RESEARCH ARTICLE



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Retention modeling of therapeutic peptides in sub-/supercritical fluid chromatography

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Abstract

Chromatographic modeling software packages are valuable tools during method optimization steps. These are well established for reversed-phase applications utilizing retention time (RT) prediction to optimize separations and receive robust methods, which is of high interest for the analysis of pharmaceuticals. In contrast to liquid chromatography, the knowledge of RT prediction in supercritical fluid chromatography is limited to a manageable number of studies. This study uses the software DryLab to predict the RTs of the peptides bacitracin (Bac), colistin, tyrothricin (Tyro), and insulin analogs. Gradient time, column temperature, and the ternary composition (terC) of carbon dioxide, methanol (MeOH), and acetonitrile (ACN) in the gradient elution are varied in a feasibility approach using a neutral (Viridis BEH) and an amino-derivatized aromatic (Torus 2-PIC) stationary phase. In the second part, chromatographic optimization is performed in silico through gradient adjustments to optimize the separation of the fingerprint of Bac. The final gradient method utilizes a Viridis BEH column (100×3.0 mm, 1.7μ m), carbon dioxide, and a modifier consisting of ACN/MeOH/water/methanesulfonic acid (60:40:2:0.1, v:v:v:v). In addition, changes in the retention order of Tyro compounds with the proportion of the terC in combination with a Torus Diol column are investigated.

KEYWORDS

chromatographic modeling, retention time prediction, ternary composition, therapeutic peptides

Article Related Abbreviations: ACN, acetonitrile; Bac, bacitracin; ICH, International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use; LSSM, linear solvent strength model; MeOH, methanol; QbD, quality by design; RP-LC, reverse phase-liquid chromatography; RT, retention time; SFC, sub-/supercritical fluid chromatography; SP, stationary phase; terC, ternary composition; tG, gradient time; Tyro, tyrothricin; WP, working point.

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1 | INTRODUCTION

Retention time (RT) prediction finds multiple applications: 1) characterization of stationary phases (SPs), 2) column screening and consecutive method optimization, 3) method transfer from one analytical system/laboratory to another, and 4) retention prediction of known compounds [1, 2]. Various models were developed over the last decades and customized to certain chromatographic modes (reverse phase [RP], hydrophilic interaction liquid chromatography, ion exchange chromatography, gas chromatography, etc.) [3]. The linear solvent strength model (LSSM) was introduced in 1979 [4], and the suitability for gradient separations of small molecules and peptides or proteins was demonstrated in the early 80s and 90s [5]. It underwent ongoing evolution and found broad application in reverse phase-liquid chromatography (RP-LC). A modified LSSM [4] is used in recent software versions, such as the DryLab software [6], with the actual mathematical equations being proprietary information. Nowadays, the preparation of 3D models through simultaneous variation of three chromatographic parameters, such as pH or ternary composition (terC) in combination with gradient time (tG) and column temperature (T), is well established [7-11].

The development of analytical methods applying quality by design (ObD) driven workflows experiences growing relevance in the pharmaceutical context. It requires to make decisions based on sound science principles and risk assessment to fulfill regulatory demands [12, 13]. The concept was initially introduced by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) in their Q8 guideline "Pharmaceutical Development" [14] for the development of pharmaceutical products. Just recently, the ICH Q14 guideline "Analytical Procedure Development" [15], which is dedicated to analytical procedures, was adopted (11/23). In 2021, the United States Pharmacopeia published the chapter < 1220 > "Analytical Procedure Life Cycle" covering the same topic. These guidelines demand a comprehensive understanding of analytical methods and accelerate the adaptation of new techniques. Upstream risk assessment, predefined objectives, multifactorial process parameter screening, and multifactorial data evaluation must be performed to define a design space, ensuring a robust procedure. Thus, the impact of several parameters is studied following the Design of Experiments principles. The complex data sets generated then demand a softwareassisted workflow. Software packages dedicated to chromatographic method development relying on either an empirical or mechanistic approach are available, such as ACD/LC simulator (ACD/Laboratories), ChromSword (Merck), DryLab (Molnar-Institute), or Fusion QbD (S-

Matrix). These are used to predict the RTs of the analyte(s) and assist in method optimization and robustness testing. The optimization of analytical methods for small molecules and even more for complex biopharmaceuticals benefits from advances in software-assisted workflows [16].

In recent years, sub-/supercritical fluid chromatography (SFC) extended its potential to the separation of complex biomolecules, such as peptides [17-19], providing orthogonal selectivity to RP-LC [20-24]. Thus, the application of modeling software packages in SFC is desirable. The general applicability of RT prediction of biogenic amines in SFC using flow rate, T, and tG was shown through an empirical approach [25]. A few studies were published on mechanistic RT predictions of small molecule pharmaceuticals. A high accuracy for non-linear prediction models at modifier concentrations > 5% for gradient elutions can be achieved in SFC [26]. However, the early eluting compounds at lower modifier concentrations led to high prediction errors. The switch from the critical to the subcritical state might play a role in this [27]. Some attempts have been made to overcome these issues by applying higher-order and more complex formulas by adding other variables [28, 29]. In contrast, above the 2% modifier, a linear function of the solvent strength was shown for the elution of steroids on a polar SP. The nonlinearity below 2% is explained through the adsorption of methanol (MeOH) and carbon dioxide, which is invariable above [30, 31]. Two other studies found a linear relation between the retention factor and a shift in the solvent composition for polynuclear aromatic hydrocarbons on phenyl-type SPs in the smaller and higher-modifier range [32, 33]. It should be noted that these studies were performed with small molecules and with a set of different column chemistries determining the driving retention effects, obstructing data comparability. In addition, these studies were performed in the low-modifier SFC mode. In conclusion, the overall knowledge about RT prediction and modeling in SFC is limited and thus needs to be supplemented. No data is available on the high-modifier SFC mode used for peptide and biopharmaceutical separations. Previously, the differing composition of MeOH and acetonitrile (ACN) used as the modifier turned out to be an efficient tool to improve peak separation and to control the peak order of peptides when combined with varying SP chemistries providing π - π or hydrogen bonding interactions [17, 19].

This knowledge was leveraged in retention modeling to optimize the separation of diverse therapeutic peptides. This study applies the LSSM model via the DryLab software for the prediction. In the first part, the model's suitability is tested by varying the input parameters in relatively narrow ranges by comparing the predicted and



FIGURE 1 Amino acid sequences of the model compounds. For tyrothricin, which is a mixture of various compounds, a linear gramicidin, and a cyclic tyrocidine are exemplarily shown. For the insulins, the common amino acid sequence is shown with differing positions indicated.

experimental RT. In the consecutive step, software-assisted method optimization is performed by applying broader input ranges and optimizing additional parameters, such as gradient points and flow rate, with the software. The chromatographic factors terC, tG, and T are varied simultaneously as inputs to create a 3D model, which enables RT prediction. Also, changing compositions of MeOH and ACN when combined with differing column chemistries are further investigated to supplement our previous study [17].

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Bacitracin (Bac) and colistin sulfate were purchased from Thermo Scientific. Tyrothricin (Tyro) CRS was supplied by the European Directorate for the Quality of Medicines. These peptides were dissolved in MeOH/water (90:10, v:v) to receive a concentration of 1 mg/ml. Insulin aspart from Novo Nordisk Pharma GmbH and insulin glulisine from Sanofi-Aventis Deutschland GmbH (Frankfurt am Main, Germany) were used (both 100 IU/ml) as is. The amino acid sequence of each is shown in Figure 1. These peptides were chosen as a representative set of analytes due to various chemical characteristics: basic/acidic sidechains, isomeric, and linear/cyclic structures in a mass range of 1000–6000 Da.

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Acetonitrile and MeOH (LC gradient grade) were bought from VWR. Milli-Q water was prepared freshly before use by a Merck Milli-Q system, and carbon dioxide (99.995%) was acquired from Air Liquide. Methanesulfonic acid (MSA; \geq 99.0%) was purchased from Sigma Aldrich.

2.2 | Instrumentation and software

The chromatographic experiments were performed using an Acquity UPC² SFC system (Waters, Eschborn, Germany) equipped with a binary pump with a dwell volume of 440 µL, a 4-port column manager with active eluent pre-heaters, an Acquity UPC² photodiode array detector (UV), an Acquity UPC² convergence manager (backpressure regulator) and an Acquity TQD (triple quadrupole mass spectrometer) with an electrospray ionization source operated in positive mode. A fixed-leak interface was used to connect the SFC system to the MS and a makeup solvent was provided through a Waters 515 make-up pump. Empower 3 software was used for system control, data acquisition, and processing. DryLab 4.3 from Molnár-Institute was used to model, display, and predict the RTs. The 2D graphs were calculated via Microsoft Excel. The following SPs were utilized for the experiments: Viridis BEH,

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FIGURE 2 Input runs ($n = 2 \times 2 \times 3 = 12$) required to create the 3D prediction model indicated as dots (A). The chosen verification runs were performed to demonstrate the suitability of the prediction model (B).

Torus 2-PIC, and Torus Diol from Waters (Eschborn, Germany). All column dimensions were 3.0×100 mm; particle size was 1.7μ m.

2.3 | Feasibility experiment

Compressed carbon dioxide was delivered by pump A, and a generic gradient from 30% to 95% modifier (B) was run, applying a flow rate of 0.6 ml/min and a backpressure of 1500 psi. Varying MeOH/ACN compositions of the modifier (terC) were combined with T and tG, as demonstrated in Figure 2A. Three levels were selected for terC ACN/MeOH (0/100, 25/75, 50/50) and two for tG (10/20 min) and T (30/50°C) each to prepare the input data required for the model. Water (2%, v/v) and MSA (0.1%, v/v) were used as additives. The levels were combined in all possible combinations, resulting in twelve corner experiments ($2 \times 2 \times 3 = 12$). An aromatic (2-PIC) and a non-aromatic (BEH) SP were chosen based on our previous work [17].

A make-up flow (0.1 ml/min) consisting of MeOH/water/formic acid (95/5/0.1, v:v:v) was added before the MS. Chromatograms were recorded at 210 nm and via MS (m/z 500 to 1500). The following MS settings were applied: cone voltage: 30 V, capillary voltage: 3.0 kV, source temperature: 120°C, desolvation gas temperature: 250°C and desolvation gas flow: 500 L/h.

Four working points (WP) inside the model (Figure 2B) were performed as real experiments to verify the software predictions: intermediate levels of T (40° C) and tG (15 min) were combined with four terCs (10/20/30/40% ACN in MeOH). The percental RT error of the chosen peaks was calculated as:

$$Error (\%) = \left| \frac{RT \ predicted - RT \ experimental}{RT \ experimental} \times 100\% \right|$$

2.4 | Optimization experiment

Consecutively, additional optimization models were prepared for Bac and Tyro with terC ACN/MeOH (15/85, 40/60, 65/35), tG (20/40 min), and T (30/50°C). Water (2%, v/v) and MSA (0.1%, v/v) again were used as additives. The BEH column was used for Bac, and five WPs were modeled with the software by adjusting the chromatographic parameters as described in Table 1. A Diol column was adapted from our earlier study [23] for Tyro due to superior performance. Again, MS detection was applied in addition to UV to all base runs for accurate peak tracking. All other parameters were kept constant, as described in section 2.3. The predicted RTs were compared to the experimental ones.

3 | RESULTS AND DISCUSSION

3.1 | Feasibility experiment: application to diverse peptides

The feasibility of the established chromatographic modeling software DryLab for the RT prediction in SFC was investigated initially. The combination of three chromatographic factors is well-approved in the DryLab software. While in RP-LC, the combination of pH, tG, and T is commonly used [7, 10, 11], this set is not applicable in SFC due to the absence of a measurable pH. Thus, the terC/tG/T model is considered a suitable alternative model for SFC. A ternary modifier composition (carbon dioxide, MeOH, and ACN) improved the peptide fingerprint resolution in our previous works [17, 19]. In addition, the terC was found to alter the elution order of the analytes when combined with an aromatic SP (2-PIC) and was now supplemented by a non-aromatic SP (BEH). The currently available software version does not allow the incorporation of the

	Input runs		WP I		WP II		WP III		WP IV		WP V	
Stationary phase			Viridis BEH, 3.0×100 mm, 1.7μ m									
Modifier	15/40/65 % ACN in MeOH		ACN/MeOH 60/40		ACN/MeOH 60/40		ACN/MeOH 60/40		ACN/MeOH 60/40		ACN/MeOH 60/40	
Additive	2% water + 0.1% MSA											
Column temperature	30/50°C		48°C		48°C		48°C		48°C		48°C	
Backpressure	1500 psi											
Flow rate [ml/min]	0.6		0.6		0.6		0.6		0.9		1.2	
Gradient	t [min] 0	B [%] 30	t [min] 0	B [%] 30	t [min] 0	B [%] 50	t [min] 0	B [%] 30	t [min] 0	B [%] 30	t [min] 0	B [%] 30
	20/40	95	55	70	55	95	3 55	55 70	55	70	55	70
Average error [%]			1.0		5.5		6.0		1.3		2.0	
R ² predicted vs. experimental RT	1.0000			000	0.9999		0.9996		0.9999		0.9999	

TABLE 1 Chromatographic parameters of the input runs and the chosen working points. The average prediction error for the compounds B1–10 and bacitracin at each tested working point is indicated.

backpressure as an additional chromatographic parameter in SFC. However, due to its impact on the density and diffusibility of the eluent, it was treated as a constant parameter for the experiments.

Ten representative peaks were chosen in the four samples, as indicated in Figure 3A. For Tyro (bottom), a complex of more than 20 linear and cyclic compounds, including isomers, MS detection was added to ensure accurate peak tracking. Retention times and the peak widths at 50% height were used as the input data for the calculation of the model. Separate models were then calculated for each SP. In this first feasibility step, the variation of the parameters for the verification runs was limited to levels enclosed by the input experiments without extrapolating these. Based on the corner experiments, the software calculates the RT for any combination of terC, tG, and T.

The peak order for the linear (peak 1+2) and the cyclic (peak 3–6) compounds in Tyro distinctly differed on the two SPs (Figure 3B), which is similar to earlier findings [24]. Also, the elution order for the two insulin analogs (peaks 9 and 10) is inverted. Comparing the elution order of the peaks on the aromatic SP, the inversion for Bac (peak 7) and colistin (peak 8) with the change in the terC is identical to the data found in our previous work [17]. The same was found for several other peaks, again proving how valuable the optimization of the terC is to control the elution order and to improve the separation when combined with an aromatic SP. Overall, the effect of solvent selectivity is well-known for small molecules and peptides in RP-LC [34–37] and also was investigated in SFC separations of small molecules [38–41].

This shift is almost absent on the hybrid silica BEH column. Nevertheless, a change in the elution order of peaks 6 and 7 was found. In contrast to the π - π -interactions that are more relevant for the 2-PIC SP, delimited through the proportion of ACN, the amount of hydrogen-bond interactions, delimited through the MeOH content, are the driving force on the hybrid silica phase [42]. The terC used with a non-aromatic SP might be helpful for separating highly different peptides but to a smaller extent.

The comparison of the predicted versus the experimental RTs shows good consistency with an average error of $\leq 1.5\%$ with both SPs (Figure 4). The pre-mixing of the modifier that requires manual volumetric proportioning of four components is a factor that contributes to inaccuracies and should be considered when evaluating the data. Attention was paid while preparing the modifiers for both the input and the verification runs, but minor deviations in the volumes were not preventable.

A tendency for the earlier eluting compounds to show higher deviations from the predicted RTs is present, especially with the 2-PIC column. The error at 40% ACN is in the higher range with the 2-PIC but in the lower range with the BEH column. The same modifier preparations were used for all experiments with both columns and can be excluded as the source of these deviations. The dominant interactions (hydrogen bonding or π - π -interactions) determining the retention differ due to the SP's derivatization and also shift with the change in the ratio of MeOH and ACN. The fit of the model does not reflect these differences. Consequently, adjustments to the calculations might be necessary to account for these aspects and improve accuracy. Nevertheless, the average error is acceptable and is comparable to ranges reported for RP-LC separations using the same model [7, 10]. Noteworthy is that the elution order between prediction and verification



🔶 1: Val-Gram C – 2: Val-Gram A – 3: Tyrocidine A – 4: Tyrocidine B – 5: Tyrocidine C – 6: Tryptocidine C – 7: Bacitracin – 8: Colistin – 9: Aspart – 10: Glulisin

FIGURE 3 Exemplary ultraviolet (UV) overlay chromatograms of colistin, bacitracin, insulin aspart and insulin glulisine (top), and tyrothricin (bottom) derived from the base run combining 0% acetonitrile (ACN), 20 min gradient time, and 50°C column temperature. Representative peaks used for the calculations are labeled. The large peak (retention time [RT] < 1.6 min) corresponds to phenol and cresol contained in the insulin formulations (A). Experimental retention times of the verification runs using 10/20/30/40% ACN in MeOH combined with 15 min gradient time and 40°C column temperature as indicated in Figure 2B. Please note that interpolated lines are just used to visualize changes in the peak order (B).

was consistent, which is of higher priority for method optimizations.

In conclusion, the prediction model used and verified in the DryLab software can be applied as is for modeling SFC separations if the high-modifier range (> 30%) is used and rather simple variations within the model are performed.

3.2 | Optimization experiment: separation of complex peptides

In contrast to the feasibility model, which just varied the input levels of tG, T, and terC through the application of intermediate levels, more comprehensive settings were applied in the second step to challenge the prediction model through extrapolation and optimize the separation of a main compound and its impurities. Bacitracin, a fermentation product containing many byproducts, was chosen as the model substance. The BEH column, which performed superior to the 2-PIC column in the feasibility experiments in terms of the number of separated peaks related to Bac (Figure 3A), was used. Apart from the main peak of Bac, ten characteristic compounds (B1–10, Figure 5A) were considered as input data (RT and peak width) for software-based predictions and modeling.

Five WPs were modeled for Bac via the software (Table 1). Now, variations of chromatographic parameters that are not directly covered in the input experiments but are based on chromatographic principles incorporated in the software's algorithm were performed. The "Gradient



FIGURE 4 Graph showing the experimental retention times versus the percental error for all compounds.

Editor" of the software allows gradient modeling via variations of tG and start% and end% of the modifier. Also, other parameters, such as flow rate, can be adjusted. As a challenge to the prediction model, all these parameters were varied in different WPs. The gradient slope was varied by changing the start and end %B separately (WP I + II). A gradient step (WP III) was inserted, and the flow rate was increased (WP IV + V). The conditions were also adjusted with the goal of improving the resolution of the peptide's fingerprint. The five chosen WPs were performed as real experiments. The experimental RTs were compared to those predicted, and the percental prediction error of all tracked peaks was calculated (Figure 6A). In addition, the R² value was calculated (Table 1) after plotting the predicted versus experimental RTs for each WP individually (Figure 6B).

Again, the potential inaccuracy due to the pre-mixing of the modifier should be considered. Since the same preparation was used for all five experiments, all were impacted equally. The WPs maintaining the start% B of the gradient (I, IV, and V) from the input experiments provide low error rates (average < 2%). In contrast, increasing the start% B (WP II) results in a much higher error (< 5%). Other authors reported similar tendencies when using gradients

from 2% to 50% modifiers as inputs to predict a 5%-50% gradient [26] with much higher errors (up to 35%). Obviously, additional factors play a role in the low modifier region, as summarized initially. For all five WPs, the earlier eluting compounds show higher errors than the later eluting compounds. The impact on the density of the modifier due to an increase in backpressure at the head of the column, resulting in higher pressure drops with increasing velocity, is a reasonable explanation. Likewise, the pressure increases, and density shifts with increasing modifier proportion along the gradient. As worked out by other authors [26, 29], pressure is a major factor impacting RT predictions in SFC, and prediction accuracy can be drastically increased when respected in the calculations [29]. As indicated in Figure 6B, the experimental data for WP I, II, and III show a slightly faster elution of all peaks than predicted. When comparing WP I, IV, and V, this is moved to slower elutions when the flow rate is increased.

The high-modifier SFC conditions used result in lower fluid compressibility and consequently smaller density shifts under subcritical conditions [27]. Thus, the retention behavior seems to resemble linear relations present in the liquid state. This explains, why the LSSM is applicable within the limits discussed. The prediction accuracy is acceptable as all peaks were impacted similarly while relative elution and elution order were maintained. For the optimization of a method, receiving a separation is far more relevant than achieving a high prediction accuracy as long as the error affects the predicted RTs of all compounds equally under a single condition. Considering the R^2 at each WP, this is the case under all conditions tested.

The chromatographic parameters were optimized in silico to separate the chosen compounds and increase the critical resolution to the maximum achievable value, resulting in WP V as indicated in Figure 7. The 3D cube shows a critical resolution > 1.0 for all peaks in red, which corresponds to the smallest resolution between any two peaks. An overall lower resolution of all peaks compared to WP V was predicted by the software for the two other red areas. Thus, these were not investigated experimentally. The prediction of WPV accurately matches the experimental run regarding the elution order of the fingerprint with minor RT shifts. The overall resolution of the fingerprint was improved compared to the input runs, and an additional major peak eluting before Bac (Figure 5B), which coeluted before, was detected. However, the resolution of the peaks predicted does not match the experimental chromatogram where broader peaks were detected (Figure 5B), even if the peak width was included as an input to the model. For sufficient separations of peptides, shallow gradients are often required. The gradient slope of the final method was much shallower than the one used for the





input experiments, with extrapolation of the model also contributing to the peak broadening.

Overall, the suitability and benefits of the softwarebased optimization using the terC/tG/T model were demonstrated with the limitations described.

An optimization model was also prepared for Tyro. However, the SPs used for the feasibility experiments performed worse than the Diol column in our previous study [24]. Thus, the Diol SP was used again for the optimization model of Tyro. Peak assignment was performed according to the MS data provided in our previous study [24]. The separation could not be improved through the addition of ACN to MeOH. Increasing the ACN content resulted in the coelution of several compounds during all the input runs. Also, no sufficient improvement could be achieved when adjusting the chromatographic parameters with the software. However, a shift in the retention order was found for some peaks (Figure 8). With the alterations in terC, inversions for the peak pairs 3/4, 1/5, 10/12, and 14/15 were found. Compounds 1 and 5 differ in the exchange of tryptophan in Val-Gramicidin A (1) for a tyrosine residue in

Val-Gramicidin C (5). The additional aromatic hydroxy function results in a relative increase in retention with the reduction in the MeOH proportion. The same explanation holds true for the peak pairs 13/15 and 14/16, where the exchange of tyrosine in 13 and 14 for tryptophan results in 15 and 16, respectively.

These findings confirm the hypothesis regarding the relevance of hydrogen bond interactions for the selectivity of hydroxy sidechain functions in peptides. Apart from the amount of π - π -interactions present when using ACN with an aromatic SP, the amount of hydrogen bond interactions may be the driving force on a Diol phase combined with MeOH, determining the selectivity. The contribution of both interactions shifts with the ratio of the MeOH and ACN in a binary mixture of these. Even when this observation was not beneficial for separating the Tyro compounds, it supplements our study on binary mixtures of ACN and MeOH combined with an aromatic SP [17]. It might be helpful for other analytes and again shows the benefits of applying varying compositions of ACN/MeOH as a major parameter in optimizing SFC peptide separations. These



FIGURE 6 Graph showing the experimental retention times versus the percental error (A) and predicted versus the experimental retention times of bacitracin and byproducts for the chosen working points (B).

can be effectively leveraged as inputs to the terC/tG/T prediction model.

4 | CONCLUDING REMARKS

In the first part of the study, a set of representative compounds covering a broad range of physicochemical characteristics was used to demonstrate the feasibility of chromatographic modeling via the DryLab software based on the LSSM for RT prediction of peptides in SFC. To our knowledge, this is the first work published on this topic. An accurate prediction was successfully proved via verification experiments.

The software model was used in the second part to successfully separate compounds within a single sample of

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FIGURE 7 Three-dimensional design space for working point V with a critical resolution \geq 1.0 indicated in red for bacitracin and its main impurities.



FIGURE 8 Overlay chromatogram of the input runs (A) and a plot showing the shift of the retention time order of tyrothricin compounds with variation in ternary composition (terC) while T (30°C) and gradient time (tG) (40 min) are kept constant (B). Please note that interpolated lines are just used to visualize changes in the peak order.

complex peptides like Bac and Tyro. The model was challenged due to parameter settings not included in the input experiments. A sufficient accuracy level of prediction was achieved in terms of peak order and relative elution. For Bac, the resolution of the fingerprint was optimized successfully and experimentally confirmed afterward. This work demonstrates the applicability of currently available modeling software for predicting high-modifier SFC separations with limitations in predicting the peak widths and slight drifts in the RTs.

Adding ACN did not improve Tyro's fingerprint on a Diol phase, but a shift in the elution order of several compounds was noticed. It was assumed that the proportion of MeOH providing hydrogen bond interaction with a diol-type SP is similarly relevant to the π - π -interaction due to ACN with an aromatic SP. These findings complement our previous results on terCs for peptide SFC separations and show again the usefulness of varying compositions of MeOH and ACN during optimization steps.

AUTHOR CONTRIBUTIONS

Jonas Neumann: Conceptualization; methodology; investigation; formal analysis; visualization; and writing original draft. Sebastian Schmidtsdorff: Formal analysis and writing—review & editing. Alexander H. Schmidt: Resources and writing—review & editing. Maria K. Parr: Supervision and writing—review & editing.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

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