Received: 11 December 2022 | Revised: 30 December 2022 | Accepted: 31 December 2022

DOI: 10.1002/jssc.202201007

RESEARCH ARTICLE

JOURNAL OF SEPARATION SCIENCE

Ternary eluent compositions in supercritical fluid chromatography improved fingerprinting of therapeutic peptides

Jonas Neumann[1,2] | **Sebastian Schmidtsdorff**[1,2] | **Alexander H. Schmidt**[2] | **Maria K. Parr**[1]

[1]Department of Biology, Chemistry and Pharmacy, Institute of Pharmacy, Freie Universität Berlin, Berlin, Germany

[2]Chromicent GmbH, Berlin, Germany

Correspondence
Jonas Neumann, Chromicent GmbH, Johann-Hittorf-Str. 8, 12489 Berlin, Germany.
Email: jonas.neumann@posteo.net

Currently, little information has been published on the application of ternary eluent compositions in supercritical fluid chromatography for separating peptides. This work investigates the benefits of adding acetonitrile to methanol as the modifier. Three cyclic antibiotic peptides (bacitracin, colistin, and daptomycin) ranging between 1000 and 2000 Da were chosen as model substances. The ternary mixture of carbon dioxide, methanol, and acetonitrile is optimized to increase the resolution of the peptide's fingerprint. In addition, varying compositions of methanol and acetonitrile were found to change the elution order of the analytes, which is a valuable tool during method development. An individual gradient method using two Torus 2-PIC columns (each 100 × 3.0 mm, 1.7 μm), carbon dioxide, and a modifier consisting of acetonitrile/methanol/water/methanesulfonic acid (60:40:2:0.1, v:v:v:v) was optimized for each of the peptides. Subsequently, a generic method development protocol applicable to polypeptides is proposed.

KEYWORDS
fingerprinting, supercritical fluid chromatography, ternary composition, therapeutic peptide

1 | INTRODUCTION

The interest in polypeptides used as therapeutics has grown distinctly in the last two decades [1]. Their high binding affinity allows addressing specific targets combined with a low prevalence of side effects compared to conventional small drug substances. Especially cyclic peptides find usage as potent therapeutics based on their favorable pharmacokinetic properties [2, 3]. The growing number of registered drugs comes along with more and more regulatory requirements concerning their chemical analysis. Reversed-phase chromatography is the central analytical technology used due to its widespread usage, versatility, and efficacy. Peptide samples often are rather complex, containing the main compound and a variety of isomers or degradation products and related substances from manufacturing [4]. Often, the separation of all compounds contained can hardly be achieved using RP-LC exclusively. Thus, orthogonal and complementary technologies are valuable to gain a more comprehensive

Article Related Abbreviations: BEH, hybrid silica; DoE, Design of Experiments; MeOH, methanol; MSA, methanesulfonic acid; RT, retention time.

knowledge of a particular sample. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use guideline Q6B accounts for this situation: "The absolute purity of biotechnological and biological products is difficult to determine, and the results are method dependent. Consequently, the purity of the drug substance is usually estimated by a combination of methods [5]."

Recently, analytical SFC underwent a renaissance due to fundamental hardware improvements. Its application field enlarged from non-polar to polar analytes such as biomolecules [6]. Separating distinctly different peptides in a single run proved the general applicability of SFC as an analytical tool for separating peptides < 5000 Da [7–11]. In addition, it turned out to be a valuable tool for the separation of isomeric peptides [12, 13]. Even proteins up to 40 kDa are accessible [14–16]. Under most of the conditions applied, the term SFC is inaccurate due to the absence of a supercritical state. Some authors instead use "enhanced-fluidity chromatography" [14], "unified chromatography", [17] or "chromatography with compressed carbon dioxide" [18] when using higher modifier proportions. Nevertheless, the term SFC is used in this work due to the instrument used, which is well known as an SFC.

The orthogonality and thus complementarity of SFC to RP-LC were demonstrated [17, 19] without one being definitively superior. The systematic comparison of SFC, RP-LC, HILIC, and mixed-mode LC showed that SFC holds drawbacks regarding the selectivity for chemical closely related linear decapeptides but higher versatility for peptides covering a broad range of polarities [19]. These findings are partially in line with the observations we made in our previous work [20]. SFC was superior for separating the cyclic compounds, while RP-LC was better for the linear peptides of tyrothricin. Similar results for a mixture of cyclic diastereomeric peptides were reported by another group [21]. Summing up, SFC is more suitable for cyclic than for linear peptides.

The combination of different column chemistries and additives was reported in several studies [22–24]. Methanesulfonic acid (MSA) or ammonia combined with water in methanol (MeOH) can be defined as a generic setup for analyzing peptides. The beneficial use of MSA due to its high acidity and ion pairing properties was demonstrated recently [24]. Acidic to slightly acidic characteristics of the mobile phase were proposed if carbon dioxide and MeOH were used as the mobile phase due to the presence of a methoxylcarbonic acid buffer system [23, 25]. Most basic peptide functionalities will be protonated under these chromatographic conditions. An increase in the number of basic functionalities increases the polarity of the peptide due to a charged state, which results in higher retention of the analyte in SFC. Depending on the basic or acidic character of the analyte, either neutral (silica) or basic (amino-derivatized) stationary phases are favored [24].

Different approaches to method optimization using Derringer's desirability functions [26] or a systematic Design of Experiments (DoE) based workflow are published [20]. So far, the main parameters controlling the selectivity of a chromatographic method are limited to column chemistry and additives. Other parameters, such as column temperature, are more considered fine-tuning parameters with a relatively small impact on selectivity in peptide separation. The gradient optimization is then the following promising parameter. Thus, more work on finding beneficial parameters to control separation needs to be done.

The benefits of a ternary composition of MeOH, ACN, and carbon dioxide are reported for separating small molecules [27–31]. So far, its use has not been systematically studied for peptides. Here, MeOH usually is used as the modifier [6]. The addition of ACN to MeOH as the modifier is rarely described for analytical separations. Preparative applications using a ternary composition are reported [32, 33], but no detailed information about the impact of the composition is given, except for an adjustment of the solvent strength and selectivity [32]. In this study, we present the beneficial application of varying compositions of MeOH and ACN as the modifier to significantly improve the separation of peptides and to control selectivity in means of peak order in some cases.

Three antibiotics, cyclic peptides (bacitracin, colistin, and daptomycin), were chosen as model analytes (Figure 1) based on their acidic, neutral, or weakly basic properties. Implementing a ternary composition is shown in a systematic workflow leading to a highly improved fingerprint and controlling the selectivity. Finally, generic conditions for separating peptides are defined. A generic workflow for method development is proposed considering the results obtained in this study and other relevant data published for peptide separation.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Bacitracin, colistin sulfate (both Thermo Scientific, Dreieich, Germany), and daptomycin (Xellia Pharmaceuticals Ltd., Budapest, Hungary) were dissolved in MeOH/water (90:10, v:v) to receive a concentration of 1 mg/ml. The solutions were stored at room temperature for a week to generate degradants. Methanol and ACN (LC gradient grade) were bought from VWR (Darmstadt, Germany). Milli-Q water was prepared freshly before use via a Merck

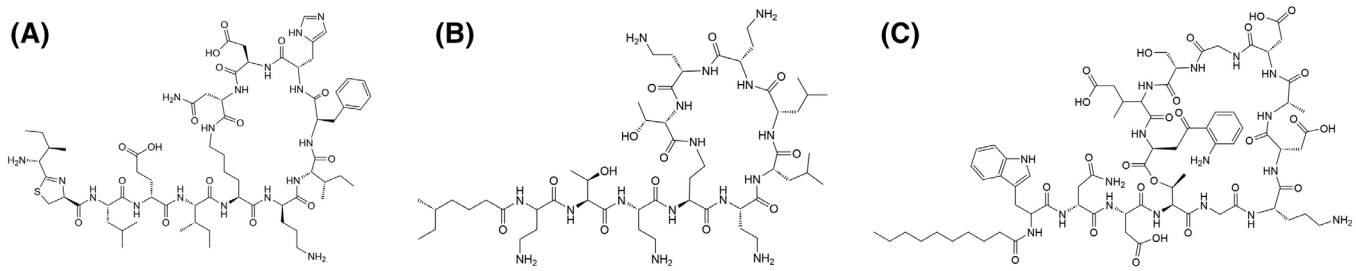

FIGURE 1 Chemical structures of bacitracin (A), colistin (B), and daptomycin (C).

Milli-Q system (Darmstadt, Germany) and carbon dioxide (99.995%) was acquired from Air Liquide (Berlin, Germany). Methanesulfonic acid (≥99.0%) was purchased from Sigma Aldrich (Taufkirchen, Germany), and aqueous ammonia solution (25%) was supplied by Merck.

The following stationary phases were used for the SFC experiments: Torus Diol, Torus 2-PIC, Torus 1-AA, and Viridis hybrid silica (BEH) from Waters (Eschborn, Germany). All columns' dimensions were 3.0 × 100 mm; particle size was 1.7 μm.

2.2 | Choice of analytes

Three therapeutic peptides used as antibiotics were chosen for the experiments based on varying chemical characteristics. Bacitracin (Figure 1A) contains an even number of basic and acidic side chains. Colistin (Figure 1B) has numerous basic functionalities and thus was considered a challenging molecule based on other studies published [24]. Daptomycin (Figure 1C) is a cyclic lipopeptide with several carboxylic and amino groups. All three peptides are head-sidechain cyclizations.

2.3 | Instrumentation and software

The chromatographic experiments were performed using a Waters Acquity UPC^2 SFC system equipped with a binary pump, a 4-port column manager with active eluent preheaters, an Acquity UPC^2 photodiode array detector, and an Acquity UPC^2 convergence manager (back pressure regulator). Empower 3 was used for system control, data acquisition, and processing. The two-dimensional graphs were visualized via Microsoft Excel.

2.4 | Development of the SFC methods

Initially, a generic gradient from 25% to 90% modifier (B) in 15 min was run, applying a flow rate of 0.6 ml/min. Methanol/water (100:2, v:v) plus 0.5% ammonia or 0.1% MSA (v:v) as the modifier were tested in combination with four stationary phases (Section 2.1). The column temperature was set to 50°C and backpressure to 1500 psi. All chromatograms were recorded at 210 nm.

Afterward, the gradient time was prolonged to improve separation and a gradient step was inserted to shorten the overall runtime: 0–3 min: 20%–35% B, 3–23 min: 35%–75% B. The number of peaks, peak width, shape, and separation of the compounds were used to assess the selectivity of the stationary phases and additives. The best combination was kept for further experiments: the 2-PIC column and eluent B consisting of MeOH/water/MSA (100:2:0.1, v:v:v).

Subsequently, the portion of MeOH was partially exchanged by ACN, and the gradient profile was adjusted to 0–20 min: 30–95 % B to evaluate the best composition of MeOH and ACN. A modifier composition consisting of ACN/MeOH/water/MSA (70:30:2:0.1, v:v:v:v) then was applied using two 100 mm columns running the following gradient: 0–3 min: 30%–65% B, 3–23 min: 65%–95% B at a flow rate of 0.5 ml/min. Step-wise, the segments of the gradient were optimized for the final methods.

2.5 | Final SFC separation

The final methods used two Torus 2-PIC columns (each 2.1 × 100 mm, 1.7 μm). The column temperature was set to 50°C and the backpressure to 1500 psi at a flow rate of 0.5 ml/min. For each analyte, an individual modifier composition and gradient were optimized. An injection volume of 3 μl was set for bacitracin and colistin and 2 μl for daptomycin.

Bacitracin: ACN/MeOH/water/MSA (70:30:2:0.1, v:v:v:v), 0–3 min: 30%–65% B, 3–20 min: 65%–70% B, 20–21 min: 70%–30% B, 21–27 min: 30% B.

Colistin: ACN/MeOH/water/MSA (60:40:2:0.1, v:v:v:v), 0–3 min: 30%–60% B, 3–20 min: 60%–70% B, 20–21 min: 70%–30% B, 21–27 min: 30% B.

	Bacitracin	Colistin	Daptomycin
1-AA	ammonia MSA	ammonia MSA	X MSA
2-PIC	ammonia MSA	ammonia MSA	X MSA
BEH	ammonia MSA	ammonia MSA	X MSA
Diol	ammonia MSA	ammonia MSA	X MSA

FIGURE 2 Evaluation of the column and additive screening. The best performance is ranked from dark to light green over yellow to red for poor performance or non-elution of the analyte. Ammonia was not tested for daptomycin (X).

Daptomycin: ACN/MeOH/water/MSA (60:40:2:0.1, v:v:v:v), 0–3 min: 30%–65% B, 3–20 min: 65%–80 % B, 20–21 min: 80%–30% B, 21–27 min: 30% B.

3 | RESULTS AND DISCUSSION

3.1 | Screening of additives and column chemistry

Different stationary phases providing neutral or basic chemistries were chosen to evaluate their impact on selectivity: diol, amino(1-AA and 2-PIC), and BEH derivatization. So far, MSA and ammonia have been demonstrated to be the most promising additives [24] and therefore were chosen. Adding 2% water to the modifier was considered mandatory [23, 34].

Using ammonia as the additive did not elute colistin. This observation matches that reported before for basic peptides [24]. The "apparent" pH of the mobile phase, which probably is slightly acidic [25], results in a charged state of the basic functionalities and, thus, strong interaction with the stationary phase. The addition of MSA, an ion-pairing reagent, masks these charged functionalities and consequently elutes the analyte [9, 24, 35].

The 2-PIC column, in combination with MSA, performed best in the case of colistin and daptomycin and was the second choice for bacitracin. The BEH column showed a slightly better-resolved fingerprint of bacitracin, but the best compromise matching all three peptides was chosen. An overview of the evaluation is given in Figure 2. Daptomycin was not available at our lab initially but was added to the experiments after excluding ammonia. In conclusion, the 2-PIC column and MSA as the additive were kept for further experiments.

3.2 | Type of modifier and ternary composition

The focus during method development for peptide separation is on optimizing column chemistry and additives, the primary parameters to control and improve selectivity. Methanol as the modifier is usually set as a fixed parameter for peptide separation [6] and the potential impact of ACN seems to be underestimated. Its lower eluotropic strength in SFC might seem disadvantageous for the elution of polar analytes such as peptides. Due to missing data on its effect on peptide separation, this factor should be investigated in detail.

Thus, the modifier composition (single versus ternary mixture) was tested via partial substitution of MeOH by ACN in 10 % steps. Using 100% ACN instead of MeOH did not elute all three analytes, even if up to 95% B was used. This lacking elution of the analytes might explain why no publications reporting the use of ACN for separating peptides can be found. However, in this study, a distinct improvement of the separation was achieved when increasing the proportion of ACN in the modifier up to 80% (Figure 3) for all analytes. At least 20 % MeOH was necessary to elute bacitracin and colistin, and 30% was needed for daptomycin.

To visualize the impact of the ternary composition on selectivity, the capacity factor (k) as a chromatographic parameter was calculated via the retention time (RT) and the dead time (0.83 min) of the system (T0):

$$capacity\ factor\ (k) = \frac{\mathrm{RT} - \mathrm{T0}}{\mathrm{T0}} \quad (1)$$

In the case of bacitracin (Figure 4A), the relative change of k for characteristic peaks (main compound and byproducts) named as "Ba, Bb, Bacitracin, By and Bz" is constant with the change in the percentage of ACN. This can be expected based on the high chemical similarity of the main peak and related substances [36]. However, the plotting of the RT difference (ΔRT) to the main peak indicates a better separation of the compounds (Figure 4B), considering that no significant increase in the peak width was observed up to 60 % ACN in the modifier.

$$\Delta\mathrm{RT} = \mathrm{RT}\,(impurity) - \mathrm{RT}\,(\mathrm{main\ compound}) \quad (2)$$

A similar outcome was observed for daptomycin and the characteristic impurities (Figure 4C). Relative to the other peaks, a shift in the elution of the peak "Dd" indicated as the green bar is observed. This shows that a change of selectivity in terms of changing peak order can be achieved by adjusting the composition of MeOH and ACN.

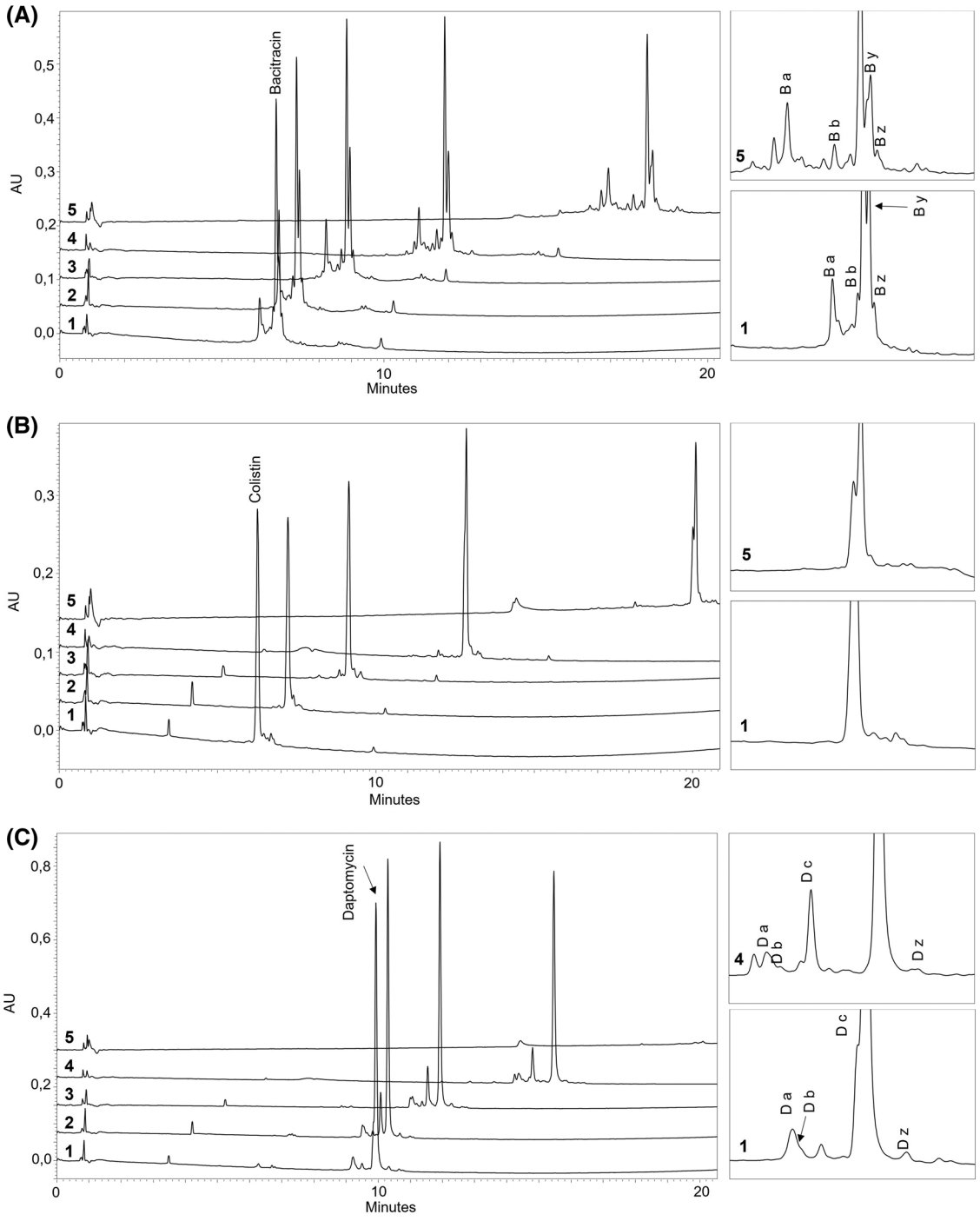

FIGURE 3 Overlay chromatograms of the separation of bacitracin (A), colistin (B), and daptomycin (C) applying varying modifier compositions of methanol (MeOH)/ACN (1:100/0, 2:80/20, 3:60/40, 4:40/60 and 5:20/80 – v:v). A zoomed chromatogram of the highest and lowest retention on the column for each peptide mixture is shown on the right. For colistin, no impurities were marked due to ambiguous peak tracking. In the case of daptomycin, no elution was possible via composition 5.

To further evaluate the effect of the ternary composition on selectivity, the capacity factors of the three main peptides, which differ significantly in their chemical properties, were also plotted (Figure 4D). Interestingly, there is an unequivocal effect on selectivity for colistin relative to either bacitracin or daptomycin, depending on the composition of ACN and MeOH. Not just a lower eluotropic strength due to the increase of the ACN content, but an additional effect must be present determining its elution. The difference in the presence of π-bonds and the protic or aprotic characteristics of the two solvents are apparent sources. These characteristics are well understood in

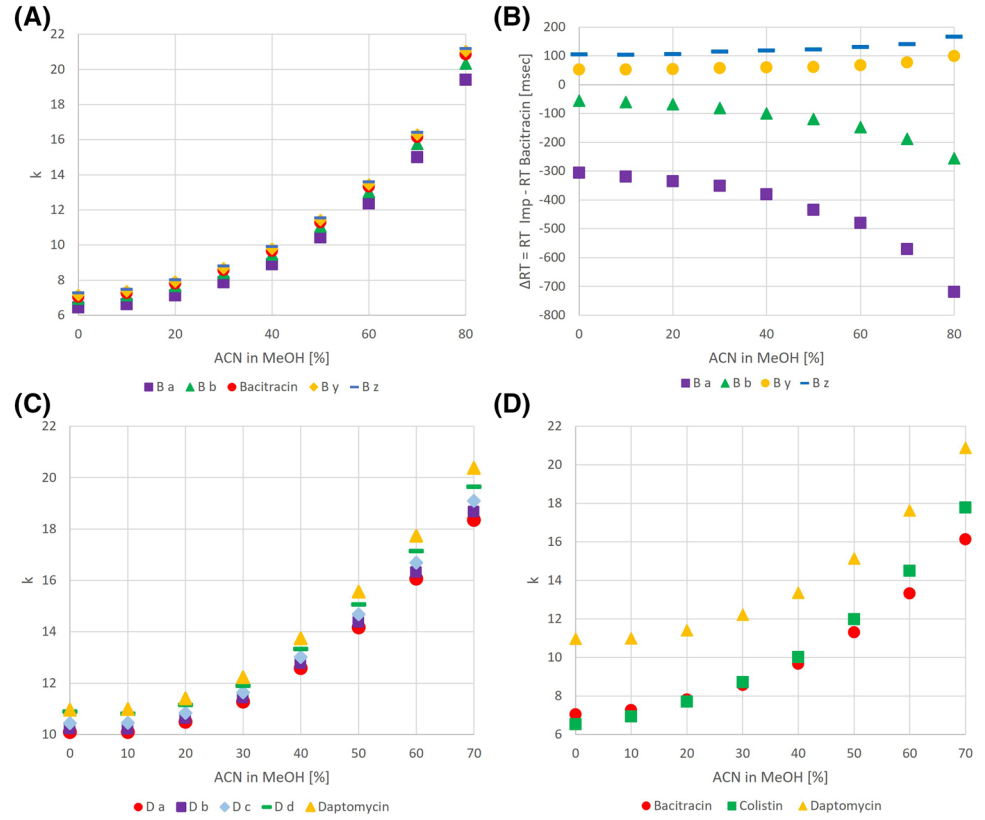

FIGURE 4 (A) Plots of the capacity factor (k) of bacitracin and related compounds versus proportions of ACN in methanol (MeOH). (B) Retention time difference (ΔRT) of the byproducts to bacitracin. (C) Plots of the capacity factor of daptomycin and byproducts versus ACN in MeOH. (D) Plots of the capacity factor of bacitracin, colistin, and daptomycin versus ACN in MeOH.

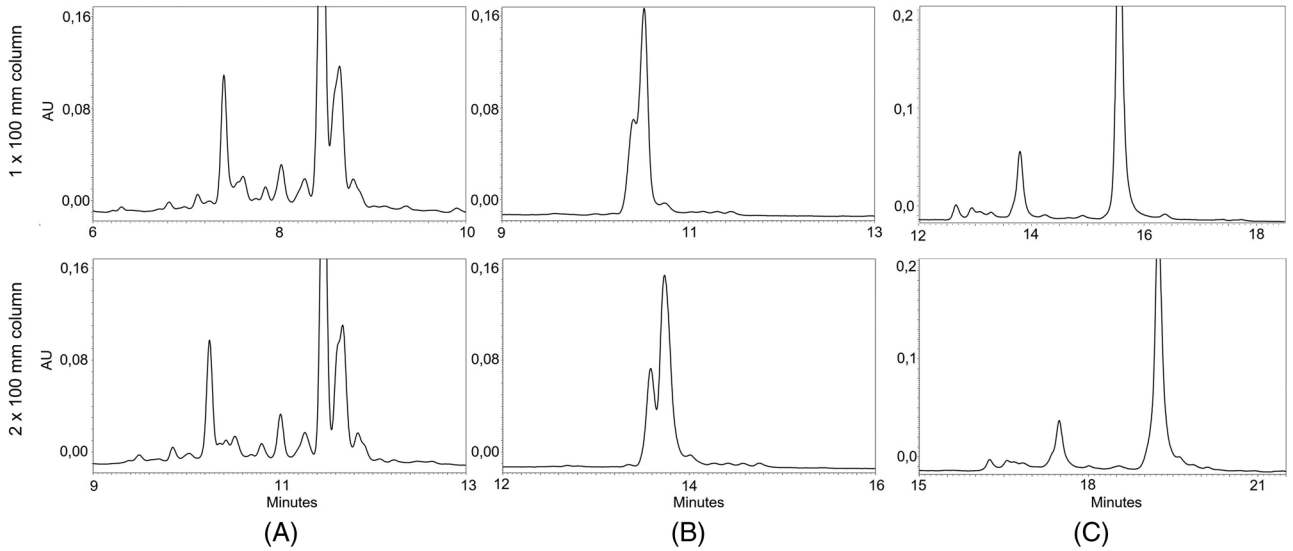

FIGURE 5 Comparison of a single 100 mm (top) and two 100 mm columns (bottom) for bacitracin (A), colistin (B), and daptomycin (C)

RP-LC, especially when working with phenyl-derivatized stationary phases [37, 38].

A 2-PIC column, which has a picoylamin derivatization and thus contains a pyridine and a diol functionality, was used in this study. With the shift in the composition of MeOH and ACN, the properties of the mobile phase to interact with the stationary phase due to either hydrogen bonds or π-π interactions are changed. The absence

of aromatic sidechains in colistin's structure and following weaker π-π interactions with the stationary phase explain the differing shift of the capacity factor with the variation in the ternary composition. In conclusion, a ternary composition of the modifier can be used to control the elution order of peptides. It should be considered as an additional, highly promising chromatographic parameter once the column chemistry and additive are defined during method development. A mixture of MeOH and ACN unequivocally outperformed the usage of just one organic solvent.

The most beneficial composition was determined in between 60 and 70% ACN in the modifier, which is a compromise between drastically increasing the resolution of the fingerprint and not increasing the overall retention too much. The selectivity for all peptides and related substances was increased distinctly. In the challenging case of colistin, an additional, prominent peak was partially separated from the main peak, which was not achievable before.

3.3 | Increasing the column length and final optimization

Step-by-step adjustments of the gradient segments were finally performed for all three peptides. An increase in the start percentage of the modifier to 65% resulted in a much earlier elution of all peaks, which came along with a drastic loss of resolution of all peaks. The insertion of a fast gradient step (0–3 min: 30%–65%) helped to shorten the runtime while maintaining the resolution of the fingerprint.

The increase in the ACN content from 0 to 70% decreased the system pressure from 4100 to 3000 psi at the end of the gradient due to the lower viscosity of the eluent. Based on the pressure limit of the instrument (6000 psi), this allowed an increase in the column length. Often longer columns and long flat gradients are used for peptide separation in RP-LC due to the high chemical similarity of the main compound and impurities expected. In our case, the slight reduction in the flow rate (from 0.6 to 0.5 ml/min) allowed a doubling of the column length. A comparison of the three peptides using one or two 100 mm columns running the same gradient (Section 2.4) is shown in Figure 5. The resolution of the colistin peak and the primarily related substance was significantly improved. Solely a slight fingerprint improvement was achieved for the other two peptides. Overall, a higher impact on resolution and selectivity was expected. The capillary used to connect the two columns was probably not optimal.

In conclusion, the employment of a longer column should be considered case by case depending on the individual analyte. A noticeable improvement in the selectivity was achieved by applying a ternary composition of carbon dioxide, ACN, and MeOH as the modifier, followed by gradient optimization for all three peptides (Figure 6).

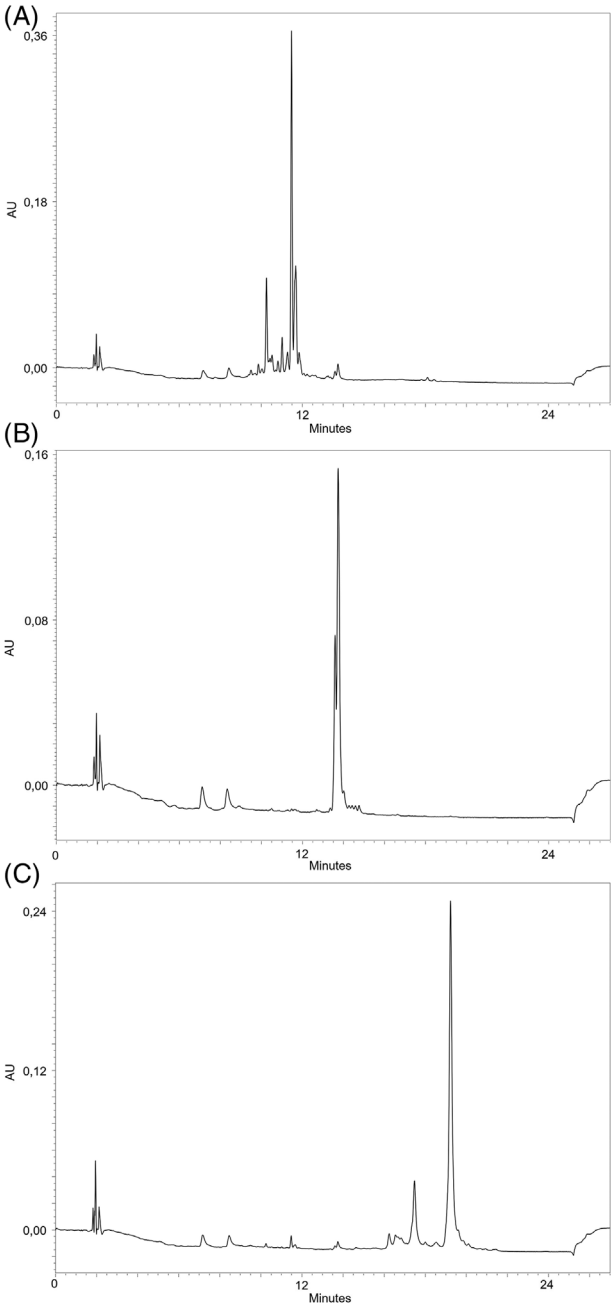

FIGURE 6 Chromatograms of the finally optimized SFC methods for bacitracin (A), colistin (B), and daptomycin (C). Please note that all peaks with a retention time (Rt) < 10 min are caused by the water contained in the sample solvent.

3.4 | Method development protocol

A generic screening and method optimization approach is proposed based on the data obtained in this and our previ-

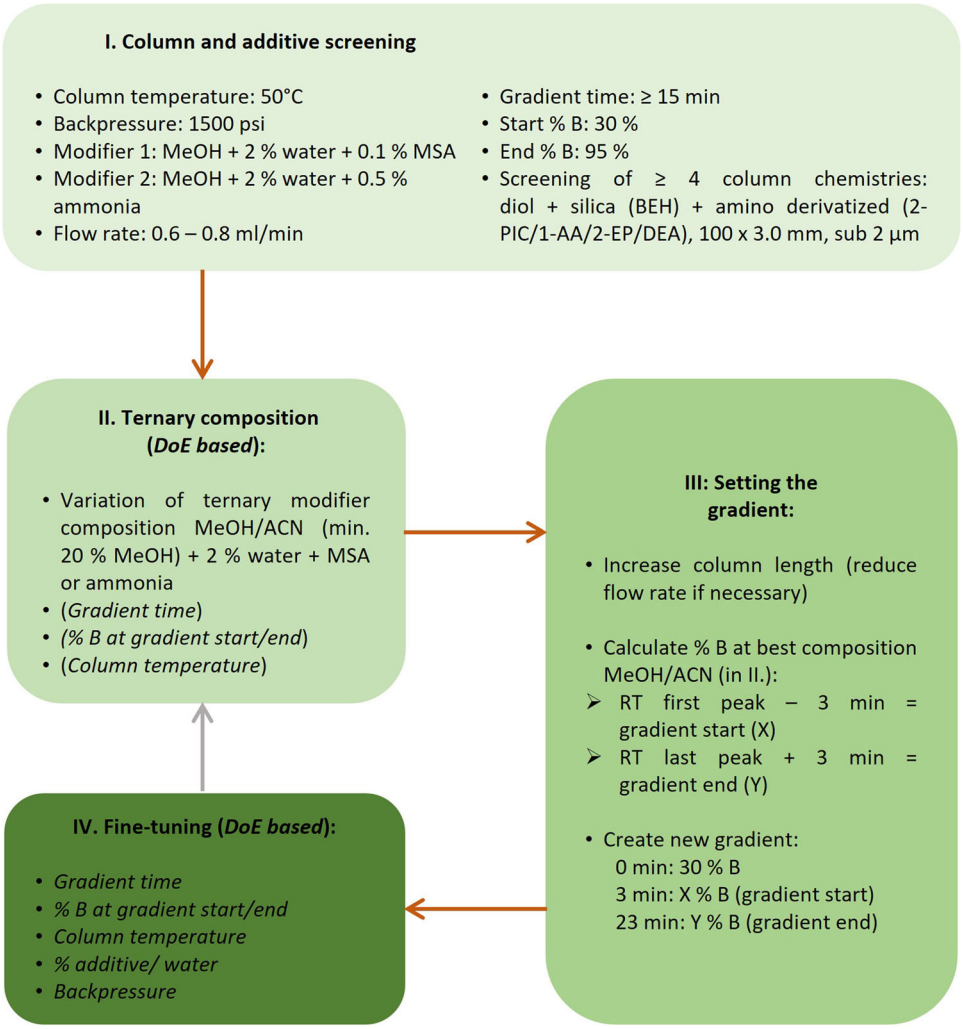

FIGURE 7 A generic method development protocol for the separation of peptides via SFC. The optional optimization via Design of Experiments (DoE) principles is included in cursive.

ous study [20] and other relevant publications [7, 13, 17–19, 21, 24, 26]. The potential impact of the chromatographic parameters on the selectivity and following resolution of the fingerprint is ranked as follows: column chemistry > additive type > composition MeOH/ACN > gradient time = start/end %B = column temperature > flow rate = additive concentration = backpressure. Concluding, method development should be performed according to this ranking. An overview is given in Figure 7.

Step I (Column and additive screening): In this study, the 2-PIC, an amino-derivatized column, was the best-performing stationary phase for two of three peptides. It was also chosen by other authors [18, 19, 24]. Other amino-derivatized columns, which proved helpful [7, 13, 18], should be added to test for better or complementary selectivity. The BEH, a hybrid silica particle, was superior for separating bacitracin and other cyclic peptides reported by another research group [21]. Diol chemistry performed the best in our previous [20] and other studies [7, 17, 19, 26]. Concluding, a set of columns covering a broad range of chemistries should be screened in combination with MSA [18-20, 24] or ammonia [17, 19, 23, 26] using MeOH as the modifier.

Step II (Ternary composition): The preferred column or columns should be kept for the experiments investigating the suitable composition of MeOH and ACN. In our case, a minimum of 20 - 30 % MeOH was necessary to ensure the elution of the analytes in a reasonable runtime. The main compounds in this study have a chain length of 10–12 amino acids. However, other authors reported that the retention increased with increased chain lengths of the peptides [24]. Concluding, a lower proportion of ACN may be applicable for larger peptides and vice versa. DoE principles should be

considered at this step. The multifactorial optimization of the ternary composition in combination with one or more other parameters (gradient time, start and end percentage of the modifier, or column temperature) is suitable.

Step III (Setting the gradient): The partial substitution of MeOH with ACN reduces the system pressure. A slight reduction of the flow rate then allows the usage of a long column or coupling of equivalent columns. A gradient is created by setting the start at 30% B and increasing it to X in 3 min, where X is calculated as the gradient composition 3 min before the elution of the first peak of interest in the run obtained in step II. This procedure successfully defined the modifier's end percentage for the fast gradient step, as described in Section 3.3. A 20 min gradient then ends at Y % B, where Y is calculated at the RT of the last eluting peak plus 3 min obtained in step II.

Step IV (Fine-tuning): The gradient time or start and end percentage of the modifier and the column temperature should now be optimized. The backpressure and concentration of the additives (water, MSA, and ammonia) are tweaked last. A step back to step II may be needed to further adjust the ternary composition's optimum to the fine-tuning parameters. Once the ternary composition and the elution window are defined, DoE principles should be included in the protocol to ensure multifactorial method optimization.

4 | CONCLUDING REMARKS

In this study, we demonstrated the beneficial application of the addition of ACN to MeOH as the modifier for the separation of peptides via SFC in detail for the first time. The fingerprint of three peptides was improved distinctly. Primarily, lowering the eluotropic strength seems to be the dominant factor resulting in a better resolution of the peaks. A secondary effect involving hydrogen bonds and π-π interactions is also supposed to be present. For some analytes, a varying selectivity in the elution order was found. This knowledge can be a valuable tool during method development steps for the separation of peptides that show a substantial difference in the chemical structures and might be beneficial to a smaller extent for separating closely related peptides. The integration of the ternary composition into a systematic method development protocol was then proposed to define generic conditions, considering our observations and previous studies of other research groups. With these findings, we contribute to the evolving field of peptide separation via SFC by investigating an often overlooked and underestimated chromatographic parameter that is worthwhile to be optimized.

AUTHOR CONTRIBUTIONS

Jonas Neumann: Conceptualization, methodology, investigation, formal analysis, visualization, and writing—original draft. Sebastian Schmidtsdorff: Conceptualization, and writing—review, and editing. Alexander H. Schmidt: Writing—review, editing, and resources. Maria K. Parr: Conceptualization, supervision, and writing—review and editing.

ACKNOWLEDGMENT

Open access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are availale on request from the authors.

ORCID

Jonas Neumann https://orcid.org/0000-0001-5414-2942
Sebastian Schmidtsdorff https://orcid.org/0000-0002-5881-5099
Alexander H. Schmidt https://orcid.org/0000-0002-4526-7820
Maria K. Parr https://orcid.org/0000-0001-7407-8300

REFERENCES

1. Lau JL, Dunn MK. Therapeutic peptides: Historical perspectives, current development trends, and future directions. Bioorg Med Chem. 2018;26:2700–7.
2. Zhang H, Chen S. Cyclic peptide drugs approved in the last two decades (2001-2021). RSC Chem. 2022;3:18–31.
3. Henninot A, Collins JC, Nuss JM. The current state of peptide drug discovery: back to the future. J Med Chem. 2018;61:1382–414.
4. D'Hondt M, Bracke N, Taevernier L, Gevaert B, Verbeke F, Wynendaele E, De Spiegeleer B. Related impurities in peptide medicines. J Pharm Biomed Anal. 2014;101:2–30.
5. International Conference on Harmonization. Harmonised tripartite specifications: test procedures and acceptance criteria for biotechnological/biological products Q6B. 1999. https://database.ich.org/sites/default/files/Q6B%20Guideline.pdf
6. Molineau J, Hideux M, West C. Chromatographic analysis of biomolecules with pressurized carbon dioxide mobile phases – a review. J Pharm Biomed Anal. 2021;193:13736.
7. Ventura M. Advantageous use of SFC for separation of crude therapeutic peptides and peptide libraries. J Pharm Biomed Anal. 2020;185:113227.

8. Tognarelli D, Tsukamoto A, Caldwell J, Caldwell W. Rapid peptide separation by supercritical fluid chromatography. Bioanalysis 2010;2:5–7.
9. Blackwell JA, Stringham RW. Effect of mobile phase components on the separation of polypeptides using carbon dioxide-based mobile phases. J High Resolut Chromatogr. 1999;22:74–8.
10. Zheng J, Pinkston JD, Zoutendam PH, Taylor LT. Feasibility of supercritical fluid chromatography/mass spectrometry of polypeptides with up to 40-mers. Anal Chem. 2006;78:1535–45.
11. Zajickova Z, Nováková L, Svec F. Monolithic poly(styrene-co-divinylbenzene) columns for supercritical fluid chromatography–mass spectrometry analysis of polypeptide. Anal Chem. 2020;92:11525–9.
12. Patel MA, Riley F, Ashraf-Khorassani M, Taylor LT. Supercritical fluid chromatographic resolution of water soluble isomeric carboxyl/amine terminated peptides facilitated via mobile phase water and ion pair formation. J. Chromatogr. A. 2012;1233:85–90.
13. Patel MA, Riley F, Wang J, Lovdahl M, Taylor LT. Packed column supercritical fluid chromatography of isomeric polypeptide pairs. J. Chromatogr. A. 2011;1218:2593–7.
14. Wang Y, Olesik SV. Enhanced-fluidity liquid chromatography–mass spectrometry for intact protein separation and characterization. Anal. Chem. 2019;91: 935–42.
15. Bennett R, Olesik SV. Protein separations using enhanced-fluidity liquid chromatography. J. Chromatogr. A. 2017;1523:257–64.
16. Zhang X, Scalf M, Westphall MS, Smith LM. Membrane protein separation and analysis by supercritical fluid chromatography—mass spectrometry. Anal. Chem. 2008;80:2590–8.
17. Molineau J, Hideux M, Hennig P, Bertin S, Mauge F, Lesellier E, West C. Analysis of short-chain bioactive peptides by unified chromatography-electrospray ionization mass spectrometry. Part II. Comparison to reversed-phase ultra-high performance liquid chromatography. J. Chromatogr. A. 2022;1663:462771.
18. Molineau J, Hideux M, Hennig P, Bertin S, Mauge F, Lesellier E, West C. Effect of dilution solvent and injection volume on the analysis of basic hydrophilic therapeutic polypeptide salts with pressurized carbon dioxide mobile phases. J. Chromatogr. B. 2022, 1213:123519.
19. Deidda R, Losacco GL, Schelling C, Regalado EL, Veuthey J-L, Guillarme D. Sub/supercritical fluid chromatography versus liquid chromatography for peptide analysis. J. Chromatogr. A. 2022;463282.
20. Neumann J, Schmidtsdorff S, Schmidt AH, Parr MK. Application of sub-/supercritical fluid chromatography for the fingerprinting of a complex therapeutic peptide. J Sep Sci. 2022;45:3095–104.
21. Wu Y, Wu Z-M, Zhang S-S, Liu L-Y, Sun F, Jiao W-H, Wang S-P, Lin H-W. Axinellasins A–D, immunosuppressive cycloheptapeptide diastereomWers, discovered via a precursor ion scanning–supercritical fluid chromatography strategy from the Marine Sponge Axinella species. Org Lett. 2022;24:934–8.
22. Govender K, Naicker T, Baijnath S, Chuturgoon AA, Abdul NS, Docrat T, Kruger HG, Govender T. Sub/supercritical fluid chromatography employing water-rich modifier enables the purification of biosynthesized human insulin. J. Chromatogr. B. 2020;1155:122126.
23. Liu J, Makarov AA, Bennett R, Haidar Ahmad IA, DaSilva J, Reibarkh M, Mangion I, Mann BF, Regalado EL. Chaotropic effects in sub/supercritical fluid chromatography via ammonium hydroxide in water-rich modifiers: enabling separation of peptides and highly polar pharmaceuticals at the preparative scale. Anal. Chem. 2019;91:13907–15.
24. Losacco GL, DaSilva JO, Liu J, Regalado EL, Veuthey J-L, Guillarme D. Expanding the range of sub/supercritical fluid chromatography: Advantageous use of methanesulfonic acid in water-rich modifiers for peptide analysis. J. Chromatogr. A. 2021;1642:462048.
25. Raimbault A, West C, Effects of high concentrations of mobile phase additives on retention and separation mechanisms on a teicoplanin aglycone stationary phase in supercritical fluid chromatography. J. Chromatogr. A. 2019;1604:460494.
26. Molineau J, Hamel Y, Hideux M, Hennig P, Bertin S, Mauge F, Lesellier E, West C. Analysis of short-chain bioactive peptides by unified chromatography-electrospray ionization mass spectrometry. Part I. Method development. J. Chromatogr. A. 2021;1658:462631.
27. Muscat Galea C, Mangelings D, Vander Heyden Y. Investigation of the effect of mobile phase composition on selectivity using a solvent-triangle based approach in achiral SFC. J. Pharm. Biomed. Anal. 2017;132:247–57.
28. West C, Lesellier E. Effects of mobile phase composition on retention and selectivity in achiral supercritical fluid chromatography. J. Chromatogr. A. 2013;1302:152–62.
29. Lesellier E. Overview of the retention in subcritical fluid chromatography with varied polarity stationary phases. J Sep Sci. 2008;31:1238–51.
30. Brunelli C, Zhao Y, Brown M-H, Sandra P. Development of a supercritical fluid chromatography high-resolution separation method suitable for pharmaceuticals using cyanopropyl silica. J. Chromatogr. A. 2008;1185:263–72.
31. Aubert MC, Lee CR, Krstuloviác AM, Lesellier E, Péchard MR, Tchapla A. Separation of trans/cis- α- and β-carotenes by supercritical fluid chromatography: I. Effects of temperature, pressure and organic modifiers on the retention of carotenes. J. Chromatogr. A. 1991;557:47–58.
32. Schiavone NM, Bennett R, Hicks MB, Pirrone GF, Regalado EL, Mangion I, Makarov AA. Evaluation of global conformational changes in peptides and proteins following purification by supercritical fluid chromatography. J. Chromatogr. B. 2019;1110–1111, 94–100.
33. Emmett MR, Kazazic S, Marshall AG, Chen W, Shi SDH, Bolaños B, Greig MJ. Supercritical fluid chromatography reduction of hydrogen/deuterium back exchange in solution-phase hydrogen/deuterium exchange with mass spectrometric analysis. Anal. Chem. 2006;78:7058–60.
34. Liu J, Regalado EL, Mergelsberg I, Welch CJ. Extending the range of supercritical fluid chromatography by use of water-rich modifiers. Org. Biomol. Chem. 2013;11:4925–9.
35. Zheng J, Taylor LT, Pinkston JD. Elution of cationic species with/without ion pair reagents from polar stationary phases via SFC. Chromatographia. 2006;63:267–76.
36. Qin F, Zhang H, Liu H, Zhang X. High performance liquid chromatography-quadrupole/time of flight–tandem mass

spectrometry for the characterization of components in bacitracin. Chromatographia. 2020;83:647–62.

37. Coym JW. Evaluation of ternary mobile phases for reversed-phase liquid chromatography: effect of composition on retention mechanism. J. Chromatogr. A. 2010;1217:5957–64.
38. Yang M, Fazio S, Munch D, Drumm P. Impact of methanol and acetonitrile on separations based on π–π interactions with a reversed-phase phenyl column. J. Chromatogr. A. 2005;097:124–9.

How to cite this article: Neumann J, Schmidtsdorff S, Schmidt AH, Parr MK. Ternary eluent compositions in supercritical fluid chromatography improved fingerprinting of therapeutic peptides. J Sep Sci. 2023;46:e2201007. https://doi.org/10.1002/jssc.202201007