the goodness of fit and evaluated using prediction errors. Optimal separation for a BSA digest was obtained using formic acid as additive for an amide column, ammonium formate (pH=3) for a Z-silica (Zorbax) column, and ammonium acetate (pH=6) for W-silica column (Waters-Atlantis). Equilibration times were also different for the different stationary phases, with the shortest time needed for the amide column.

RPLC experiments were performed as a benchmark to test the modelling procedures, as well as to aid in identifying the peptides in the protein digest sample. The best fit to the data was obtained with the exponential model, as expected, but the mixed-mode and quadratic models also performed adequately. By computing the F -statistic for regression we noted that the third parameter of these latter two models did not have a significant influence on the model performance. Therefore, these models behave like the exponential model and the added complexity has no significant benefits.

The goodness of fit values indicated that the adsorption model was the most suitable to describe retention of peptides using the three HILIC columns. At least four input gradients were needed to obtain reliable model coefficients for the quadratic and Neue-Kuss models, whereas three input gradients were sufficient for the mixed-mode, adsorption and exponential models. The adsorption model gave the lowest AIC values with the smallest standard deviations.

We were able to predict the retention times of peptides on all three stationary-phases with errors below 2%. The amide column had the smallest average errors in prediction with the adsorption model (0.08%), followed by the W-silica column with average prediction errors of 0.78%. The Z-silica column showed higher prediction errors for all the models, exhibiting a systematic error. On this latter column the prediction error for the adsorption model was 1.76%, while the lowest errors were observed for the exponential model with 1.36%.

There have been previous studies for retention models applied in HILIC separations. Česla et al. [18] have concluded that for the isocratic separation of malto-oligosaccharides in HILIC the mixed-model provided the best fit of the data, yielding the lowest AIC values and prediction errors. Tyteca et al. [17] proposed the same model for isocratic separations of acidic, basic and neutral small molecules. However, for gradient separations they found the Neue-Kuss model to be more suitable, because it allowed analytical integration to obtain gradient retention times. The use of a large number of measurements used in the above mentioned experiments could possibly explain the better functioning of the Neue-Kuss empirical model. However, for a limited number of scouting gradients Pirok et al. [19] showed a poor performance of the Neue-Kuss model, with the adsorption model providing a better fit and yielding lower prediction errors for a variety of small molecules.

Based on the results reported previously in a study involving small-molecule analytes [19] and the results reported in this paper, we recommend that the adsorption model be used to describe retention in HILIC, unless specific information is available to support the suitability of other models.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.chroma.2019.460650](https://doi.org/10.1016/j.chroma.2019.460650).

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