Potential of Supercritical Fluid Chromatography for the Separation of Therapeutic Peptides under Consideration of Current Pharmaceutical Guidelines

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Declaration of independence

I hereby declare that I alone am responsible for the content of my doctoral dissertation and that I have only used the sources or references cited in the dissertation.

Jonas Neumann

Outline

Abbreviations

2-PIC	2-Picolylamine
AA	Amino acid
ACN	Acetonitrile
ATP	Analytical target profile
CE	Capillary electrophoresis
CO_2	Carbon dioxide
DEA	Diethylamine
DoE	Design of experiments
DP	Drug product
DS	Drug substance
EFLC	Enhanced fluidity liquid chromatography
EMA	European medicines agency
EtOH	Ethanol
FA	Formic acid
FDA	U.S. food and drug administration
HILIC	Hydrophilic liquid interaction chromatography
HRMS	High-resolution mass spectrometry
ICH	International conference on harmonization of technical requirements for registration of pharmaceuticals for human use
IEX	Ion exchange chromatography
IP	Ion pair
LC	Liquid chromatography
LSSM	Linear solvent strength model
MeOH	Methanol
MMC	Mixed mode chromatography
MODR	Method operable design region
MS	Mass spectrometry

MSA	Methanesulfonic acid
NADES	Natural deep eutectic solvent
NP	Normal phase
PF	Pharmacopeial forum
Ph. Eur.	European pharmacopeia
QbD	Quality by design
RA	Risk assessment
R&D	Research and development
RP	Reversed-phase
SEC	Size exclusion chromatography
SFC	Sub-/supercritical fluid chromatography
SP	Stationary phase
TFA	Trifluoroacetic acid
UC	Unified chromatography
UHPLC	Ultra high-performance liquid chromatography
USP	United States pharmacopeia
WHO	World health organization

Summary

This work explores the potential of supercritical fluid chromatography for the analysis of peptide drugs. The regulatory aspects of peptides are more complex and challenging than for small drug molecules. The same applies to the analytical characterization of peptides. After technical innovations and the introduction of a new instrument generation, supercritical fluid chromatography nowadays is a suitable alternative to reversed-phase chromatography, which is considered the gold standard in peptide analysis. However, the scientific data on the analysis of peptides with supercritical fluid chromatography is limited.

In the first part of this thesis, supercritical fluid chromatography is used to develop a method to separate the variety of cyclic and linear peptides characteristic for tyrothricin. A software-based design of experiments approach is utilized to optimize an analytical method that provides superior separation of cyclic peptides compared to the reversed-phase separation. Even isomeric peptide pairs are efficiently separated.

Subsequently, the potential of a binary mixture of methanol and acetonitrile combined with additives as the modifier for the separations of other cyclic peptides is investigated. The proportion of the mixture is optimized to improve the resolution and control the elution order of the analytes. Paired with an aromatic stationary phase, this chromatographic parameter offers an effective method optimization opportunity.

This knowledge is furthermore extended to larger and more hydrophilic peptides in the following experiment. A single method for the purity analysis of human insulin and six analogs is optimized. A crown ether is also investigated as a promising additive to control the elution order of the insulins in dependence to its concentration.

In the last study, the applicability of an established chromatographic modeling software for the in-silico optimization of peptide separations in supercritical fluid chromatography is demonstrated. Optimizing a binary mixture of methanol and acetonitrile serves as an effective parameter of the model. Using such software tools offers enormous time savings and, at the same time, meets the current requirements for analytical method development according to the quality by design concept.

These already published studies are finally put into the context of the life cycle concept of analytical methods that will soon be demanded by the ICH Q14 guideline "Analytical procedure development". Existing knowledge, in the form of scientific publications, is employed as the basis for a general risk assessment applicable to peptide separations with supercritical fluid chromatography. From this, a generic method development strategy is derived and presented. This way, the current state of science and technology is taken into account to define an efficient workflow. This is particularly relevant in early pharmaceutical development phases, from where only a small fraction of initial drug candidates reaches market maturity. At the same time, this approach enhances the drug knowledge in early development phases and ultimately serves patient safety.

The present work makes an important contribution to demonstrating the potential of supercritical fluid chromatography for the analysis of peptide drugs. It serves as a concise example of how supercritical fluid chromatography can be interpreted as a new technique in the ICH Q14 guideline setting. The approach presented can be easily applied to any other analytical technique or analyte class.

Zusammenfassung

In dieser Arbeit wird das Potential der Superkritischen Fluid Chromatographie für die Analytik von Peptidwirkstoffen untersucht. Die regulatorischen Aspekte von Peptiden sind komplexer und herausfordernder, als es für kleine Wirkstoffmoleküle der Fall ist. Selbiges gilt für deren analytische Charakterisierung. Nach technischen Neuerungen und Einführung einer neuen Gerätegeneration ist die Superkritische Fluid Chromatographie heutzutage eine geeignete Alternative zur Umkehrphasen Chromatographie, welche als der Goldstandard in der Peptidanalytik gilt. Die wissenschaftliche Datenlagen zur Analytik von Peptiden mit der Superkritischen Fluid Chromatographie ist momentan jedoch limitiert.

Im ersten Teil dieser Arbeit wird eine Methode für die Superkritische Fluid Chromatographie entwickelt, um die Vielzahl an zyklischen und linearen Peptiden zu trennen, welche charakteristisch für Tyrothricin sind. Durch einen softwaregestützten Design-of-Experiments Ansatz wird eine Methode optimiert, welche der Umkehrphasen Trennung überlegen ist. Hiermit können sogar isomere Peptidpaare effizient getrennt werden.

Anschließend wird das Potential einer binären Mischung aus Methanol und Acetonitril plus Additiv als schwacher Eluent zur Trennung weiterer zyklischer Peptide untersucht. Anhand variierender Kompositionen wird die Trennung verbessert und die Elutionsreihenfolge dieser Peptide gesteurt. In Kombination mit einer aromatischen stationären Phase bietet dieser chromatographische Parameter eine effektive Methodenoptimierungsmöglichkeit.

In dem folgenden Experiment wird dieses Wissen auf größere und hydrophilere Peptide übertragen. So wird eine Multianalyt-Methode für die Reinheitsanalytik des Humaninsulins und seiner sechs Analoga entwickelt. Anschließend wird ein Kronenether als ein vielversprechendes Additiv genutzt, um in Abhängigkeit zu seiner Konzentration die Elutionsreihenfolge der Insuline zu bestimmen.

In der letzten Studie wird die Anwendbarkeit einer etablierten chromatographischen Modellierungssoftware zur in-silico Optimierung von Peptidtrennungen in der Superkritischen Fluid Chromatographie gezeigt. Die Variation

der binären Mischung aus Methanol und Acetonitril dient hier als effektiver Parameter des Models. Der Einsatz einer solchen Software bietet enorme Zeitersparnisse und stellt gleichzeitig sicher, dass die aktuellen Anforderungen an die analytische Methodenentwicklung nach dem Quality-by-Design Konzept erfüllt werden.

Diese bereits veröffentlichen Studien werden abschließend in den Kontext zum Lebenszykluskonzept analytischer Methoden gesetzt, welches zukünftig durch die ICH Q14 Leitlinie „Analytical procedure development" gefordert wird. Bestehendes Wissen, in Form von wissenschaftlichen Publikationen, wird als Grundlagen für eine allgemeine Risikobewertung für die Trennung von Peptiden mittels Superkritischer Fluid Chromatographie genutzt. Hieraus wird ein generischer Methoden-entwicklungsansatz abgeleitet. Der aktuelle Stand von Wissenschaft und Technik wird somit berücksichtigt und erlaubt die Definition möglichst effizienter Arbeitsabläufe. Diese sind vor Allem in frühen Entwicklungsphasen von Arzneistoffen relevant, bei denen nur ein Bruchteil anfänglicher Wirkstoffkandidaten die Marktreife erreicht. Gleichzeitig erweitert dieser Ansatz das Produktwissen während dieser Phasen und dient letztendlich der Patientensicherheit.

Die vorliegende Arbeit leistet einen wichtigen Beitrag dazu, das Potential der Superkritischen Fluid Chromatographie für die Analytik von Peptidwirkstoffen zu zeigen. Sie dient als anschauliches Beispiel, wie diese als eine neue Technik, im Sinne der ICH Q14 Leitlinie interpretiert werden kann. Der vorgestellte Ansatz lässt sich problemlos auf jede andere analytische Technik oder Analytenklasse übertragen.

1   Introduction

Peptides are emerging as powerful and selective pharmaceutical substances with manifold advantages spanning various therapeutic domains, including oncology and endocrinology. Regulatory demands are growing along with the rising number of peptide drug approvals. These require the application and combination of several state-of-the-art analytical techniques and the rapid adaption of innovations in the field of chemical analysis. Analyzing peptides poses inherent challenges, demanding sophisticated instrumental setups. Chromatographic methods dominate the peptide field, with reversed-phase liquid chromatography (RP-LC) standing out as the most prominent.

Supercritical fluid chromatography (SFC) experienced a renaissance following technological advancements that effectively addressed reproducibility concerns, rendering it robust and consistently reliable. These innovations supported SFC's emergence in the field of pharmaceutical analysis, broadening its utility beyond chiral and preparative separations. While early applications focused primarily on relatively lipophilic small molecules, recent scientific efforts have extended SFC's range to polar biomolecules. Nevertheless, a need remains to explore and expand the understanding of the full potential of this technique.

The development and routine use of analytical procedures and the adaption of new techniques in the pharmaceutical field are currently undergoing tremendous changes driven by the adoption of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q14 guideline "Analytical procedure development" and the United States Pharmacopeia (USP) chapter <1220> "Analytical procedure lifecycle".

This work investigates the potential of SFC's versatility and orthogonality for peptide separations and expands the currently limited knowledge on this topic. It demonstrates how peptides as challenging analytes can benefit from SFC as a new technique when aligned with today's pharmaceutical guidelines.

2   Theoretical background

2.1   Relevance of peptide therapeutics

Peptide therapeutics have a rich history in medical research, dating back to the discovery of insulin in the early 20th century [1-4]. Although pancreatic extracts could be purified to obtain endogenous insulin in the 1920s, the identification of its AA sequence took until the 1950s [1, 2]. The successful insulin synthesis paved the way for understanding its three-dimensional structure in the 1960s [1, 2]. Recombinant insulin became commercially available in the following decade, revolutionizing its production on a large and cost-efficient scale [1, 2]. Today, insulin and its analogs remain crucial in the treatment of diabetes mellitus and are included in the World Health Organization (WHO) Model list of essential medicines [5].

Insulin, while perhaps the most famous, is not the only peptide of therapeutic significance. **Figure 1** provides an overview of chosen events in the timeline of peptide drug discovery. Other endogenous hormones like bradykinin and oxytocin were discovered and synthesized successfully in the 1950s [2, 4, 6]. Based on the natural structures of these hormones, analogs (e.g. desmopressin derived from vasopressin) were developed to enhance the pharmacodynamic profiles [2, 4, 6]. Additionally, peptides with antimicrobial properties, such as tyrothricin and vancomycin, garnered substantial scientific interest at that time [7].

The potential therapeutic applications of peptides are as diverse as the biological functions they serve. Endogenous peptides are synthesized in various organs, including the hypothalamus, pancreas, adenohypophysis, and thyroid gland. Peptide hormones, such as insulin, oxytocin, and angiotensin, play pivotal roles in regulating complex human metabolic processes [4, 8]. Today, peptide-based drugs are employed in fields ranging from oncology and endocrinology to urology and gastroenterology, among many others [3, 4, 8].

Nature forms a rich reservoir for new peptide lead structures found in bacteria, fungi, insects, mammalians, etc. [3, 4]. This natural diversity continually contributes to the discovery of a growing number of bioactive compounds. Likewise, new peptide

substance classes are found, for example, cyclotides (cyclic and disulfide-rich) and lanthipeptides (linear and thioether-containing). These compounds are highly relevant as potential antimicrobials satisfying the need for new substances to overcome the increasing microbial resistance to existing products [9]. For example, teixobactin is a promising candidate for treating gram-negative bacteria. So far, no resistances were observed [10]. Just recently, zosurabalpin, an antibiotic agent active against carbapenem-resistant *Acinetobacter baumannii*, attracted great scientific interest [11].
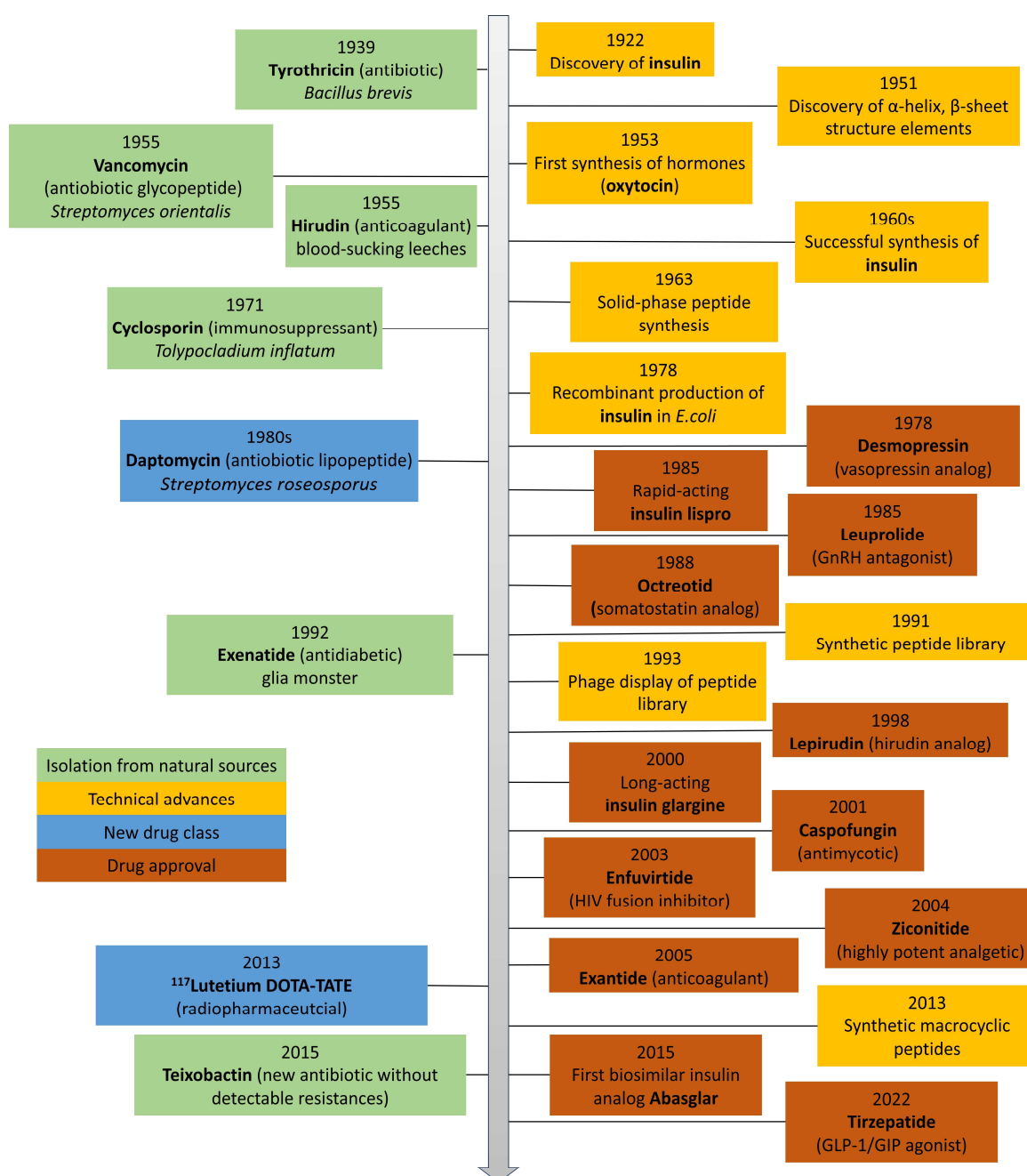

Figure 1: *Timeline of chosen events in peptide discovery [1-4, 6, 8, 12].*

Besides discovering new lead structures, peptide conjugates led to innovative therapeutic options: pegylation, lipidation, radiolabeling, or conjugation with small molecules or proteins [3, 4, 6, 8]. For example, the combination of zoptarelin and doxorubicin was granted orphan drug status for ovarian and endometrial cancer by the U.S. Food and Drug Administration (FDA). In this case, the well-established cytotoxic agent doxorubicin is attached to a peptide that binds specifically to hormone receptors, which are overexpressed in tumor cells. This targeted conjugate allows for the concentration of the cytotoxic agent within the tumor cells, enhancing its therapeutic effectiveness [13]. Similarly, Lutathera (^{177}Lu-DOTA-TATE) represents a first-in-class medication where a peptide is linked to a chelator, forming a complex with a radioisotope of lutetium. This conjugate allows the selective radiotherapy of somatostatin receptor-expressing cancer cells. Apart from their use as radiotherapeutics, such conjugates are relevant in diagnostics [14].

The growing number of innovations underlines the vast potential of peptide therapeutics and has led to a noticeable surge in drug approvals over the past decade (**Figure 2**). As of 2019, peptide therapeutics accounted for 5 % of the global pharmaceutical market, as reported by Muttenthaler et al. [4]. The database *PepTherDia* (last accessed: 03/2024) lists 119 approved peptide drugs and diagnostics in Europe, Japan, and North America [15].
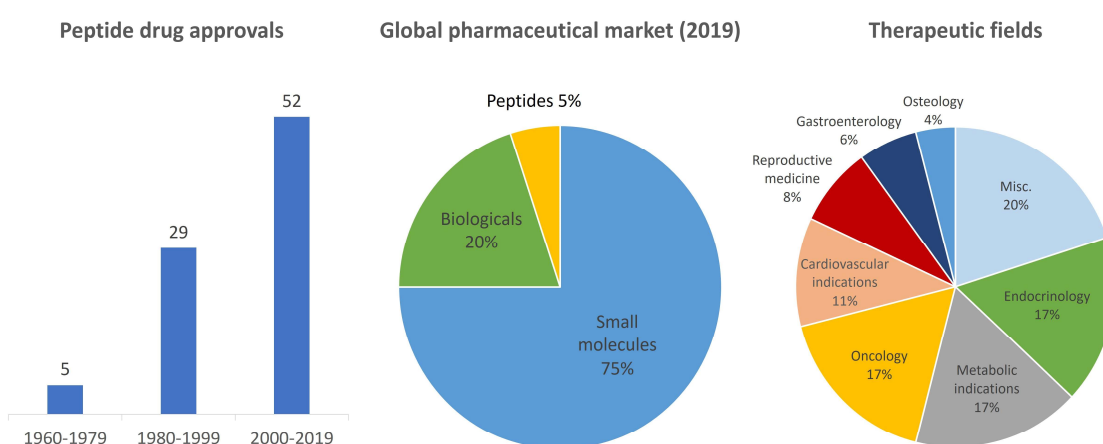

Figure 2: The relevance of peptide therapeutics indicated by the growing number of drug approvals accounting for 5 % of the global budget for pharmaceuticals in 2019 used in diverse therapeutic fields. Adapted from [4].

Generally, peptide drugs combine low side effects and immunogenicity with high potency and selectivity [16]. The pharmacokinetic drawbacks are low plasma stability and oral bioavailability, which require a parenteral application, leading to reduced patient compliance. Several strategies were established to overcome these drawbacks: AA substitution (proteinogenic or non-proteinogenic), terminus modification (N-acetylation or C-amidation), pegylation, or the insertion of lipophilic tails [3, 8, 16, 17].

Today, 21 proteinogenic L-configuration AAs, including selenocysteine, are known to be directly encoded by eukaryotes. In addition, pyrrolysine is found in several bacteria strains. Other non-standard AAs, like ornithine and homoserine, are not encoded in the DNA but result from metabolism pathways. Until now, over 800 non-standard AAs have been found to serve as building blocks for non-ribosomal peptides in bacteria, fungi, or other organisms [18]. The insertion of such non-proteinogenic or non-natural AAs (D-configuration, *N*-alkylation, α-/β-substitution, or proline analogs) is a probable option to modify the pharmacokinetic profile of a peptide drug. Another effective strategy is the cyclization of a peptide, which combines improvements in pharmacokinetics and -dynamics. The stabilization of the conformation is provided through head-to-tail, side chain-to-side chain, tail-to-side chain, or head-to-side chain cyclization. This enhances the stability of the secondary structure through the formation of loops, α-helices, or β-sheets [8]. As Zhang and Chen [19] summarized in 2022, approximately two-thirds of the approved peptide drugs are cyclic, underlining the relevance of these structure elements.

Producing therapeutic peptides involves various methods, including synthetic, semisynthetic, gene expression, or fermentation process steps. Among these, solid-phase peptide synthesis is well established for a chain length of up to 50 AAs [3, 20]. Solid-phase peptide synthesis starts by attaching the first AA to a resin through its carboxylic functionality. Subsequently, individual AAs are added one by one, following the desired sequence. Protecting groups are bound to the N-terminus to prevent the formation of impurities or self-coupling [20]. Repetitive cycles of bonding, protection, washing, and deprotection are performed to receive the target compound with a high purity. Thus, the educt and reagent quality is highly relevant to prevent the formation of unwanted byproducts.

Another approach to obtain the target peptide is the direct extraction of the active compound from their natural sources [21]. Alternatively, fermentation processes are preferred for larger molecules (greater 50 AAs), such as insulin [3]. Recombinant methods utilize bacteria, fungi, or mammalian cells to produce the desired compounds. The purification of the final product is a crucial step in the production process. Regardless of the method, several byproducts are expected and must be removed. Here, a combination of chromatographic techniques is required, which is both time and resource-intensive [22].

Traditionally, molecules consisting of 10 to 100 AAs were called polypeptides, and longer ones were defined as proteins. Nowadays, molecules greater than 50 AAs are also named proteins. The common characteristic of both is the presence of linked AAs. A major aspect of peptides is the lack of a tertiary structure, while the secondary structure is present. Indeed, these rules are not very strict. For instance, insulin (6 kDa, 51 AAs) is a compound described as a polypeptide as well as a protein in the scientific literature. This is a matter of long-running scientific and regulatory controversy. The FDA addressed this by defining a protein as a peptide consisting of 40 or fewer AAs and a protein as a polypeptide of more than 40 AAs. In addition, chemically synthesized polypeptides are defined to be 41 to 99 AAs in length [23].

2.2   Regulatory aspects

From a regulatory point of view, the differentiation between peptides and proteins matters, as other rules apply to each class. The ICH Q6B guideline defines: "proteins and polypeptides are produced from recombinant or nonrecombinant cell-culture expression systems" [24]. While the European Medicines Agency (EMA) follows this definition, the FDA differentiates based on the size of the molecules and the route of manufacture [25, 26]. A myriad of guidelines applies to pharmaceuticals in general and a few to peptides or proteins in particular. With the rise of biotherapeutic products, general drug-related guidelines were consecutively supplemented by dedicated ones to biological products, for example the ICH Q1A [27] was supplemented by ICH Q5C [28]. **Table 1** summarizes essential guidelines and monographs related to peptides and proteins.

The definition of release specifications for a drug substance (DS) or product (DP) entering the market is a crucial step in the registration process, especially regarding the drug purity. From a quality-related perspective, this requires comprehensive the knowledge about the presence and identity of relevant byproducts, intermediates, and degradants that are either process or product-related [20, 29]. In contrast to the regulatory framework for small molecules [30, 31], which is well-established and straightforward, the official ICH Q6B guideline regarding biotechnological and biological products [24] demands the applicant to provide scientific justification for setting the acceptance criteria. However, synthetic and antibiotic peptides are explicitly not covered by this guideline. The USP accounted for synthetic peptides by releasing the dedicated monograph <1503> [26] in 2021. The EMA already published a similar concept paper [32]. What should be noted is that the USP monograph only considers the DS. Nonetheless, additional specifications are required for the DP, which should be separated into release and shelf-life specifications [24]. The EMA closed the gap for antibiotics with the "Guideline on setting specifications for related impurities in antibiotics" [33], which is not exclusively applicable to peptides but explicitly refers to them. Also, a reference is made to set limits according to the pharmacopeias for monographed substances.

Table 1: *Relevant pharmaceutical guidelines and monographs for peptide products applicable in the EU and USA.*

Guideline name

- ICH Q1A(R2) - Stability testing of new drug substances and products [27]
- ICH Q1B - Stability testing: Photostability testing of new drug substances and products [34]
- ICH Q2(R2) - Validation of analytical procedures [35]
- ICH Q5C - Quality of biotechnological products: Stability testing of biotechnological/biological products [28]
- ICH Q6B - Test procedures and acceptance criteria for biotechnological/biological products [24]
- ICH Q8(R2) - Pharmaceutical development [36]
- ICH Q9(R1) - Quality risk management [37]
- ICH Q10 - Pharmaceutical quality system [38]
- ICH Q11 - Development and manufacture of drug substances (Chemical entities and biotechnological/biological entities) [39]
- ICH Q12 - Technical and regulatory considerations for pharmaceutical product lifecycle management [40]
- ICH Q14 - Analytical procedure development [41]
- EMA - Guideline on the chemistry of active substances [42]
- Ph. Eur. 2034 - Substances for pharmaceutical use [43]
- Ph. Eur. 2.2.55 - Peptide mapping [44]
- Ph. Eur. 5.10 - Control of impurities in substances for pharmaceutical use [45]
- EMA - Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: Quality issues [46]
- EMA - Guideline on non-clinical and clinical development of similar biological medicinal products containing recombinant human insulin and insulin analogues [47]
- EMA - Guideline on setting specifications for related impurities in antibiotics [33]
- EDQM- Technical guide for the elaboration of monographs on synthetic peptides and recombinant DNA proteins [48]
- EMA - Draft guideline on the development and manufacture of synthetic peptides [32]
- USP <1055> Biotechnology-derived articles - Peptide mapping [49]
- USP <1086> Impurities in drug substances and drug products [50]
- USP <1220> Analytical procedure lifecycle [51]
- USP <1503> Quality attributes of synthetic peptide drug substances [26]
- USP <1504> Quality attributes of starting materials for the chemical synthesis of therapeutic peptides [52]
- FDA - Guidance for industry - ANDAs for certain highly purified synthetic peptide drug products that refer to listed drugs of rDNA origin [53]
- FDA - Guidance for industry - Quality considerations in demonstrating biosimilarity of a therapeutic protein product to a reference product [54]
- PF 49(5) - Proposal for a new general chapter <1060> Mass spectrometry-based multi-attribute method for therapeutic proteins [55]

The critical quality attributes of a pharmaceutical product need to be evaluated during the pharmaceutical development [36]. These define acceptance criteria that form the specification as the foundation of the quality of the pharmaceutical product. These quality matters are directly connected to a drug's efficacy (labeled content) and safety (limit of impurities). Due to the highly selective pharmacodynamic profile of a peptide, extensive impurity profiling is necessary to ensure its quality. A high potency can be expected not just for the main compound itself but also for its impurities [56]. Thus, the characterization of the impurities plays an outstanding role in the development process and shall be emphasized early to assure that observed effects are related to the correct structure. This helps to avoid false positive or negative test results due to impurities affecting the drug action [56-58]. Later on, this knowledge helps to prevent fluctuations in the pharmacological profile due to a higher batch-to-batch consistency of the production process [24]. Likewise, this is relevant to provide stable products and define adequate distribution, storage, preparation, and application conditions. Thus, the stability-indicating profile [28] must be evaluated through forced degradation studies that are crucial for developing stable products [59].

General valid limits for impurities in peptides are still missing apart from those defined in individual monographs in the pharmacopeias. According to the general Ph. Eur. chapter 2034 [43], a reporting (> 0.1 %) and identification (> 0.5 %) threshold is set for synthetic peptides. However, biological, biotechnological, and fermentation products are out of the scope. In contrast, the FDA considers limits on a case-by-case basis, as defined in the USP chapter <1503> [26]. According to the FDA's approval path for generic products containing synthetic peptides [53], impurities are limited to < 0.5 % and need to be identified > 0.1 %, as long as immunogenicity and biological activity are not affected. In contrast, proteins and polypeptides produced from recombinant or nonrecombinant cell-culture expression systems fall under the requirements of the ICH Q6B guideline [24], which lacks explicit numeric values.

When dealing with peptides or proteins, as in the case of antibiotics, the pharmaceutical activity is often not related to a single compound but to a composition of different substances. As a result, the entirety of these is defined as the DS. Such compounds are often produced through fermentation processes and underly a

particular variance. Thus, the distribution of the individual compounds is of interest, and the fingerprint is a major quality attribute next to the impurity profile.

In conclusion, all relevant guidelines [24, 26, 28, 33, 53] demand an extensive evaluation of the drug and its impurity profile by applying a set of orthogonal and complementary methods and enforce the adoption of state-of-the-art technology. To streamline and fasten the adoption process of such techniques, an international guideline became effective in 2023 (ICH Q14 [41]), and a new monograph was released for the US market (USP chapter <1220> [51]). Both define knowledge management, risk assessment (RA), and sound science as the key enablers in the development of analytical procedures.

2.3   Analytical challenges in peptide separations

To fulfill the regulatory requirements regarding the quality (identity, assay, and impurities) of peptides, these are typically analyzed utilizing chromatographic techniques. Predominantly, reversed-phase liquid chromatography (RP-LC) is used. Ion exchange chromatography (IEX), hydrophilic interaction chromatography (HILIC), or capillary electrophoresis (CE) are potential alternative or complementary techniques but often lack the required resolution and sensitivity compared to RP-LC [60]. For larger molecules like insulin, where aggregates play an essential role [61], size exclusion chromatography (SEC) is also applied. Peptide mapping, used for the characterization of enzymatically digested proteins, utilizes a combination of the abovementioned techniques to analyze the resulting peptides.

While identity and assay are relatively simple to be determined, purity testing is much more challenging. Developing a suitable analytical method for the determination of impurities demands comprehensive knowledge about the presence of compounds next to the main product. Especially in early development steps, the number and identity of compounds are unknown, and reference material is often unavailable. Apart from process byproducts, degradants need to be considered [24, 28, 29]. Stability and forced degradation studies [59] enforce the formation of impurities that might form during storage. These studies are usually performed during early development stages when authentic long-term stored samples are unavailable. Thus, extensive chromatographic studies are required, and orthogonal and complementary techniques are of significant interest.

In the case of complex peptide therapeutics like antibiotics, the characterization of the fingerprint of all substances is essential. Combining several techniques again is desirable to create a comprehensive physicochemical picture of the drug. This also helps to avoid hidden and coeluting peaks that otherwise might be missed. The same is relevant during natural product isolation and screening, where highly complex mixtures must often be separated.

Chromatographic peptide separations are challenging due to the high chemical similarity of the analytes and their impurities. These include isomeric forms and

insertions, deletions, or exchanges of single AAs [29, 62]. Thus, long and flat gradients or even isocratic steps are often required to ensure a proper separation. Mass spectrometry (MS) is popular besides UV detection. Due to their low mass resolution capabilities, the widely-used single quadrupole MS systems cannot distinguish between isomers and isobaric compounds [63]. Frequently observed deamidation products of peptides form masses of the main compound incremented by +1 Da. Overlaps with isotopes of the main product then cannot be identified via single quadrupole MS systems and must be separated chromatographically, especially when present at low levels [62]. Tandem (triple quadrupole) MS instruments can help to overcome these issues through analyte fragmentations, which provide more sophisticated structure data. Also, more cost-intensive high-resolution MS (HRMS) systems are suitable for the characterization of peptides. Otherwise, online or offline 2D-LC provides increased peak capacity and orthogonality, which aids in the detection of hidden compounds [63-65].

Eluent additives (phosphate buffers) that often provide superior chromatographic performance in RP-LC are nonvolatile and not MS-compatible. Therefore, these must be removed before MS detection [66, 67]. The substitution for formic acid (FA) or trifluoroacetic acid (TFA) is an alternative that, as a drawback, frequently results in worse chromatographic performance or causes ion suppression during MS detection.

Supercritical fluid chromatography (SFC) might help to overcome some of these issues by using MS-compatible additives and providing orthogonal selectivity to RP-LC [68, 69]. These properties make it a promising candidate as an alternative or 2D coupling technique to RP-LC [70, 71].

2.4 Supercritical fluid chromatography

Supercritical fluid chromatography relieved a renaissance in analytical applications in the last decade due to technical advances that enable more robust and reproducible separations [72, 73]. Its advances over RP- or normal phase (NP) LC have made it viable for preparative and chiral separations [74-76]. The possibility of solvent reduction attracted additional attention, as it can help to fulfill today's requirements in green chemistry, especially for the preparative scale-up [77]. For SFC compressed carbon dioxide (CO_2) is used as the main part of the mobile phase. Besides its low critical point (**Figure 3**), which made it the most dominant solvent in SFC, its advantages are miscibility to many organic solvents, UV transparency, low costs, easy availability, low toxicity, and non-flammability.

In the early years of SFC, CO_2 was used with low amounts (1-5 %) of modifier added. Methanol (MeOH) is the most popular modifier, but ethanol (EtOH), 2-propanol, or acetonitrile (ACN) also see application. Such modifiers increase the mobile phase polarity and density, which enables the analysis of highly lipophilic compounds. Successively, the modifier content was increased, and additives (ammonium hydroxide, ammonium formate, TFA, FA, water, etc.) were introduced to enable the elution of basic and acidic compounds [78-80]. At the same time, this increased the accessible polarity range further and further. This way, highly polar AAs, sugars, lipophilic prostaglandins [81, 82], or even water and fat-soluble vitamins [83] can be covered in a single chromatographic run.

The expansion of the polarity range nowadays makes SFC an exciting alternative to RR-LC for pharmaceutical applications, as most small molecule drugs are rather polar to meet Lipinski's "Rule of Five" [84]. In addition, biopharmaceuticals, such as highly polar peptides and nucleotides, gained interest as analytes for SFC [79, 85, 86]. Today, gradients from 0-100 % modifier are run using up to 5 % water in MeOH, including an additive as the modifier [87, 88]. Due to high modifier percentages, the supercritical state is not maintained over the major part of the analysis (**Figure 3**) [72]. Thus, the term supercritical fluid chromatography is not clearly accurate. Nevertheless, "SFC" is accepted when related to the instrumental setup of these applications: a CO_2 and a

modifier pump combined with a backpressure regulator maintaining the supercritical or liquid state. Alternatively, "unified chromatography (UC)" is used when running from the low to the high modifier range [69, 86, 89-92].
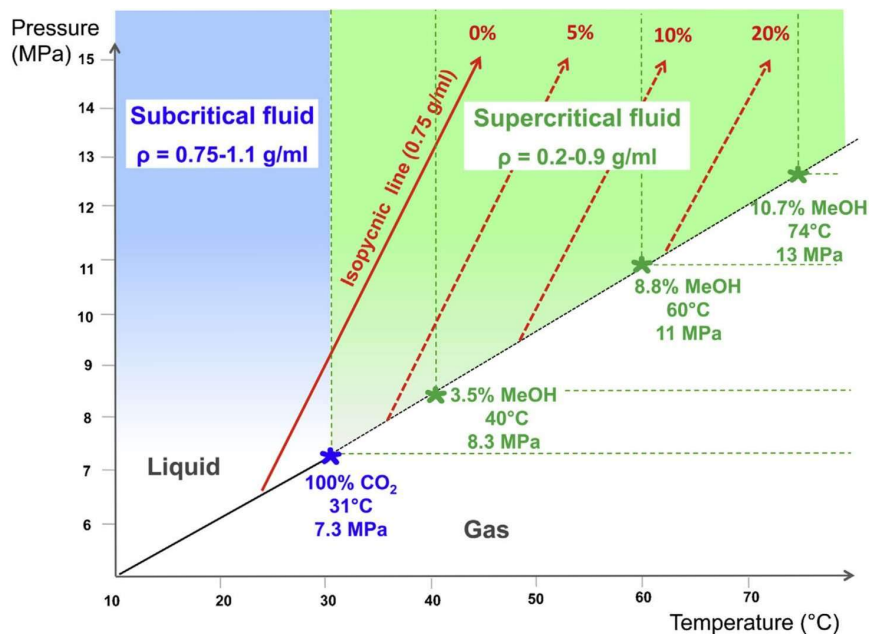

Figure 3: *Phase diagram of pure CO_2 (blue) or in mixtures with MeOH (green). The dashed red arrows indicate isopycnic lines. Reprinted with permission from [72].*

Besides SFC, enhanced fluidity liquid chromatography (EFLC) should be named as it exhibits differing characteristics [93] but relies on identical instruments. It is defined as adding gas to a liquid as the eluent to lower viscosity and backpressure. Adding water to MeOH is limited to about 7-10 % due to immiscibility with CO_2 [78, 94]. In EFLC, a lower proportion of CO_2 (up to 40 %) is added to a HILIC-like eluent consisting of MeOH, ACN, water, and additives. This allows the elution of highly polar compounds like oligosaccharides [95] or glycoproteins [96].

Recent studies even further expanded the polarity range of SFC by applying a dual gradient mode [89, 97]. Adding a second modifier pump provides a dynamic modifier composition that bridges the miscibility gap between high-modifier SFC and EFLC. This dual gradient UC can be described as the transition from SFC over EFLC to HILIC in a single run (**Figure 4**).
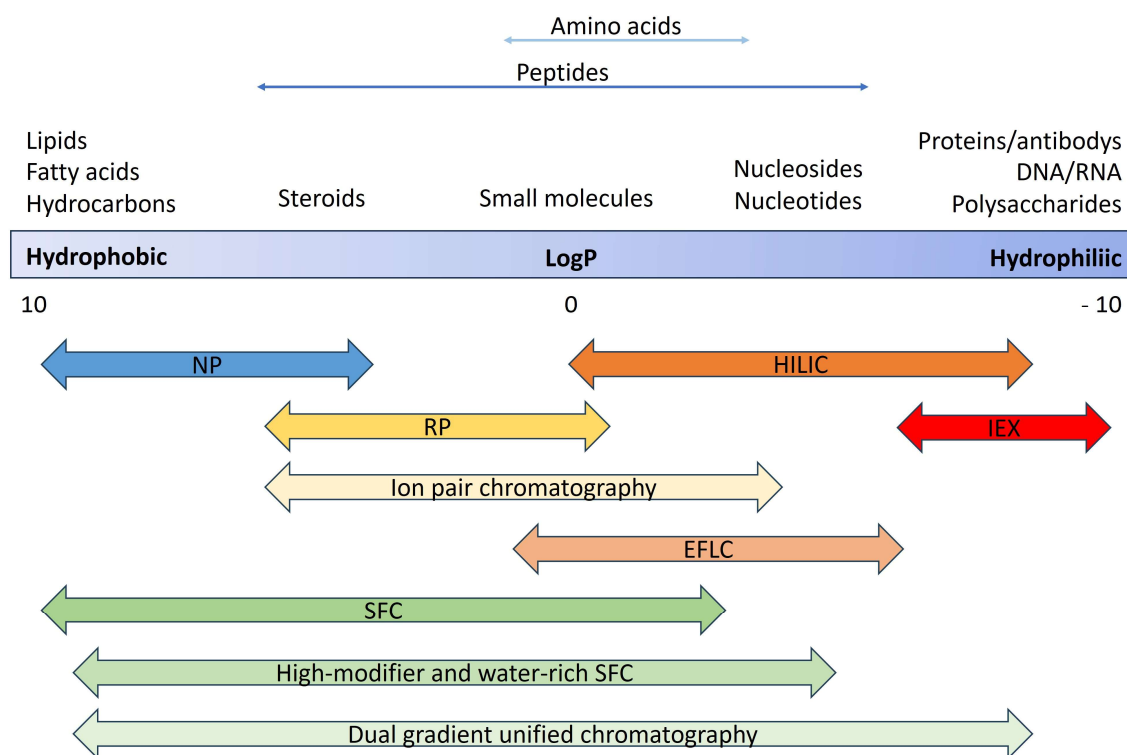
Amino acids

Peptides

Lipids
Fatty acids
Hydrocarbons   Steroids   Small molecules   Nucleosides Nucleotides   Proteins/antibodys DNA/RNA Polysaccharides

Hydrophobic   **LogP**   **Hydrophiliic**

10   0   - 10

NP   HILIC

RP   IEX

Ion pair chromatography

EFLC

SFC

High-modifier and water-rich SFC

Dual gradient unified chromatography

Figure 4: Schematic polarity range accessible via SFC instruments in contrast to other separation modes.

The range of applicable column chemistries for SFC is as wide as the range of analytes. Diverse stationary phases (SP) were adapted from other chromatographic modes (NP, HILIC, RP, or IEX) [98]. Successively, these were supplemented by SPs dedicated to SFC. The column characterization protocol established by West et al. [99, 100] assists in selecting an adequate SP for a specific analyte based on the favored retention interactions (**Figure 5**).

The retention mechanisms in SFC are complex, and the driving effects are determined through the combination of additives [101, 102], solvent type, and SP [103]. Thus, grasping the mobile phase characteristics is essential for understanding the retention mechanisms. The formation of alkyl carbonic acids when using pressurized alcohols and CO_2 was proposed by Gohres et al. [104]. In addition, hydrogen carbonate salts in water-rich modifiers were shown to offer chaotropic effects. These enhance the peak shape of polar compounds [105]. Apart from MeOH, CO_2 can also react with the analyte or the SP, whichs alters the retentive interactions [106].
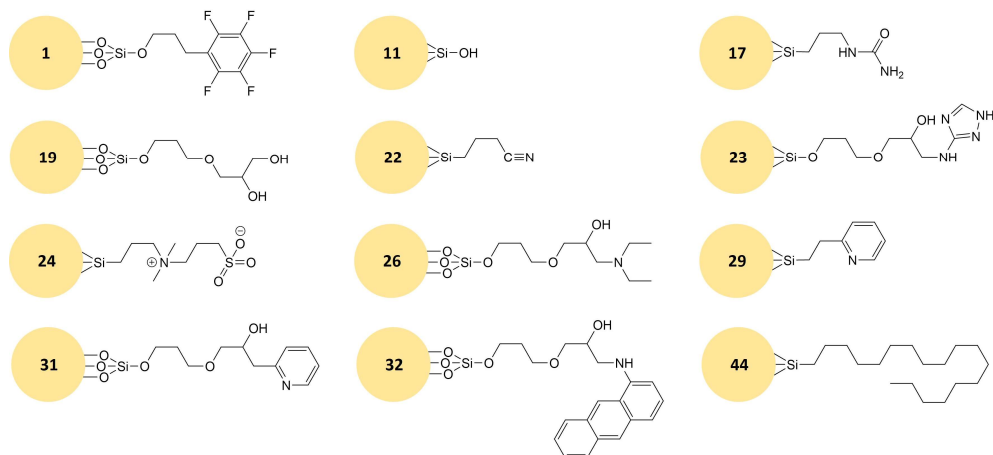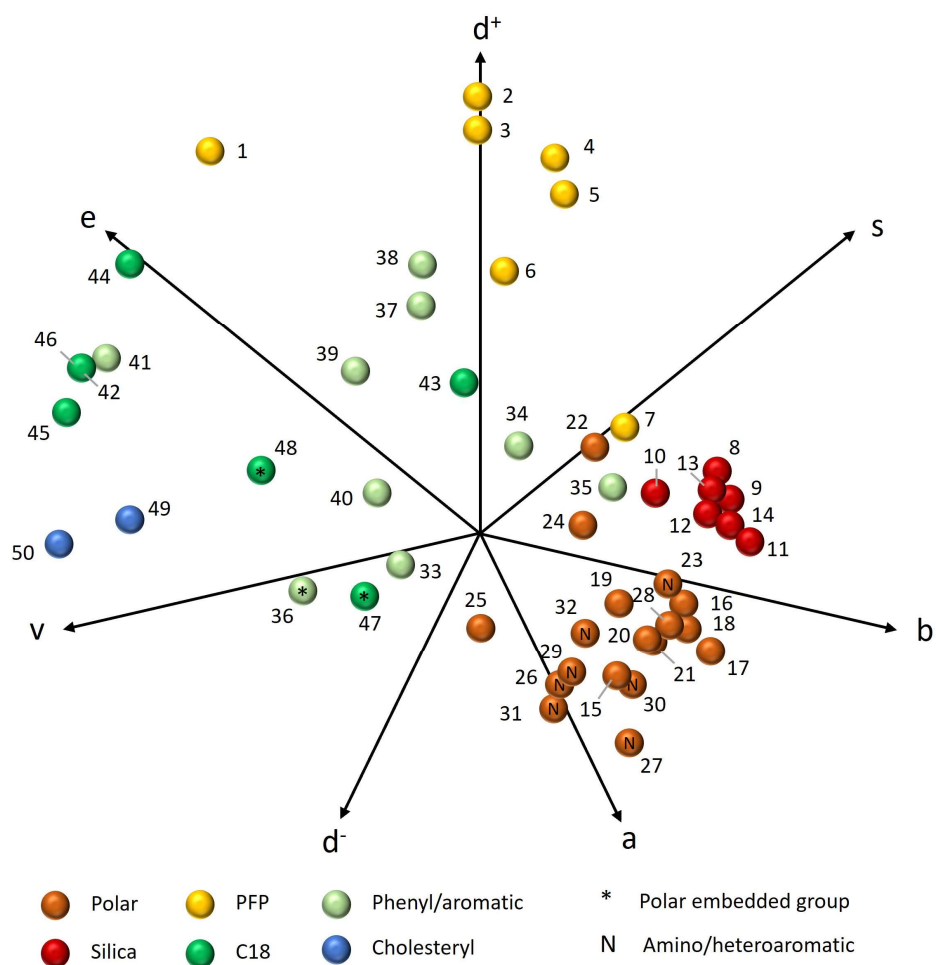
Figure 5: *Characterization of commercially available stationary phases covering a broad range of ligands. The axes correspond to the Abraham descriptors [107] accounting for π-π and Van der Waals (e), dipole-dipole (s), hydrogen bonding (a, b), and dispersive interactions (v). These were supplemented for anionic (d⁻) and cationic (d⁺) interactions. Adapted from [99]. Exemplary structure formulas of selected SP ligands are shown at the bottom. See Appendix for more detailed information on the individual dots.*

Another work reported an "apparent pH" of 4-5 for a mixture of CO_2/MeOH 90:10 (v/v) without additives [102]. The addition of TFA resulted in an "apparent pH" of 1-2. In contrast, the basic range could not be reached. Even after adding high amounts of isopropyl amine (> 100 mM), the "apparent pH" did not exceed 5-6. This study provides essential data to understand the relevance of protonated amino- and carboxyl- functionalities that determine the retention of polar analytes under SFC conditions. In conclusion, additives are necessary to control the charged state of chemical functionalities of analytes and SPs [80].

Ion pair (IP) formation also plays a significant role in this concept [108]. In contrast to RP-LC, where IP reagents are added to increase the retention of charged compounds, the opposite is present in SFC. Alkyl sulfonates can be added to decrease the retention of amino compounds [109, 110]. Methanesulfonic acid (MSA) and TFA engage in this concept as they are both strong acids providing protonated carboxy and amino groups. They are also anionic counter ions and can form IPs with basic functionalities of the analyte or the SP [72, 102, 111].

Apart from the interactions between analyte and additive, those between additive and SP must be respected. Adsorption effects of the additive [101], as well as the modifier depending on the polarity of the SP [112], are present and impact the interaction of the SP and the analyte [101, 103]. The adsorption of MeOH was studied by Glenne et al. [112], and water adsorption was discussed to provide partition and adsorption mechanisms that resemble those in HILIC [72, 113]. Both are altering the retention of an analyte. These effects are even more complex in gradient mode separations, as the adsorption is dynamic throughout the gradient. It is modified when water increases the adsorbed layer thickness and thus modulates selectivity [103, 105]. Overall, water increases the polarity of the modifier, allowing the elution of polar compounds with a higher efficiency [114], which is best explained by the chaotropic effects of hydrogen carbonate [105].

In summary, the interactions present in SFC running a modifier gradient from 0-100 % are a mixture of RP, NP [115], HILIC [115], and IEX [116], depending on the nature of the SP and modifier, including the additives. These complex interactions are not yet all understood, and much more research is necessary. Nevertheless, the

studies summarized form the fundament for further research on the possibilities of SFC in the separation of biopharmaceuticals, such as peptides.

The comparison of RP-LC or SFC and the question of which technique is better was studied by several researchers [68, 69, 111, 117-119]. However, neither one was superior in all cases. Both hold their advantages and drawbacks over each other. Instead, they must be considered orthogonal and complementary.

2.5   The current state of peptide separations in SFC

One of the first studies dealing with the SFC analysis of peptides was published in 1988 and described the successful retention and elution of ciclosporin A [120]. A decade later, another work studied a set of five peptides with differing chain lengths from 5 to 9 AAs. The necessity of sulfonic acids providing IP and acidic characteristics was demonstrated to elute the analytes, where TFA or sulfonates failed [121]. At the beginning of the 2000s, another experiment successfully utilized SFC for various peptides (2 to 20 AAs) and for the purification of gramicidin [76]. Subsequently, SFC was used to separate mono- and dimeric forms of gramicidin [122, 123]. This knowledge was then transferred to the separation of proteins up to 40 kDa [124].

While NP-type (cyano or silica) SPs were adopted for the previous studies, a modern SFC-dedicated (pyridine-type) SP was combined with TFA for the SFC-MS analysis of longer chain peptides up to 40 AAs by Zheng et al. [125]. The authors identified basic functionalities to determine the strong retention of large peptides. In 2011, Patel et al. reported the advantages of pyridine-type SPs for the separation of isomeric peptide pairs [126]. In a follow-up work, the same authors showed the beneficial addition of 5 % water to MeOH [127]. Gramicidin as a model substance was again used to verify the benefits of water as an additive, with gradient elutions being more robust than isocratic separations [128]. In a systematic approach, Shao et al. evaluated the impact of the SP, modifier, additive type, column temperature, and backpressure on selectivity for five cyclic ciclosporins [129].

Molineau et al. reviewed the available literature until 2020 [79]. It can be stated that until then, most publications demonstrated the general applicability of SFC for the separation of chemically diverse peptides. Starting in 2021, more refined investigations that used state-of-the-art instrumentation were published. These provide more comprehensive data on diverse combinations of additives and modern SFC-dedicated SPs. Also, peptides showing highly similar structures were more often studied.

Losacco et al. found neutral SPs (diol, silica, and sulfobetaine) to perform best with a basic additive (ammonium hydroxide) and basic SPs (DEA and 2-PIC) with acidic additives, respectively [111]. The importance of an extensive column screening was

further demonstrated by other authors involving diverse SP chemistries and additives [90, 111, 130, 131]. It should be noted that the favored SPs of these studies can be found in the range of silica and polar SPs shown in **Figure 5**. The addition of sulfonic acids improved the separation of AAs [92, 132] and peptides [111], especially larger basic ones [133]. In most newer studies, water was used as an additive [68, 69, 90, 92, 105, 111, 132, 133]. Hydrogen carbonate formed under these conditions provides buffering and chaotropic effects, which are highly beneficial in separating polar analytes [105]. In combination with the mobile phase acidity, which determines the protonation of the amino- and carboxy-functionalities of analyte and SPs [80, 102, 111], this explains the benefits of IP reagents.

As in small molecule analysis [98], comparing SFC and RP-LC and the question of which one is better is of central interest for peptides. In 2022, Molineau et al. [69] compared the suitability of SFC and RP-LC for the purity testing of 43 peptides (3 to 5 AAs). In contrast to RP-LC, SFC offered a more suitable retention time window for the small peptides and retained those unretained in RP-LC. The purity profile of 12 peptides was compared afterward. Both techniques performed equally, considering the number of peaks > 1 %. However, more peaks between 0.1 and 1 % were detected with RP-LC due to superior efficiency and UV-sensitivity. It should be noted that different column formats for SFC (150 x 4.6 mm, 2.7 μm superficial porous particles) and RP-LC (100 x 2.1 mm, 1.7 μm fully porous particles) were employed. Nevertheless, the evaluation of the MS data showed that some impurities and isomeric peak pairs were resolved with SFC, but not with RP-LC and vice versa.

Soon after, a more comprehensive study was published comparing RP, HILIC, mixed mode chromatography (MMC), and SFC [68]. This time, more equal column formats (SFC: 100 x 3.0 mm, 1.7 μm, and LC: 100 x 2.1 mm, 1.7 μm) were used to separate 13 peptides (3 to 30 AAs). A higher versatility for a broader polarity range and advantages for small peptides were observed for SFC. Regarding UV sensitivity, all modes were equal, but a higher MS sensitivity was noted for SFC. However, SFC performed worse than the other modes for the separation of six highly similar decapeptides.

In contrast to these results, Ventura et al. efficiently separated a 39-mer peptide and three impurities differing in a single AA position with SFC [132].

A high level of orthogonality of SFC and RP-LC for diverse peptides is reported in several works [68, 69, 111] that is highly beneficial during stress testing [111]. As a rule of thumb, the retention in both modes increases with the chain length of the peptide when acidic additives are used. While in SFC, elution certainly increases with the chain length, the elution order in RP-LC instead follows the increase in the isoelectric point of the analytes. In both modes, selectivity changes significantly when a switch from basic to acidic additives is performed [68, 111].

The preparative purification of peptides and proteins via SFC is desirable to substitute the proportion of water in RP-LC for CO_2. Less solvent evaporation at lower temperatures fastens these process steps and enhances sample stability. The maintenance of the structure from pre- to post-purification is mandatory. Schiavone et al. showed that bradykinin and insulin (1 to 6 kDa) regained their higher-order structure post-purification, but larger proteins up to 17 kDa did not [134]. Another working group successfully purified insulin and other smaller peptides (4 to 9 AA), saving 75 % of runtime and 50 % of waste compared to RP-LC [135, 136]. These studies underline the tremendous potential of preparative SFC in terms of sustainability.

In conclusion, fewer than 40 papers dealing with peptide separations in SFC have been published. The technique is applicable in the analytical and preparative scale, providing orthogonality to RP-LC. The combination of polar SPs and water in MeOH forms the best conditions for method optimizations so far. Just a few studies evaluated the potential of SFC for the separation of peptides of high similarity, with inconsistent results reported. The same applies to the comparability of SFC and RP-LC. Thus, the overall knowledge is limited and needs to be expanded.

These facts form the starting point for the studies presented in this paper, which are intended to further elaborate the potential of SFC for the separation of peptide therapeutics. A set of antibiotic peptides was chosen as appropriate model substances. Initially, tyrothricin, a complex of about 20 compounds, including cyclic, linear, and isomeric peptides covering a mass range from 1200 to 1900 Da, is utilized. These structures are majorly characterized by hydrophobic sidechains with the linear fraction forming gramicidin. Consecutively, bacitracin, colistin, and daptomycin (1000 to 2000 Da) are added to enclose peptides rich in acidic and basic sidechains. Insulin and its

analogs are then finally included to increase the mass range to hydrophilic small proteins.

3   Manuscripts

3.1   Manuscript I: Application of sub-/supercritical fluid chromatography for the fingerprinting of a complex therapeutic peptide

Jonas Neumann, Sebastian Schmidtsdorff, Alexander H. Schmidt, Maria K. Parr

Abstract: The application area of supercritical fluid chromatography expanded tremendously over the last years and more polar analytes such as biomolecules have become accessible. The growing interest in biopharmaceuticals and associated regulatory requirements demand alternative analytical tools. The orthogonal nature of supercritical fluid chromatography compared to reversed-phase liquid chromatography meets these needs and makes it a useful option during research and development. In this study, we present a systematic approach for the development of a supercritical fluid chromatography method for fingerprinting of tyrothricin, a complex therapeutic peptide covering a mass range from 1200 to 1900 Da. The substance was chosen due to the presence of cyclic and linear peptides and isomeric or highly similar amino acid sequences. Different column chemistries covering neutral, basic, and zwitterionic functionalities in combination with acidic, basic, and neutral additives were screened. Subsequently, Design-of-Experiments principles were utilized to perform optimization of the chromatographic parameters. The final mass spectrometry-compatible gradient method using a diol stationary phase, carbon dioxide, and a modifier consisting of methanol/water/methanesulfonic acid (100:2:0.1, v:v:v) was found to provide orthogonality and superior resolution to other methods published. Isomeric peptide compounds coeluting in reversed-phase liquid chromatography were resolved by applying the final method.

RESEARCH ARTICLE

JOURNAL OF SEPARATION SCIENCE

Application of sub-/supercritical fluid chromatography for the fingerprinting of a complex therapeutic peptide

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The application area of supercritical fluid chromatography expanded tremendously over the last years and more polar analytes such as biomolecules have become accessible. The growing interest in biopharmaceuticals and associated regulatory requirements demand alternative analytical tools. The orthogonal nature of supercritical fluid chromatography compared to reversed-phase liquid chromatography meets these needs and makes it a useful option during research and development.

In this study, we present a systematic approach for the development of a supercritical fluid chromatography method for fingerprinting of tyrothricin, a complex therapeutic peptide covering a mass range from 1200 to 1900 Da. The substance was chosen due to the presence of cyclic and linear peptides and isomeric or highly similar amino acid sequences. Different column chemistries covering neutral, basic, and zwitterionic functionalities in combination with acidic, basic, and neutral additives were screened. Subsequently, Design-of-Experiments principles were utilized to perform optimization of the chromatographic parameters. The final mass spectrometry-compatible gradient method using a diol stationary phase, carbon dioxide, and a modifier consisting of methanol/water/methanesulfonic acid (100:2:0.1, v:v:v) was found to provide orthogonality and superior resolution to other methods published. Isomeric peptide compounds coeluting in reversed-phase liquid chromatography were resolved by applying the final method.

KEYWORDS
fingerprinting, orthogonal separation, peak capacity, supercritical fluid chromatography, therapeutic peptide

1 | INTRODUCTION

Analytical SFC experienced a remarkable comeback over the last few years. Its application area was expanded tremendously from rather nonpolar to more polar molecules allowing the separation of a broad polarity range using a single gradient [1, 2]. Polar biomolecules (nucleotides and peptides) thus became accessible [3]. The growing interest in peptides used as therapeutics [4] comes with a growing need for analytical tools that can be used in quality control. Currently, RP-LC is the central technique in this area. Complementary knowledge about the presence of additional compounds is of primary interest, especially in the early stages of the development of pharmaceuticals. An orthogonal separation of a specific sample providing this information often is hardly achieved by applying RP-LC exclusively but can be generated by SFC [5].

Various working groups reported the use of SFC for the analysis of peptides. Cyclosporine [6], gramicidin [7–9], mixtures of chemical diverse peptides [10–13], or isomeric peptide pairs [14, 15] were used to demonstrate its potential. Even longer chain peptides up to 40 amino acids [9, 12] or larger glycoproteins up to 80 kDa were separated successfully [16, 17]. Most recently, an optimization approach of a purity method for a set of 76 linear and cyclic short-chain peptides (< 800 Da) was published. The authors utilized Derringer's desirability functions to define the overall best conditions for a single method covering all 76 peptides based on a limited number of experiments [18]. The orthogonality of RP-LC and SFC was studied subsequently for 43 of these peptides by the same group demonstrating an advantage of SFC over RP-LC due to less coelution and stronger retention. On the other hand, smaller additional peaks were detected via RP-LC [19].

In SFC, carbon dioxide is used as the major part of the eluent. Methanol (MeOH) is often added as a modifier. Water up to 7 % can be added to the modifier to increase the modifier's eluotropic strength and enhance the chromatographic performance for highly polar substances [20]. Different additives (ammonia, ammonium acetate, and others) were demonstrated to improve the separation of biomolecules [21–23]. Carbonate and hydrogen carbonate ions in the effluent were detected when a composition of water, carbon dioxide, and ammonia was used. The carbonate ions were proposed to cause a chaotropic effect enhancing the chromatographic separation of biomolecules [21]. Recently methanesulfonic acid (MSA) became a popular additive [22, 24], even if its successful usage was already reported in 1999 [13]. Its high acidity and potential to mask amino functionalities of peptides were hypothesized to enable separation of the same by SFC through synergistic effects if combined with amino derivatized stationary phases [24].

The general applicability of SFC for distinctly different peptides was proven and its beneficial use for the purification or isolation of single compounds of interest was demonstrated [23, 25]. However, very few studies have focused on methods providing sufficient selectivity to fulfill today's requirements for the application as a purity control method or the identification of a fingerprint of a complex therapeutic peptide.

With this work, we contribute to the application of SFC in peptide analysis and demonstrate its advantages. The complex peptide antibiotic tyrothricin, manufactured through fermentation processes, was chosen as an ideal model. It contains two groups of peptides: linear gramicidins and cyclic tyro- and tryptocidines (Figure 1) [26, 27]. The high number of structures possessing a high chemical similarity and covering a mass range of 1200–1900 Da made this complex of polypeptides a good candidate for this study. A systematic method development approach was applied. After defining the goals of the chromatographic method, the potential impact of the individual chromatographic parameters was ranked and investigated accordingly. Peak capacity, resolution and peak-to-valley ratio were applied as indicators for the chromatographic performance and optimized via a Design-of-Experiments (DoE) workflow. Finally, the optimized method was compared to a published RP-LC method [28], demonstrating its orthogonality and advantages.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Tyrothricin chemical reference substance supplied by the European Directorate for the Quality of Medicines (Strasbourg, France) was dissolved in MeOH/water (90:10, v:v) to receive a concentration of 2 mg/ml and was used as a stock solution. MeOH and ACN (LC gradient-grade) were bought from VWR (Darmstadt, Germany). Milli-Q water was prepared freshly before use via a Merck Milli-Q system (Darmstadt, Germany) and carbon dioxide (99.995%) was derived from Air Liquide (Berlin, Germany). TFA (LC-grade, ≥99.0 %) and MSA (≥99.0%) were derived from Sigma Aldrich (Taufkirchen, Germany), aqueous ammonia solution (25%) and ammonium acetate (for analysis) were supplied by Merck (Darmstadt, Germany).

2.2 | Stationary phases

The following stationary phases were used for the SFC experiments: Torus Diol, Torus 2-PIC, Viridis BEH 2-EP from Waters (Eschborn, Germany), and a Nucleodur HILIC from Macherey-Nagel (Dinslaken, Germany). The
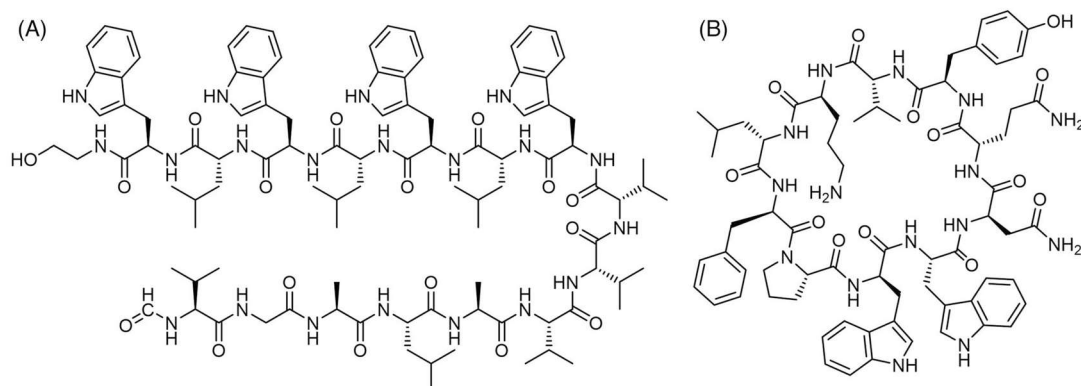
FIGURE 1 Chemical structures of the linear Val-Gramicidin A (A) and the cyclic Tyrocidine C (B) [27]

dimension of all columns was 3.0 × 100 mm. The particle size was 1.7 μm for all columns from Waters and 1.8 μm for the HILIC column. An Acquity CSH C18 column, 2.1 × 100 mm, 1.7 μm, from Waters (Eschborn, Germany) was used for the LC experiment.

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 pre-heaters, an Acquity UPC^2 photodiode array (PDA) detector, an Acquity UPC^2 convergence manager (back pressure regulator) and an Acquity TQD (triple quadrupole mass spectrometer with an ESI source). A fixed-leak interface was used to connect the SFC system to the MS and a make-up solvent was provided through a Waters 515 make-up pump. An Acquity UPLC H-Class system equipped with a quaternary pump, a column manager with active eluent pre-heaters and an Acquity PDA detector hyphenated to the same Acquity TQD mass spectrometer was used for the comparison of the SFC and UHPLC method. Empower 3 was used for system control, data acquisition, and processing. Fusion QbD from S-Matrix (Eureka, USA) was used to visualize the DoE. The 2-D graphs were calculated via Microsoft Excel.

2.4 | Development of the SFC method

Initially, a generic gradient from 25 to 95% B in 15 min was applied at a flow rate of 0.6 ml/min using MeOH/water (100:2, v:v) as eluent B. As additives 0.1% ammonia (v:v), ammonium acetate (m:v), TFA (v:v), or MSA (v:v) were used. The column temperature was set to 50°C and back-

pressure to 1500 psi. Four stationary phases were screened (see Section 2.2). Tyrothricin (1 mg/ml) was injected. In the next step, the gradient time was prolonged to 25 min (25–80% B) to receive a better separation. The gradient was applied using the four columns and additives tested before. Due to inferior performance TFA was excluded. UV detection at 220 nm was used for all experiments. The number of peaks, peak width and shape and the overall separation of the compounds were used to assess the stationary phases and additives. The best combination was kept for further optimization: the diol column and eluent B consisting of MeOH/water/MSA (100:2:0.1, v:v:v).

In the first optimization experiment, the impact of the flow rate was studied and thus varied from 0.5 to 1.1 ml/min. Backpressure and column temperature were maintained. A gradient from 25 to 64% B was run in 20 min. To better compare the performance of the individual runs, a normalized sample concentration was prepared via dilution of the stock solution to study the impact of flow rate and gradient time (10, 20, and 30 min) on the peak capacity. The ratio of the analyte concentration was adjusted equivalently to the flow rate, for example, 0.5 mg/ml was injected at a flow rate of 0.5 ml/min.

In the following experiment, the flow rate was kept constant at 0.7 ml/min. The backpressure was varied from 1500 to 2500 psi, the column temperature from 30 to 50°C and the gradient time between 23 and 30 min reaching 55% B.

2.5 | Final SFC method

A Torus Diol column (2.1 × 100 mm, 1.7 μm) was used for the final method. The column temperature was set to 41°C and the backpressure to 1500 psi at a flow rate of 0.7 ml/min. Carbon dioxide was used as eluent A.

Eluent B (modifier) consisted of MeOH/water/MSA (100:2:0.1, v:v:v) and 1 μl of tyrothricin (1.0 mg/ml) was injected. The following gradient profile was applied: 0–30 min: 25–55% B, 30–31 min: 55–25% B, 31–35 min: 25% B. Chromatograms were recorded at 220 nm using UV detection. A make-up (0.1 ml/min) consisting of MeOH/formic acid (100/0.1, v/v) was added for the SFC-MS experiments via the fixed leak interface. The MS was operated in positive ESI mode. The total ion chromatogram was recorded (m/z 500–1500) and used for peak tracking. 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.

2.6 | Orthogonal UHPLC method

For method comparison, a method was adapted [28]. A CSH C18 column (2.1 × 100 mm, 1.7 μm) was set to 40°C at a flow rate of 0.35 ml/min. Water/ACN/TFA (95/5/0.1, v/v/v) and water/ACN/TFA (5/95/0.1, v/v/v) were used as eluent A and B, respectively. The following gradient profile was applied: 0–10.5 min: 40–90% B, 10.5–11.9 min: 90% B, 11.9–12.6 min: 90–40% B, 12.6–16.8 min: 40% B. Chromatograms were recorded at 220 nm using UV detection. For MS detection, the parameters described in Section 2.5 were used.

3 | RESULTS AND DISCUSSION

3.1 | Definition of the method goals

In the beginning, goals were set for the future method: improvement of the resolution of the fingerprint, UV and MS compatibility, and orthogonality to existing methods [26–28].

The potential impact of the chromatographic parameters on the selectivity and thus the resolution of the fingerprint was ranked as follows: column chemistry > additive type > gradient time/end% B > column temperature > flow rate > backpressure. The parameters were studied based on this ranking. Column chemistry and potential additives were screened first.

3.2 | Screening of additives and column chemistry

Different stationary phases providing neutral, basic, and zwitterionic chemistries were chosen to evaluate the impact on the selectivity: diol, amino (2-EP and 2-PIC), or sulfobetaine (HILIC) derivatization. Two peak groups

(Figure 2C,D) were separated via the diol and zwitterionic derivatized stationary phase. Due to the integration of MS detection in the final step (see Section 3.3), these were identified as cyclic or linear peptides. The linear gramicidins containing alkyl or aromatic sidechains, mainly from leucine, tryptophan and valine, were eluted as the first group (retention time [Rt]: 6–8 min). The cyclic compounds containing more polar sidechains from tyrosine, asparagine or glutamine were stronger retained (Rt: > 10 min). The amino-functionalized stationary phases (Figure 2A,B) showed an inferior selectivity resulting in an elution in a smaller Rt window, which disqualified these columns for further optimization steps. Much higher retention was observed on the zwitterionic than on the diol functionality, resulting in broader peaks, which is in line with the results reported in other studies earlier for other peptides [24]. Due to the higher retention, a better resolution of the peaks of the linear group was achieved, which on the other hand, caused a worse separation of the cyclic peptides. The overall separation and the number of smaller peaks not detected on the sulfobetaine phase made the diol the better suited stationary phase.

Based on the literature summarized in the introduction, additives covering basic, neutral and acidic as well as ion-pairing characteristics were selected: ammonium acetate, TFA, ammonia, and MSA. The addition of water in a small amount was considered mandatory to improve the peak shape. An increase from 2 to 5% was also tested, but no distinct effect except slightly earlier elution and increased backpressure were observed. TFA (Figure 2A3) showed the worst performance based on the number of peaks and overall resolution detected on all columns. The other additives were comparable. Ammonium acetate (Figure 2A4) caused a baseline drift and thus was excluded. MSA (Figure 2A2) and ammonia (Figure 2A1) were comparable with MSA, resulting in a group of smaller well-separated peaks (Rt: 8.5–9.5 min) between the two groups of major peaks. Thus, MSA combined with the diol column was chosen for further optimization.

3.3 | Optimization of chromatographic parameters

The goal of the following experiment was to evaluate the impact of the flow rate and the gradient time. Initially, the effect on the selectivity of the column temperature was ranked higher than the flow rate. As the column temperature influences the backpressure and thus would have limited the flow rate to a smaller range if varied simultaneously, it was decided to study the temperature in the subsequent step. While a Van-Deemeter plot traditionally
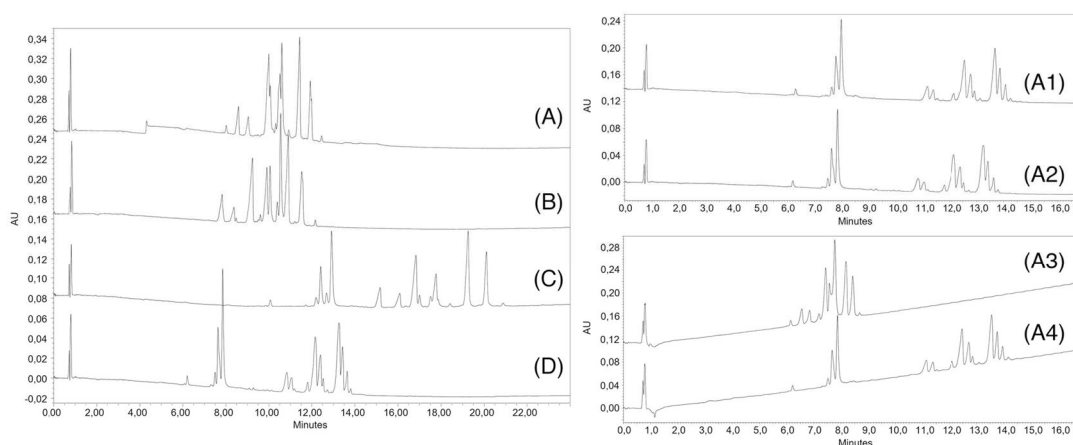
FIGURE 2 SFC-UV chromatogram of tyrothricin separated on different stationary phases ([A] 2-PIC, [B] 2-EP, [C] HILIC, and [D] Diol) using 2% water and 0.1% methanesulfonic acid (MSA) (v/v) as the mobile phase additives in modifier methanol (MeOH). A comparison of the different additives ([A1] ammonia, [A2] MSA, [A3] TFA, and [A4] ammonium acetate) in combination with the diol column is shown

is used for the characterization of isocratic methods, the consideration of the peak capacity was demonstrated to be a viable parameter for the characterization of gradient methods [29–31]. A minimum of 0.5 ml/min was necessary to maintain sufficient pressure and thus compression of the mobile phase. An unstable baseline was observed when the flow was further reduced. A flow rate of 1.1 ml/min was estimated as the maximum based on the pressure limit of the instrument used.

Representative peaks, resolved sufficiently in all chromatograms, were chosen (Figure 3A), and the peak width of the selected peaks at 50 % height ($W_{50\%}$) was calculated. The following equation, including the gradient time (tG), was used to calculate the peak capacity (P) according to Wren [32]:

$$P = 1 + \frac{tG}{1.679 \times W50\%} \tag{1}$$

The factor of 1.679 was used to calculate the width at 13.4% height from the $W_{50\%}$ peak width. This conversion was necessary to compensate for the lacking baseline separation of some peaks. The mean peak capacity was then plotted against the flow rate (Figure 3B). An increase in the mean peak capacity at higher flow rates was noted. At the same time, a logarithmic relation between the flow rate and the mean peak area was observed (Figure 3C). This effect is well known for concentration-dependent detectors such as UV [33, 34]. At a lower flow rate, the analyte remains longer in the flow cell and the response is increased. On the other hand, an increase in the detector response (either peak height

or area) results in an increased peak width. Concluding, the impact of the response on the peak width cannot be distinctly differentiated from the impact of the flow rate.

An adjustment of the sample concentration (see Section 2.4) was performed to compensate for the increasing UV response. The variation of the peak response was minimized to about 5% between the individual runs to aid comparability. As a result, the mean peak areas varied in a much smaller range, with 0.5 ml/min still giving the highest response (198 000 area and 28 000 height units) and 1.1 ml/min showing the lowest (184 000 area and 25 000 height units). This was assessed to be the more productive approach considering the goal to use peak capacity as an indicator of the performance. The optimization of the method in terms of maximizing the number of peaks and increasing their resolution, which is not impacted by a broadening of the peaks due to a higher response, was focused on. An increase in the method's sensitivity was less important at this point.

The 3-D response surface plot of the mean peak capacity (Figure 4A) shows that contrary to the observations made before, when the same sample concentration was injected for every flow rate, an increase in the peak capacity was found based on the usage of a normalized sample concentration. The lower the flow rate, the higher the mean peak capacity. The calculation of the peak capacity of peak 4 (Figure 4B) and 14 (Figure 4C), which are representative of either the first or the second group, showed converse effects of the flow rate on both peaks. A lower peak capacity caused by a broader peak was estimated for the later eluting peak. Different reasons can be hypothesized for this observation. The cyclic or linear shape and the varying
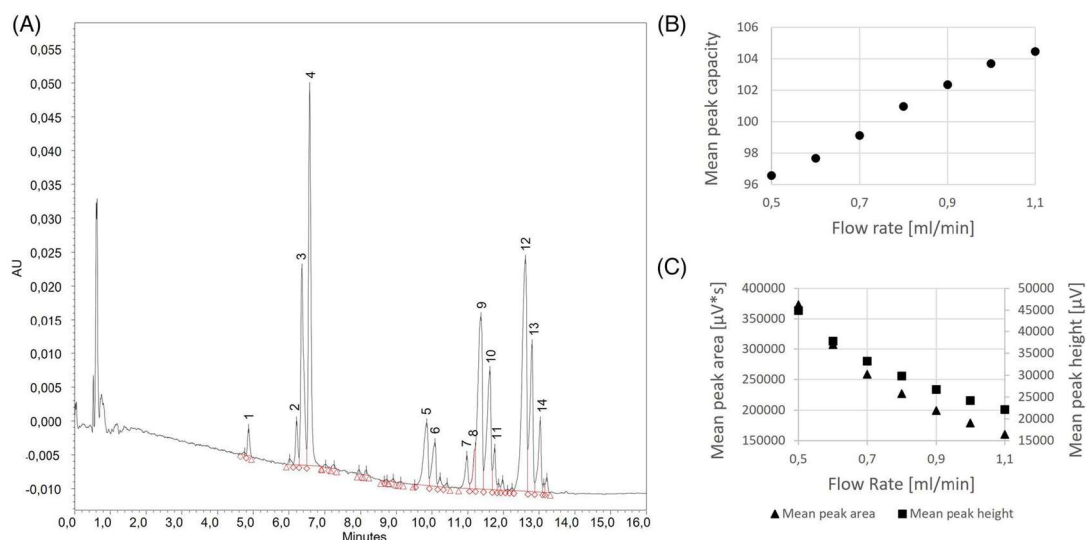
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FIGURE 3 A representative SFC-UV chromatogram is shown. The peaks 3, 4, 5, 6, 7, 9, 10, 12, and 14 provide sufficient resolution to calculate the peak width at 50% height (A). Mean peak capacity plotted against the flow rate (B). Mean peak height and area plotted against the flow rate (C)
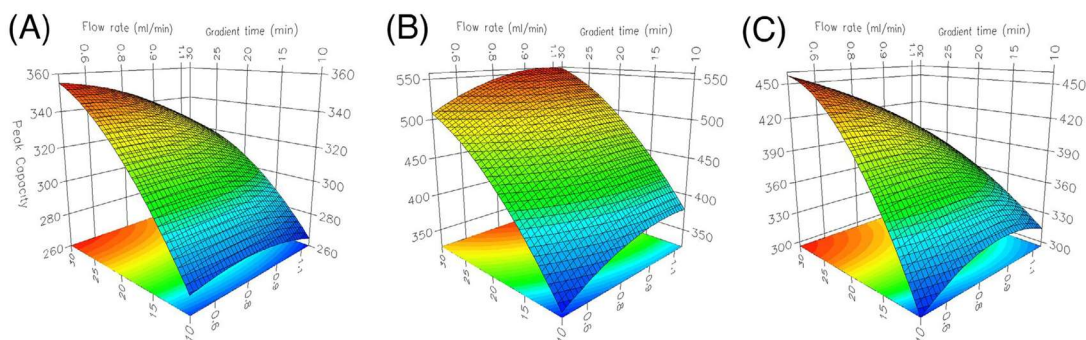

FIGURE 4 Response surface of the peak capacity ([A] mean of representative peaks, [B] peak 4, and [C] peak 14) plotted against the flow rate and the gradient time

polarity of each component caused by the different amino acid sequences can result in other interactions with the stationary phase, which may result in varying retention characteristics. A slight fronting of the late eluting peaks can also indicate the presence of coeluting peaks. An increase in the peak width and hence a decrease in the peak capacity would then convert to an improved separation. Following this idea, a further increase in the flow rate should be beneficial to resolve these peaks. However, due to the limitation of the instrument, a further increase in the flow rate was not possible.

For the development of a purity method, a certain signal-to-noise ratio needs to be achieved for relevant impurities. Thus, a reduction of the flow rate may be considered before increasing the sample load on the column. On the other hand, the sample load can also be reduced to increase the resolution of the peaks and maintain the signal intensity simultaneously.

Utilizing MS was also considered as an alternative to evaluating the peak width. The instrument design demands a flow split to minimize the carbon dioxide decompression before entering the MS. Adding a make-up flow prevents the analyte's precipitation and reduces the eluent's decompression. A variation of the split ratio based on the flow rate is known [35], which would add more factors affecting the analyte's response. After revisiting these factors, MS detection was discarded to evaluate peak capacity. Alternative evaporative detection
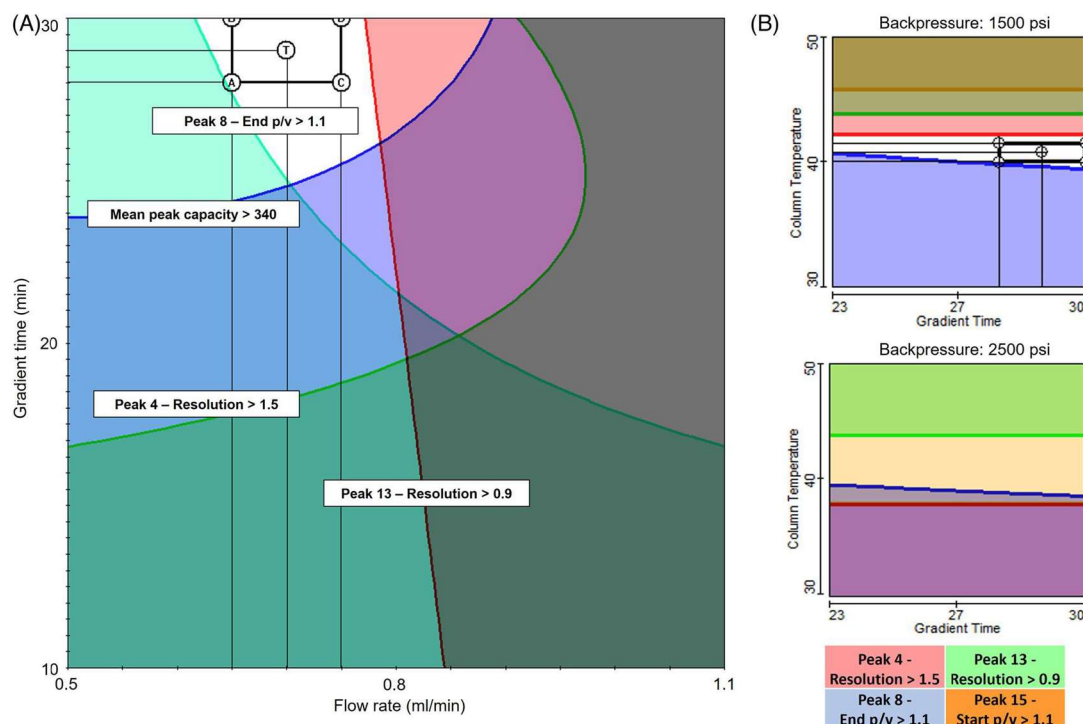
FIGURE 5 Visualization of the impact of the chromatographic parameters gradient time and flow rate (A) and column temperature, backpressure, and gradient time (B) on the separation of critical peak pairs. Colored areas indicate conditions under which the defined goals were not achieved (note that mixed colors are possible). Therefore, white areas indicate conditions under which the best overall separation was achieved. The black box indicates conditions used for further optimization

techniques, for example, evaporative light scattering detection, also demand a split of the flow and consequently are impacted by the same factors.

However, additional performance indicators than peak capacity, which do not necessarily reflect a better resolution, then were used. Thus, the resolution between peaks 3 and 4 was chosen as an indicator for the separation of the first group and the resolution between peaks 12 and 13 for the second group. In addition, the end peak to valley ratio of peak 8 with 9 was used. Minimum values to be reached were set and maximization of these was defined as goals for the DoE (Figure 5A). For the improvement of the separation of the first group, lower rates were beneficial, but higher flow rates increased the second group's resolution. An intermediate flow rate (0.7 ml/min) was estimated as a reasonable compromise to be applicable for further optimization in combination with a prolonged gradient time.

The impact of the backpressure and the column temperature was investigated in the following experiment. The same goals as in the previous experiment were set and complemented by the peak-to-valley ratio of a newly

detectable peak. As indicated in the plot (Figure 5B), the decrease in the column temperature resulted in the separation of the additional peak 15 in the first group, which was maximized at 30°C. On the other hand, a coelution of peaks 8 and 9 is present at these conditions, which were separated at higher temperatures. An increase in the backpressure led to the coelution of peak 3 and 15, which were better separated at 1500 psi. Again, a balance between an improvement of the first and second group had to be found and 41°C combined with 1500 psi were set as the final parameters.

3.4 | Use as orthogonal and MS compatible technique: RP-LC versus SFC selectivity

A comparison between LC and SFC was performed to highlight the potential of using SFC as an orthogonal method. Each sample was analyzed via the newly developed SFC and the adopted LC method [28]. UV and MS
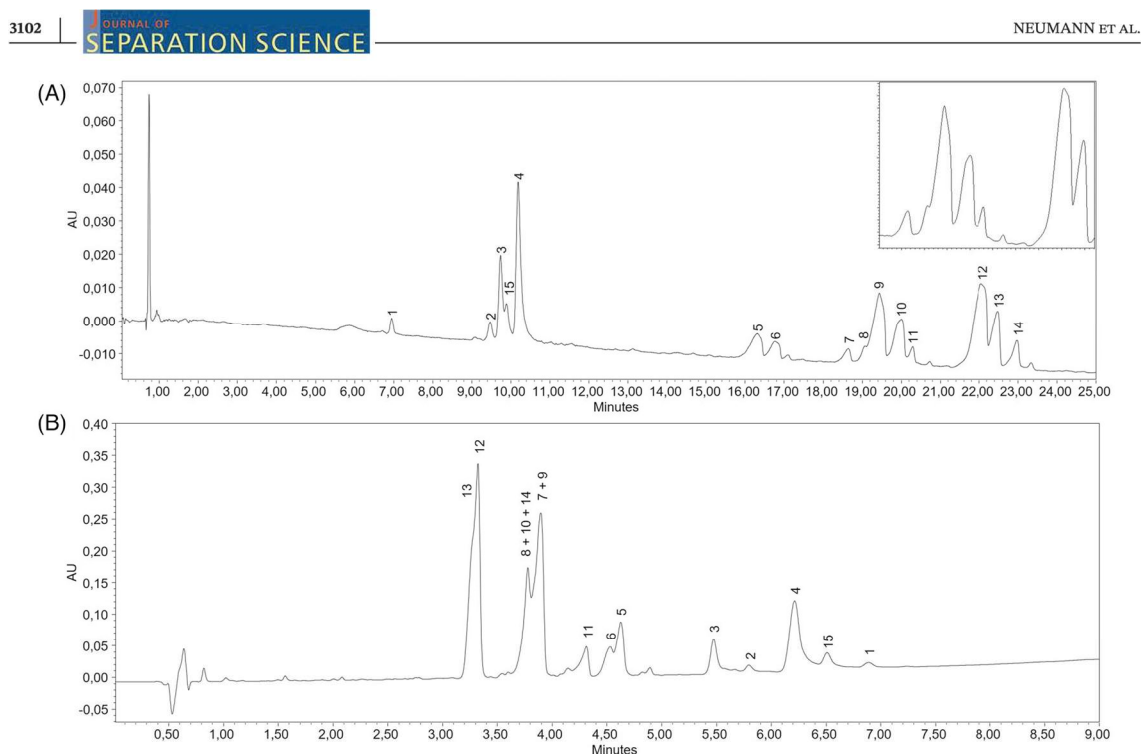
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FIGURE 6 UV chromatograms of tyrothricin analyzed via the optimized SFC (A) and the adapted RP-LC method (B)

TABLE 1 Overview of the characteristic peaks detected via SFC-MS

Peak	Peptide	m/z [M+2H]$^{2+}$ expected[a]	m/z [M+2H]$^{2+}$ detected	Rt [min]
1	Val-Gramicidin B	922.0415	922.1	6.93
2	Ile-Gramicidin C	937.0468	937.2	9.45
3	Val-Gramicidin C	930.0390	930.4	9.72
15	Ile-Gramicidin A	948.5548	948.6	9.89
4	Val-Gramicidin A	941.5470	941.9	10.17
5	Tyrocidine A	635.8351	635.9	16.30
6	Tyrocidine A1	642.8430	642.9	20.28
7/9	Tyrocidine B/B'	655.3406	655.5	18.63/19.42
8/10	Tyrocidine B1/B1'	662.3484	662.5	19.06/19.99
11	Tryptocidine B	666.8486	667.1	20.28
12	Tyrocidine C	674.8460	674.7	22.04
13	Tyrocidine C1	681.8539	682.1	22.46
14	Tryptocidine C	686.2540	686.1	22.97

[a] Data obtained from Vosloo and Rautenbach [30].

detection were utilized to assign the peaks in both methods (Figure 6).

The major peaks were assigned based on the MS data published by Vosloo and Rautenbach [26]. The two peak pairs 7/9 and 8/10 (Table 1), showing the same mass signal, were detected via the SFC method. The authors describe the presence of constitutional isomers due to a swap of tryptophan and phenylalanine at positions 3 and 4 of the amino acid sequence for tyrocidine B and B' (m/z 655.5) and tyrocidine B$_1$ and B$_1$' (m/z 662.2). Concluding, separation of these isomers was achieved easily, which was not possible in other studies utilizing RP-LC [26–28].

In addition, a splitting of peaks 5, 6, 9, 10, and 12 was found using SFC. During the evaluation of the peak capacity, the presence of coeluting peaks was presumed for some peaks of the second group, which now became more evident. An increase in the overall resolution of the fingerprint was initially set as the primary goal. The optimization was consequently performed by balancing the first and second groups. Nevertheless, the observations made during the DoE-based optimization let us conclude that an increase in the flow rate may result in a better separation of these compounds if separating these individual isomers would be of main interest.

Orthogonality was achieved when comparing the elution order of the RP-LC and the SFC method. The first group is better resolved via RP-LC, but the second group can be resolved much better using SFC. This demonstrates the complementarity of both modes and the vast potential of the parallel application during research and development studies. In addition to the orthogonality, a much better overall separation of the peaks and an improvement of the overall peak shape were achieved via the SFC method. In conclusion, the goals initially defined were attained. Thus, developing a new SFC method for the fingerprinting of tyrothricin was successful.

4 | CONCLUDING REMARKS

In this study, we demonstrated SFC to be superior to RP-LC for the separation of the complex peptide mixture in tyrothricin. The application of DoE principles proved to be beneficial during the optimization steps of the method to account for the contrary conditions favored by either the cyclic or the linear group. Peak capacity was shown to be an excellent indicator to rate the performance of experiments in the early steps performed during method development if complemented by others like resolution or peak-to-valley ratios. Isomeric peak pairs, which were not resolved using RP-LC, were efficiently separated. Nevertheless, RP-LC is in favor of the separation of the earlier eluting peaks, which shows that both techniques should be established in a complementary manner or that the use of SFC should be considered if separation cannot be achieved via RP-LC.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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 and editing and resources.

Maria K. Parr: Conceptualization, supervision, and writing - review and editing.

DATA AVAILABILITY STATEMENT
Data are available on request from the authors.

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3.2 Manuscript II: Ternary eluent compositions in supercritical fluid chromatography improved fingerprinting of therapeutic peptides

Jonas Neumann, Sebastian Schmidtsdorff, Alexander H. Schmidt, Maria K. Parr

Abstract: 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 x 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.

RESEARCH ARTICLE

JOURNAL OF SEPARATION SCIENCE

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

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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

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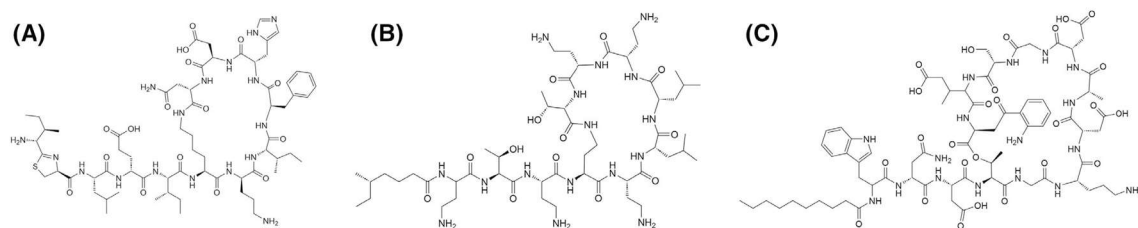
FIGURE 1 Chemical structures of bacitracin (A), colistin (B), and daptomycin (C).

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 pre-heaters, 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	ammonia	X
	MSA	MSA	MSA
2-PIC	ammonia	ammonia	X
	MSA	MSA	MSA
BEH	ammonia	ammonia	X
	MSA	MSA	MSA
Diol	ammonia	ammonia	X
	MSA	MSA	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):

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

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.

$$\Delta RT = RT\ (impurity) - RT\ (main\ compound) \qquad (2)$$

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.
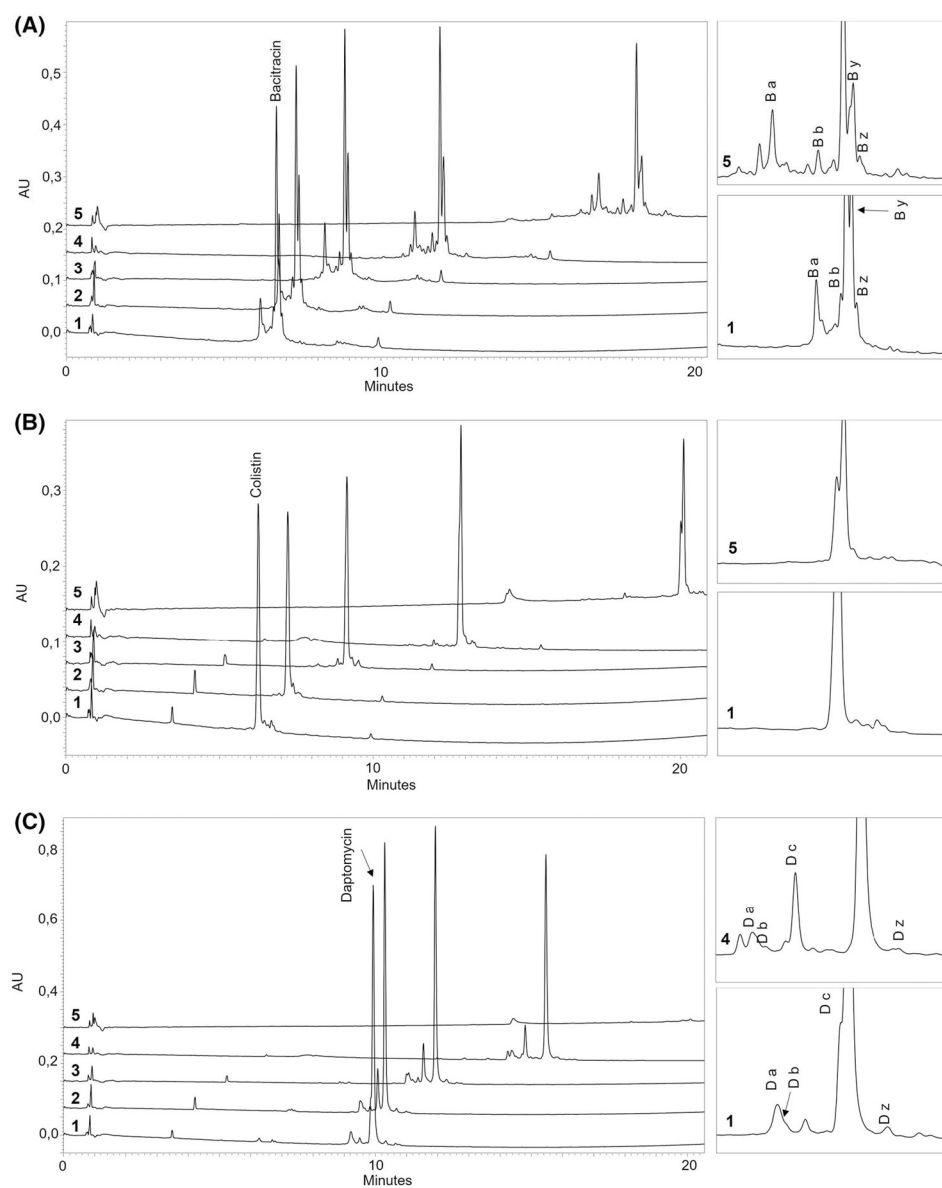
FIGURE 3 Overlay chromatograms of the separation of bacitracin (A), colistin (B), and daptomycin (C) applying varying modifier compositions of methanol (MeOH)/ACN (1:100/0, 2:80/20, 3:60/40, 4:40/60 and 5:20/80 – v:v). A zoomed chromatogram of the highest and lowest retention on the column for each peptide 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
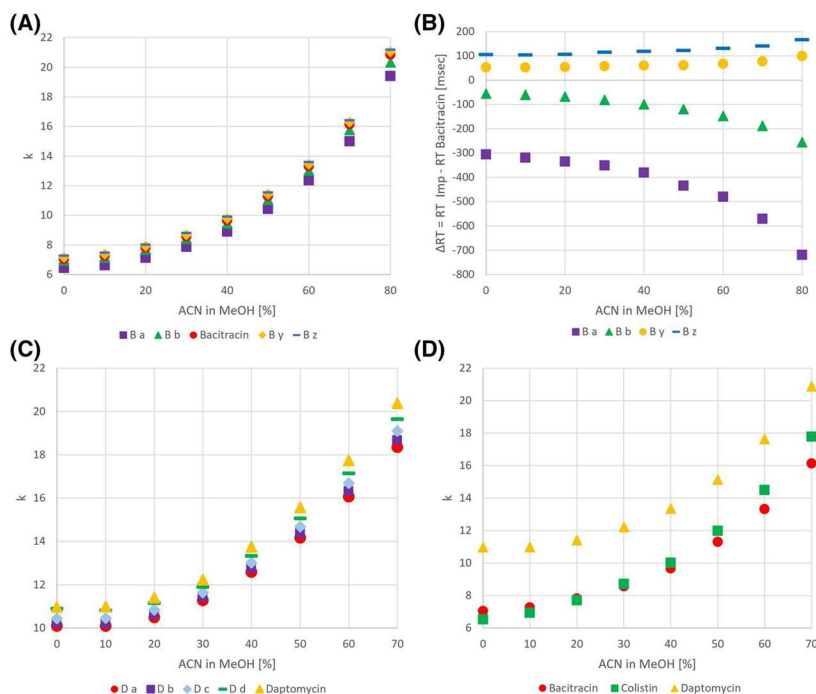
FIGURE 4 (A) Plots of the capacity factor (*k*) of bacitracin and related compounds versus proportions of ACN in methanol (MeOH). (B) Retention time difference (ΔRT) of the byproducts to bacitracin. (C) Plots of the capacity factor of daptomycin and byproducts versus ACN in MeOH. (D) Plots of the capacity factor of bacitracin, colistin, and daptomycin versus ACN in MeOH.
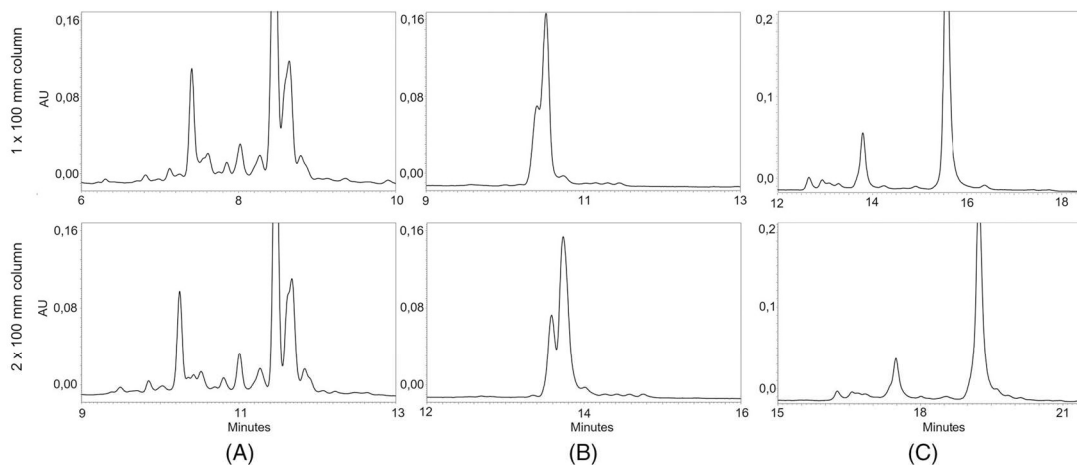

FIGURE 5 Comparison of a single 100 mm (top) and two 100 mm columns (bottom) for bacitracin (A), colistin (B), and daptomycin (C)

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
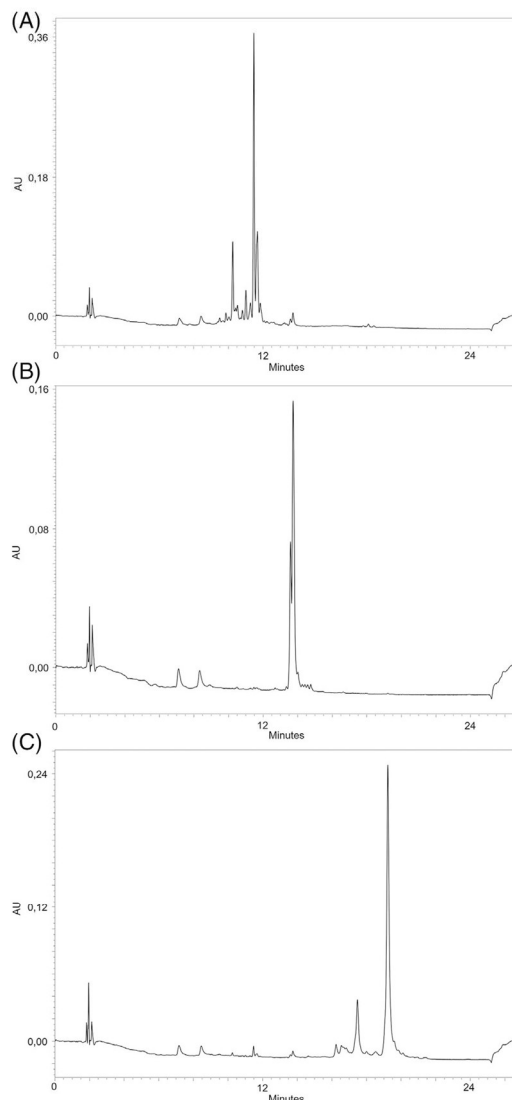

FIGURE 6 Chromatograms of the finally optimized SFC methods for bacitracin (A), colistin (B), and daptomycin (C). Please note that all peaks with a retention time (Rt) < 10 min are caused by the water contained in the sample solvent.

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).

3.4 | Method development protocol

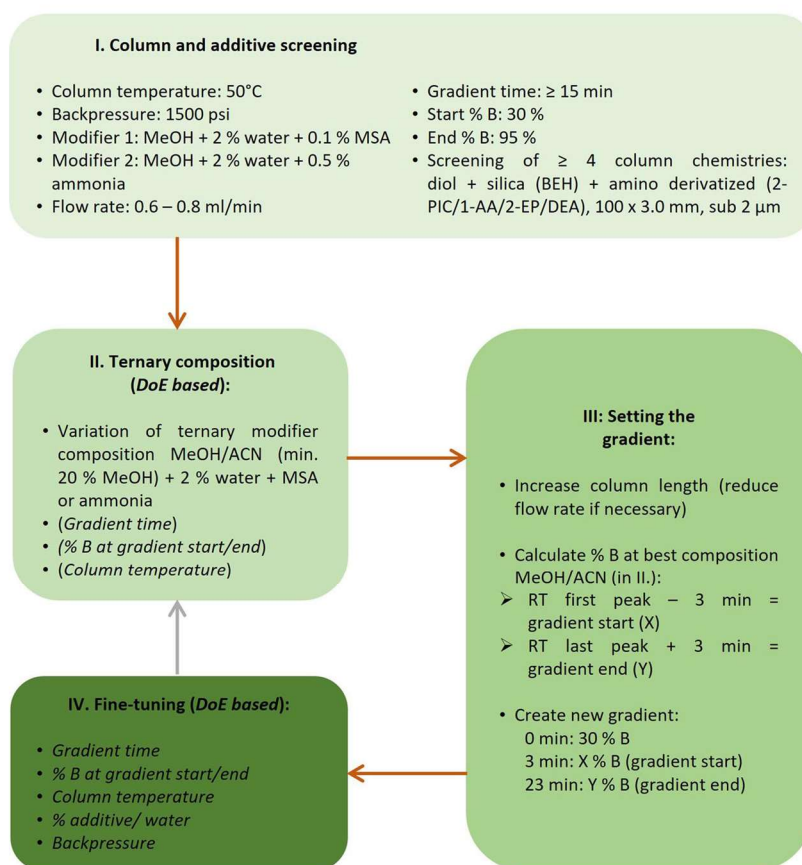A generic screening and method optimization approach is proposed based on the data obtained in this and our previ-

I. Column and additive screening

- Column temperature: 50°C
- Backpressure: 1500 psi
- Modifier 1: MeOH + 2 % water + 0.1 % MSA
- Modifier 2: MeOH + 2 % water + 0.5 % ammonia
- Flow rate: 0.6 – 0.8 ml/min

- Gradient time: ≥ 15 min
- Start % B: 30 %
- End % B: 95 %
- Screening of ≥ 4 column chemistries: diol + silica (BEH) + amino derivatized (2-PIC/1-AA/2-EP/DEA), 100 x 3.0 mm, sub 2 μm

II. Ternary composition (*DoE based*):

- Variation of ternary modifier composition MeOH/ACN (min. 20 % MeOH) + 2 % water + MSA or ammonia
- (*Gradient time*)
- (*% B at gradient start/end*)
- (*Column temperature*)

III: Setting the gradient:

- Increase column length (reduce flow rate if necessary)

- Calculate % B at best composition MeOH/ACN (in II.):
  ➢ RT first peak − 3 min = gradient start (X)
  ➢ RT last peak + 3 min = gradient end (Y)

- Create new gradient:
  0 min: 30 % B
  3 min: X % B (gradient start)
  23 min: Y % B (gradient end)

IV. Fine-tuning (*DoE based*):

- *Gradient time*
- *% B at gradient start/end*
- *Column temperature*
- *% additive/ water*
- *Backpressure*

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.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT
Data are availale on request from the authors.

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3.3 Manuscript III: Controlling the elution order of insulin and its analogs in sub-/supercritical fluid chromatography using methanesulfonic acid and 18-crown-6 as mobile phase additives

Jonas Neumann, Sebastian Schmidtsdorff, Alexander H. Schmidt, Maria K. Parr

Abstract:

The purity analysis of therapeutic peptides can often be challenging, demanding the application of more than a single analytical technique. Supercritical fluid chromatography nowadays is a promising alternative to reversed-phase liquid chromatography, providing orthogonal and complementary information. This study investigated its applicability for the separation of human insulin, its analogs and degradation products. A previously published method development protocol for peptides up to 2000 Da was successfully applied to the higher molecular weight insulins (6 kDa). A single gradient method was optimized for all insulins using a Torus DEA column (100 × 3.0 mm, 1.7 µm), carbon dioxide and a modifier consisting of methanol/acetonitrile/water/methanesulfonic acid (65:35:2:0.1, v/v/v/v). Consecutively, the crown ether 18-crown-6, which is well known to complex charged lysine sidechains and other amino functionalities, was added to the modifier to evaluate its impact on selectivity. A decreased retention and a shift in the elution order for the insulins were observed. An inverse effect on retention was found when combined with a neutral stationary phase chemistry (Viridis BEH).

RESEARCH ARTICLE

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Controlling the elution order of insulin and its analogs in sub-/supercritical fluid chromatography using methanesulfonic acid and 18-crown-6 as mobile phase additives

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The purity analysis of therapeutic peptides can often be challenging, demanding the application of more than a single analytical technique. Supercritical fluid chromatography nowadays is a promising alternative to reversed-phase liquid chromatography, providing orthogonal and complementary information. This study investigated its applicability for the separation of human insulin, its analogs and degradation products. A previously published method development protocol for peptides up to 2000 Da was successfully applied to the higher molecular weight insulins (6 kDa). A single gradient method was optimized for all insulins using a Torus DEA column (100 × 3.0 mm, 1.7 μm), carbon dioxide and a modifier consisting of methanol/acetonitrile/water/methanesulfonic acid (65:35:2:0.1, v/v/v/v). Consecutively, the crown ether 18-crown-6, which is well known to complex charged lysine sidechains and other amino functionalities, was added to the modifier to evaluate its impact on selectivity. A decreased retention and a shift in the elution order for the insulins were observed. An inverse effect on retention was found when combined with a neutral stationary phase chemistry (Viridis BEH).

KEYWORDS
crown ether, design of experiments, insulin

1 | INTRODUCTION

The rise of peptides and proteins used as pharmaceuticals over the last decade [1, 2] came along with growing regulatory needs to ensure their quality. Biosimilars were introduced into the EU market in 2006 after the first originator patent expired. With Abasglar, the first biosimilar of the insulin analog Lantus (insulin glargine

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[GLA]) was introduced in 2014, followed by a biosimilar of insulin lispro (LIS) in 2017. Today, several biosimilars of insulin aspart (ASP), GLA, and LIS are available. Specific guidelines were released, for example, by the European Medicines Agency dealing with the quality aspects of biosimilars in general [3] or insulins in specific, respecting "Quality, safety and efficacy to the reference medicinal product" [4]. The impurity profile is an essential characteristic of pharmaceutical substances and products to be controlled. Therefore, a comprehensive and extensive analysis applying a combination of orthogonal and state-of-the-art techniques is required to quantify and qualify the impurities of a biosimilar [3]. Intermediates and byproducts arising from production or degradants formed over the shelf-life must be monitored according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use guidelines Q5C [5] and Q6B [6]. These requirements apply to drug substances and products, as well as to originator and biosimilar products.

Often, reverse-phase liquid chromatography (RP-LC), size-exclusion chromatography, and ion-exchange chromatography are the dominant techniques used to analyze peptides and proteins, allowing the determination of mono- and multimeric impurities. However, the comprehensive evaluation of monomeric impurities is often challenging due to the high chemical similarity of the main compound and its impurities [7]. A comparability study on the originator and biosimilars of insulin glargine showed several compounds coeluting with the main peak [8]. As in this case, non-volatile buffer salts are often used to ensure chromatographic performance. Mass spectrometry is often coupled to LC for characterization purposes but is obstructed by these buffers and requires substitution for TFA or formic acid. As a drawback, these often result in worse chromatographic performance regarding resolved peaks [9]. Alternatively, online or offline 2D-LC can remove non-volatile buffers before characterization through LC-MS [10].

Supercritical fluid chromatography (SFC) recently gained growing attention [11] in analytical [12–17] and preparative [18, 19] peptide separation. The broad polarity range accessible by applying a single gradient is highly beneficial, for example, in vitamin analysis or metabolomics studies [20, 21]. In addition, the new concept of a dual gradient mode was investigated by adding a second high water content gradient through dynamic mixing of the modifier, which further increased the polarity range of capable analytes [22, 23]. This now allows the elution of monomeric amino acids (AAs) and small peptides up to proteins in a single run and will assist in generating comprehensive knowledge about the impurity profile of pharmaceuticals, especially during research and development phases. The orthogonality of SFC to RP-LC [13–15, 17] also makes it an exciting perspective as an on- or offline coupling technique for 2D chromatographic separations, even if today's interface designs require further optimization [24, 25]. This proves that SFC is a state-of-the-art technique that should be considered an alternative to RP-LC. It should be noted that the supercritical state often is not maintained under the conditions used. Thus, "supercritical" is not accurate, and "unified chromatography" [14, 16, 26] found growing usage to account for this discussion. Nevertheless, SFC is used in this work for sub- and supercritical.

However, the general knowledge based on the publications dealing with SFC separations of peptides and proteins is still limited. Additional research is necessary to push the boundaries of what is possible in this application field. In this work, we use human insulin (HUM) and its analogs to investigate the potential of SFC, on the one hand, to separate all insulins from each other and, on the other hand, to separate each insulin from byproducts or impurities. The method development protocol proposed in our previous work [12] for peptides up to 2000 Da is successfully applied to the higher molecular weight insulins (6 kDa), supported by a Design of Experiments (DoE) approach. Consecutively, the crown ether (CrE) 18-crown-6 (18C6), which so far found little application as a mobile phase additive in SFC, is found to provide interesting elution properties based on the column chemistry used.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

HUM and LIS from Lilly Deutschland GmbH, detemir (DET), degludec (DEG), and ASP from Novo Nordisk Pharma GmbH, GLA and glulisine (GLU) from Sanofi-Aventis Deutschland GmbH were used (all 100 I.U./ml), either as refill cartridges or ready-to-use pens. All were end-of-shelf-life batches, stored at room temperature for at least six months and were expected to contain relevant degradants. The AA sequence of all insulins is shown in Figure 1. Methanol (MeOH) and acetonitrile (ACN) in 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 (≥99.0%) and 18C6 (≥99.0%) were purchased from Sigma Aldrich, and aqueous ammonia solution (25%) was supplied by Merck.
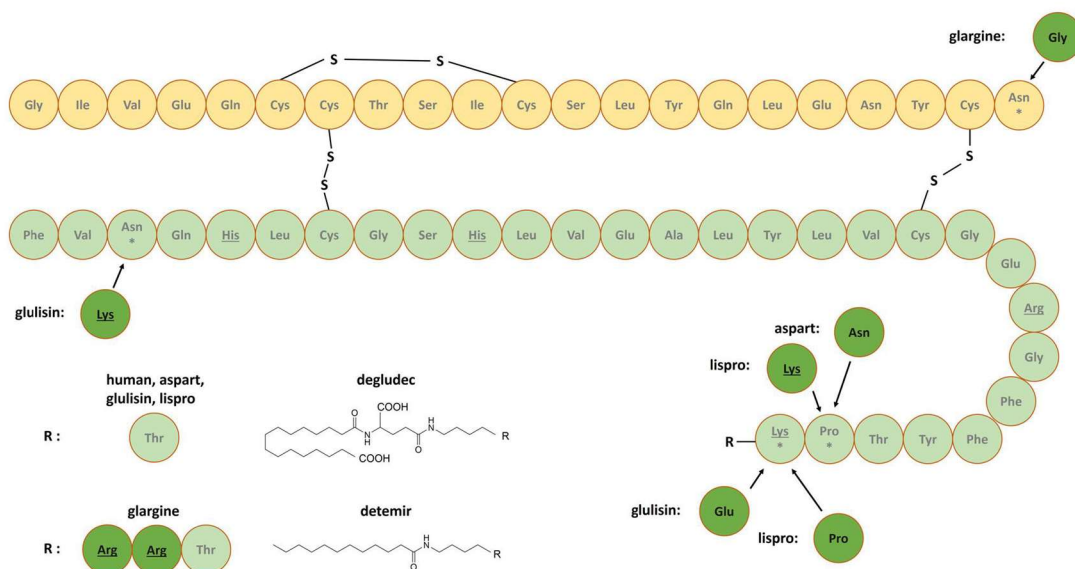
FIGURE 1 Amino acid sequences of insulin human and its analogs. The positions modified in the analogs (dark) are marked (*), and basic sidechains are underlined.

2.2 | Instrumentation and software

The chromatographic experiments were performed using an Acquity UPC^2 SFC system (Waters) equipped with a binary pump, a 4-port column manager with active eluent pre-heaters, an Acquity UPC^2 photodiode array detector (UV), and an Acquity UPC^2 convergence manager (backpressure regulator). Empower 3 was used for system control, data acquisition, and processing. Fusion QbD from S-Matrix (Eureka) was used to prepare and visualize the DoEs. The 2D graphs were calculated via Microsoft Excel. The following stationary phases (SPs) were utilized for the experiments: Torus Diol, Torus DEA, Torus 1-AA, and Viridis BEH, all from Waters. All columns' dimensions were 3.0 × 100 mm; particle size 1.7 µm.

2.3 | Method development

We adopted the method development workflow proposed in our previous work [12] for the higher molecular weight insulins. Due to mixing incompatibility, all insulin solutions had to be injected (1.5 µl) separately. A generic gradient from 30 to 95% modifier (B) was run in 20 min, applying a flow rate of 0.6 ml/min. Methanol/water (100:2, v:v) plus 0.1% methanesulfonic acid (MSA) (v:v) as the modifier was tested in combination with four SPs (Section 2.2). The column temperature was set to 50°C and the

backpressure to 1500 psi (approx. 100 bar). The best combination was kept for further experiments: the DEA column and a modifier consisting of MeOH/water/MSA (100:2:0.1, v:v:v). Consecutively, two DoEs were performed as described in chapter 3.2.

2.4 | Final method

The final method utilized a Torus DEA column (2.1 × 100 mm, 1.7 µm). The column temperature was set to 55°C and the backpressure to 1500 psi at a flow rate of 0.6 ml/min running a segmented gradient: 0–5 min: 30–62% B, 5–20 min: 62–75% B with the modifier (B) consisting of MeOH/ACN/water/MSA (65:35:2:0.1, v:v:v:v). All chromatograms were recorded at 210 nm.

3 | RESULTS AND DISCUSSION

3.1 | Screening of additives and column chemistries

The elution order of all insulins was similar for the neutral (Diol and BEH) and the amino-derivatized (1-AA and DEA) SPs but differed for both groups (Figure 2). Glargine, modified by adding two basic arginines to HUM, eluted the last on the neutral columns. That is in line with the results
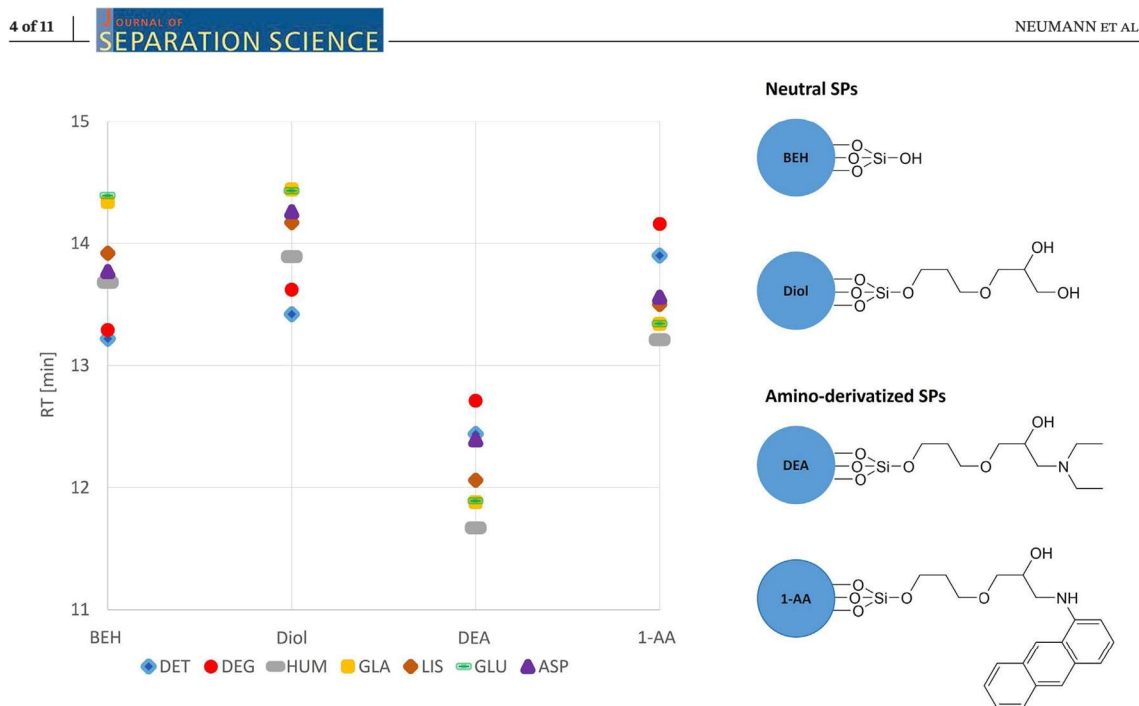
FIGURE 2   Elution order of the insulins observed during the screening on various column chemistries as shown on the right.

of previous studies, which reported that the presence and number of basic sidechains is a major factor determining the retention of peptides in SFC [12, 15, 17, 27, 28]. The addition of MSA provides acidic conditions resulting in protonated amino functionalities and ion-pair formation with MSA. In the case of DEA, repulsion effects between the protonated amino functionalities of the SP and the analytes also need to be considered [17, 26]. The higher number of basic functionalities in GLA then translates into an earlier elution within the group of non-esterified insulins due to the repulsion. The same peak order was detected using DEA and 1-AA, even if the low basicity of 1-aminoanthracene probably will not result in a charged state of the amino functionality. Detemir and DEG, carrying fatty acid esterifications, eluted earlier than the other insulins on the neutral SP. That can be explained through steric shielding provided by the lipophilic derivatization, resulting in weaker interactions with the SP. DEG contains an additional free carboxy functionality and a glutamate acid linker. The higher polarity compared to the myristic acid moiety present in DET may explain the later elution of DEG than DET. Interestingly, both eluted later than all other insulins on the amino-derivatized SPs. Stronger lipophilic interactions between the ion-pair of the protonated amino functionality of the SP and the lipophilic tail of DEG and DET or the shielding of the repulsion effects are potential explanations. Notably, a weaker retention of all insulins on the DEA was found. Again, this

may be explained by the repulsion effect. Overall, BEH and DEA performed equally, considering the peak shape and number of smaller peaks. Both performed superior in comparison to the Diol and the 1-AA column. The much earlier elution of the insulins and more prominent shoulders eluting before the main peak, especially for DEG, made the DEA the column of choice for the optimization.

Ammonia was also tested as an additive in combination with the BEH column. Based on previous works [15, 17, 27], a stronger overall retention due to the size of insulin was expected. The lack of MSA as an ion-pairing reagent and the additional deprotonation of the carboxylic functionalities under the usage of ammonia should further increase the retention. Effectively, broader peaks combined with fewer impurities peaks set in a later elution window at the end of the gradient were found for all insulins using ammonia. Thus, this combination was excluded from further evaluation.

Chromatograms and retention time (RT) data of the screening runs are provided in the Supporting Information (see Figure S1, Figure S2 and Table S1).

3.2 | Method optimization

Adding ACN up to 70% to MeOH as the modifier significantly improved the resolution of the fingerprint for smaller peptides (1200 to 1900 Da) caused by a reduced

elution strength in our previous work [12]. Due to the higher molecular weight of insulin, 60% ACN was set as the maximum for the method optimization. Since the future method shall be applicable to all insulins, DEG was chosen as the suitable candidate to reduce the number of optimization runs. It was considered representative for all insulins due to the highest retention and the most dominant shoulder eluting before the main peak. Consecutively, two DoEs were performed (see Table S2).

A linear gradient was run using varying modifier compositions (MeOH/ACN: 100/0, 80/20, 60/40, and 40/60). The column temperature was simultaneously varied (40/50/60°C) to account for a DoE-based workflow. The ranges were set to stay within the instrument's pressure range and the SP's recommended temperature range. A full-factorial approach was applied, resulting in twelve experiments. As observed in our previous study, the addition of ACN resulted in a notable increase in resolution. The range from 20% to 40% ACN in MeOH was most beneficial to improve the resolution of the fingerprint (Figure 3A). As expected from our previous work [12], a lower proportion of ACN was applicable due to the higher chain lengths of the insulins. At higher percentages, peak deformation and weak resolving power were found. The maximization of the number of peaks and the separation of the characteristic impurities via the peak-to-valley ratio (p/v) were chosen as the goals for data evaluation and visualization. An overlay of the contour plots of each response is shown in Figure 3B. Conditions not meeting the goals, as indicated in the individual boxes, are marked as colored areas in the plot. Thus, the white area represents the combination of parameter settings that fulfill the goals. As indicated ("T"), a composition of MeOH/ACN 65/35 and a column temperature of 55°C was the best combination to continue the optimization.

In the second full-factorial DoE, the start%B of the gradient step (X: with experiments at 55/60/65/70%) was varied in combination with the gradient time (Y: 15/25 min), resulting in eight runs. In our previous work [12], the insertion of a gradient step was found to shorten the runtime while maintaining resolution. Thus, the following gradient was prepared: 0–5 min: 30–X% B, 5–Y min: X–80% B. DEG and HUM, the most and least retained insulins within the set of drugs, were used for these experiments to ensure that the final gradient provides a sufficient retention window for all insulins. The improvement in the fingerprints' resolution of DEG and HUM was set as the goal (Figure 3C) via the p/v ratios of characteristic peaks (Figure 3D). The final gradient was defined as 0–5 min: 30%–62% B, 5–20 min: 62%–75% B.

The optimized method was then applied to all insulins (Figure 4). The number of peaks in all samples increased notably compared to the chromatograms obtained during the screening. The method development protocol proposed in our previous work was successfully applied to the higher molecular weight insulins. Again, the combination of ACN and MeOH in the modifier proved to be highly beneficial in increasing the resolution of the fingerprint, resulting in a single method for all samples.

3.3 | Addition of a CrE

Crown ethers (Figure 5A), macrocyclic oligomers of ethylene oxide, are known for their characteristics as complexing agents for cations, such as charged amino groups (Figure 5B). Used as mobile phase additives, they increased the retention of amino compounds [29–34] and peptides or proteins [35, 36] in RP-LC, with 18C6 being the most popular. The affinity of a CrE to a specific molecule highly depends on its ring size. For example, 18C6 and dicyclohexyl-18C6 are reported to favor different molecules [29, 33]. An inverse effect on retention was observed under SFC conditions when combined with ethanesulfonic acid to analyze AAs [27]. Nevertheless, extensive studies on the application in peptide separations by SFC are missing until now.

After optimizing the chromatographic parameters, the potential of 18C6 to affect the selectivity of the insulins was tested. A reduction of retention was desirable while maintaining the resolution. This may also allow for the accessibility of larger peptides in SFC analysis. The addition of MSA was considered necessary to generate an acidic environment in the eluent [17], which provides protonated amino functionalities of the analyte and enables coordination by the CrE. Thus, the concentration of MSA was maintained. First, the chromatographic impact of the addition of 18C6 on the individual insulin and its degradants was evaluated. A distinct reduction in the retention with an increasing concentration of 18C6 (0 to 4 mM) was found for all analytes. These findings are in line with the results reported for the elution of AAs in SFC [27]. The charged amino functionalities of the analyte are better masked by the CrE than by pure MSA, causing a reduction of the interaction with the SP. An inferior separation was obtained for each insulin and its impurities, as shown in the case of GLU. Solely a slight improvement was achieved for ASP, where adding 2 mM 18C6 increased the separation of a peak pair eluting before the main peak (Figure 6). However, most analytes are impacted equally by the CrE, and lower retention translates into a worse separation.

The elution order of insulin and its analogs can be modified by adding 18C6 from HUM < LIS < GLA < DET < ASP < DEG < GLU over HUM = LIS < ASP < GLA < DET < DEG < GLU to LIS < HUM < ASP < GLA < DET < DEG < GLU
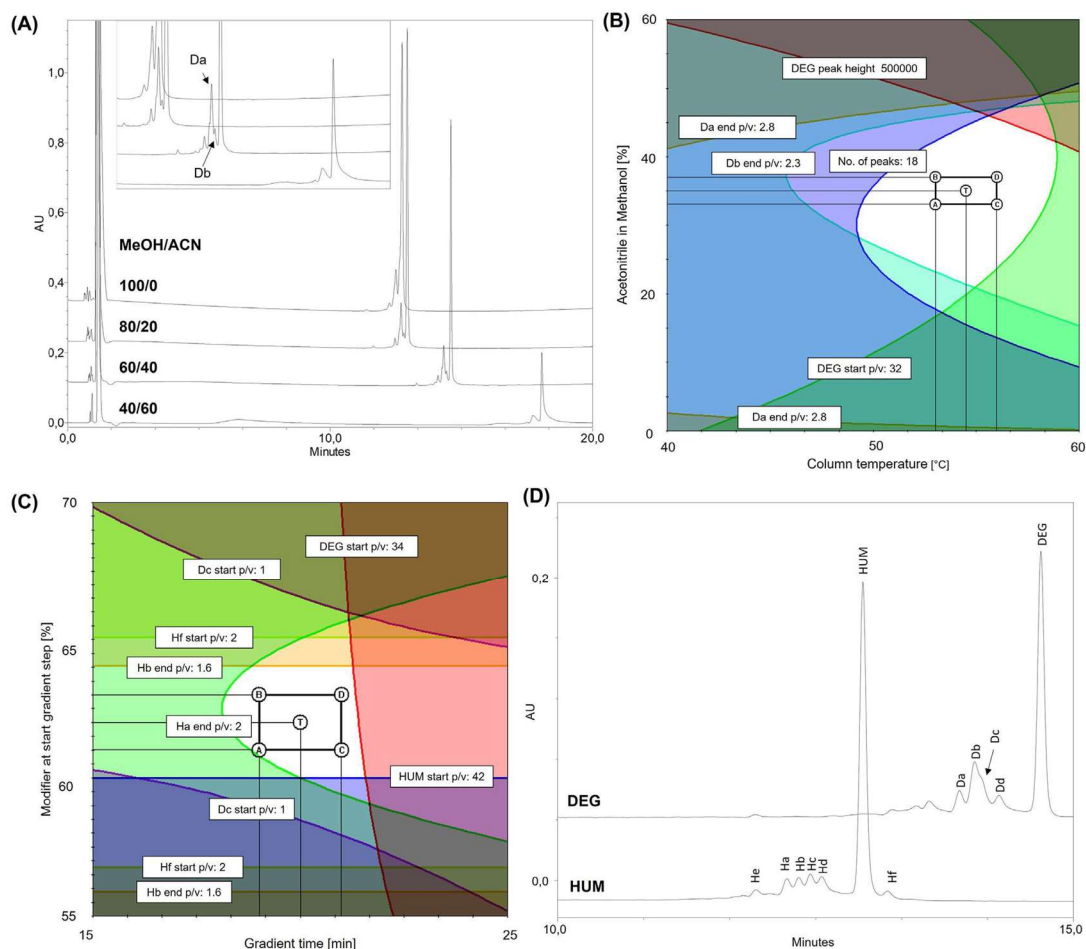
FIGURE 3 Overlay chromatogram applying varying modifier compositions of MeOH/ACN (v:v) to separate insulin degludec (column temperature: 50°C). A zoomed chromatogram is added in the box indicating relevant peaks for the separation (A). Visualization of the impact of the column temperature and modifier composition (B) and gradient "Y" time and start%B at gradient step "X" (C) on the separation of critical peaks. Colored areas indicate conditions under which the defined goals were not achieved (note that mixed colors are possible). Therefore, white areas indicate conditions for achieving the best overall separation. The black box indicates conditions used for further optimization. Exemplary overlay chromatogram of insulin degludec and human insulin combining 60% modifier "X" and 25 min gradient time "Y" (D).

on the DEA column (Figure 7). Lispro and ASP are shifting relatively to the others. In LIS, the AA sequence of HUM is modified by exchanging the Lys positions and the adjacent proline. Lys is preferred in the guest-host complex with 18C6. A shift of its position and binding to 18C6 likely impacts the molecule's conformation in the mobile phase, causing the change of selectivity. In the case of ASP, no modifications of Lys are involved, but the adjacent proline, which is well known for its impact on the secondary structure of peptides and proteins [37]. The actual conformation of the molecules under SFC

conditions is unknown, but it can be concluded that the conformation might impact the accessibility of Lys. However, GLU, which also involves the shift of the position of a Lys, does not change in elution order. The arginine-richer GLA also remains unaffected, proving 18C6's affinity for Lys sidechains over arginine. That is in line with other studies showing a higher binding affinity to Lys than to arginine or histidine either as a single AA or in a peptide sequence under MS conditions used in peptidomics [38, 39]. The same was found for the liquid state in RP-LC [35].
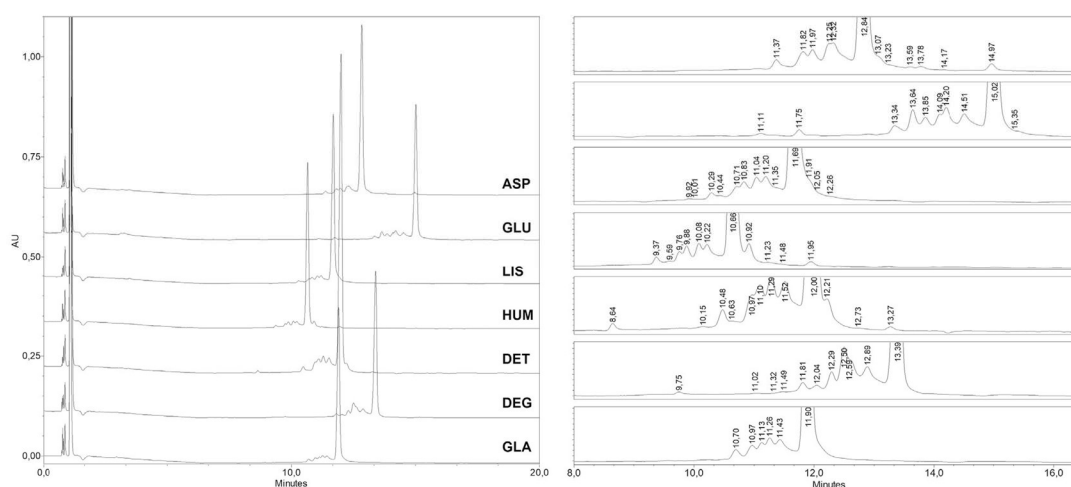
FIGURE 4 Overlay chromatogram of each insulin separated via the optimized method using a DEA column. A zoomed chromatogram is shown on the right.
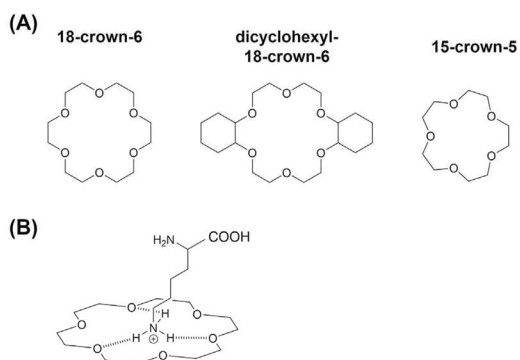

FIGURE 5 Crown ethers (A) and a guest-host complex of lysine with a charged sidechain and 18-crown-6 (B).

The experiment was repeated by applying the same chromatographic conditions, using the BEH column to investigate the effect of the CrE further. Interestingly, the RTs of all insulins were increased, but no change in the elution order was observed by adding 18C6 (Figure 7). Similar results were described before for a normal phase separation of oxindoles and isatins after adding 15-crown-5 [40]. The formation of hydrogen bonds between the residual silanols of the SP and the ether's oxygens was hypothesized. As a result, the cavity of the ether is still accessible to the analyte, but the complex is also bound to the SP, causing higher retention. The inverse effects, either increased or decreased retention based on the type of complex formed, were also described in RP-LC [41]. The formation of three hydrogen bonds between the Lys

sidechain and the cavity of the CrE was explained in the gas phase during gas chromatography analysis [39], allowing free electron pairs of the CrE's oxygens to form additional hydrogen bonds to the SP. In the case of the DEA column, complexes of the analyte (A) + 18C6 or SP + 18C6, and for the BEH column, a complex of 18C6 + SP + A, may be formed, as displayed in Figure 8. The fact that the elution order was not changed on the BEH column can be explained via steric effects. If the complex of 18C6 + SP + A is formed at the N-terminus of the peptide, other AA positions are not accessible and thus do not affect the relative elution. As a result, the retention is increased equally for all insulins.

In conclusion, using 18C6 as an additive in SFC in combination with an amino-derivatized column is a valuable tool to control the selectivity and elution order of different peptides when Lys modifications are present. Our findings may also add value to the separation of synthetic peptides and intermediates. A protected amino function should not be affected by the CrE compared to an unprotected one. In addition, the increase in retention by combining CrE with the BEH column might be helpful for the separation of smaller peptides.

4 | CONCLUDING REMARKS

We successfully applied our method development approach using DoE principles to HUM and six analogs with molecular weights up to 6 kDa. SFC provided sufficient resolution to be applicable as a potential alternative or complementary method for the determination of
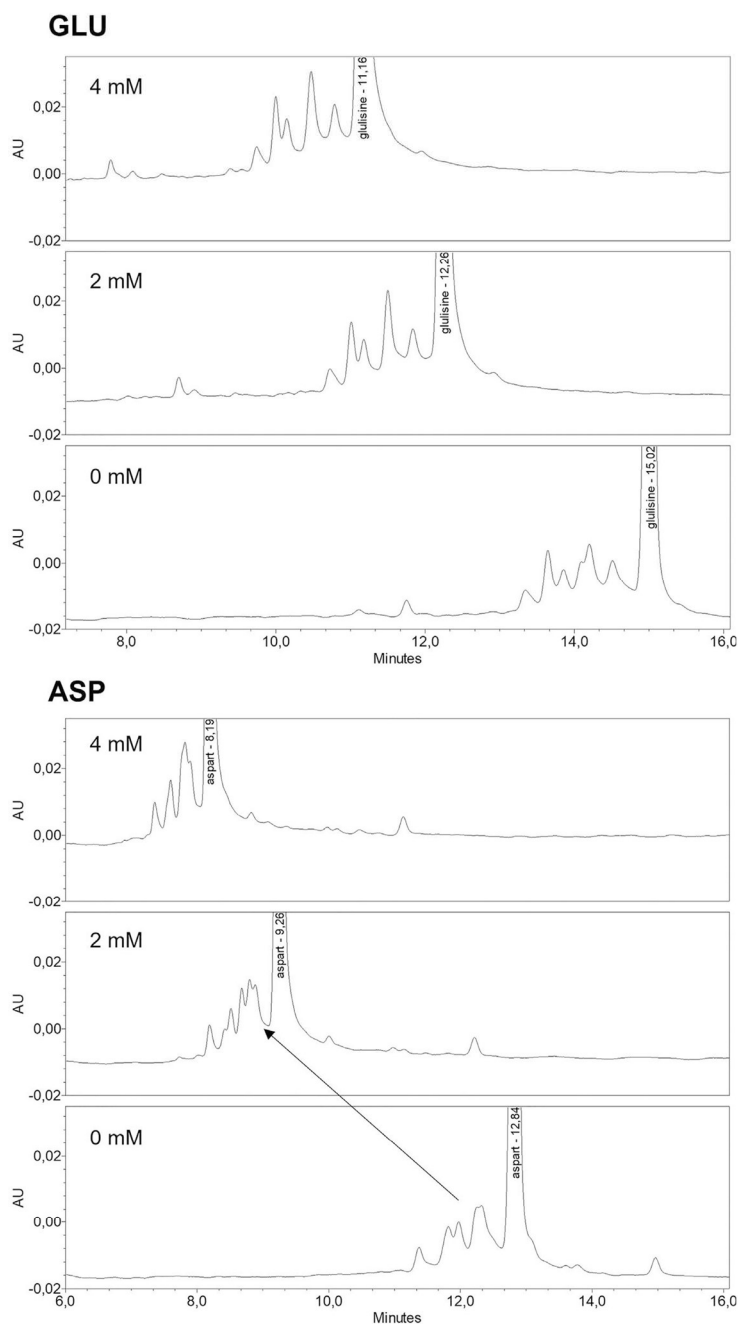
FIGURE 6 Shift in the fingerprint of insulin glulisine and aspart caused by adding 18-crown-6 using a DEA column.

impurities at this mass range. Again, a composition of ACN and MeOH proved beneficial for the separation of peptides in SFC. Additionally, the combination of MSA and 18C6 as additives and an amino-derivatized SP were found to be a suitable tool to control the elution order of the different insulins depending on the position of

Lys. However, from the perspective of purity testing, it did not increase the separation of the main peak and the impurities. When combining 18C6 with a neutral column chemistry, higher retention was achieved, which might be beneficial for smaller peptides showing overall less retention. Our findings will help further understand
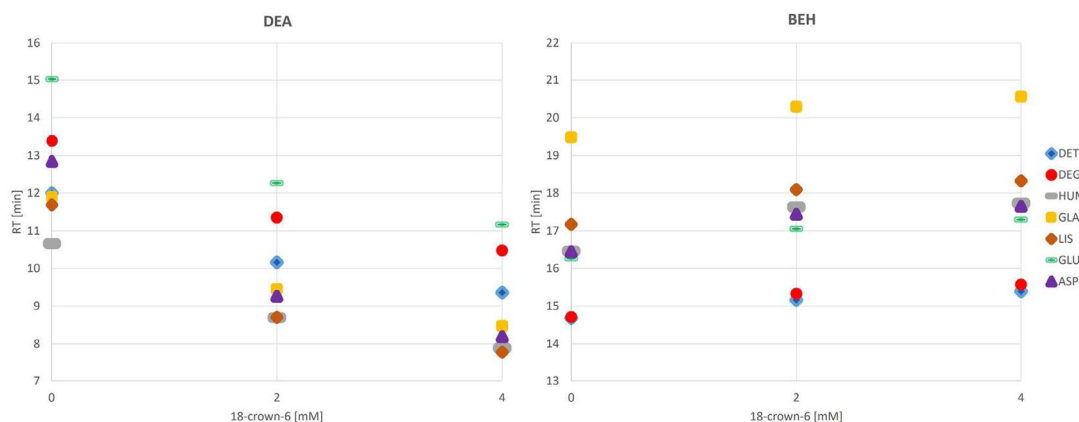
FIGURE 7 Impact of increasing crown ether concentrations on the selectivity for the insulins using a DEA (left) or BEH (right) column.
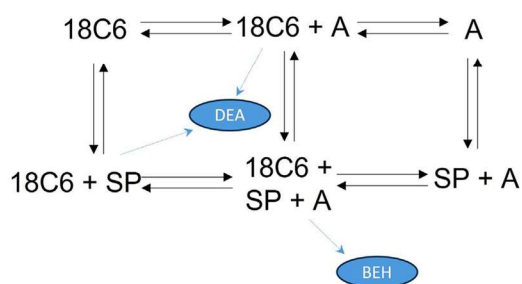

FIGURE 8 Possible complexes between the analyte (A), stationary phase (SP), and 18-crown-6 (18C6) adapted from [41].

the potential SFC as a state-of-the-art technique for the separation of peptide therapeutics.

AUTHOR CONTRIBUTIONS

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

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Supporting Information

Controlling the elution order of insulin and its analogs in supercritical fluid chromatography using methanesulfonic acid and 18-crown-6 as mobile phase additives

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Figure S-1 Chromatograms of the column screening
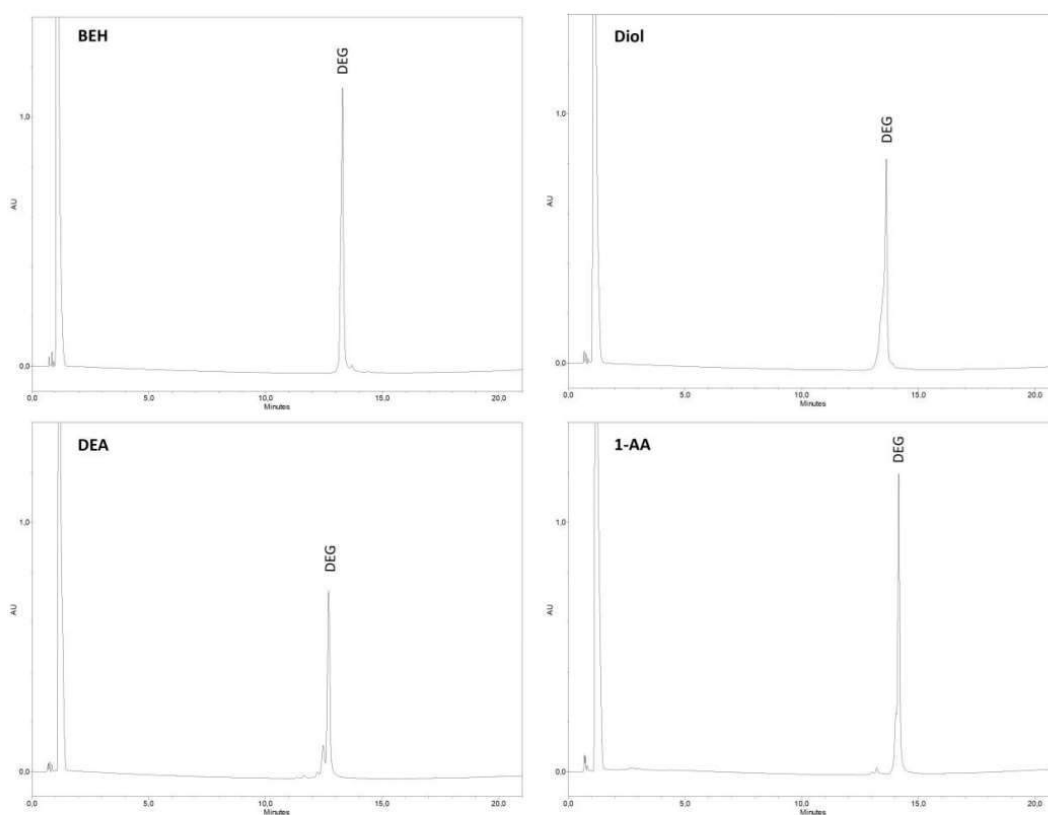
Table S-1 Retention time data of the column screening

Analyte	BEH Rt [min]	Diol Rt [min]	DEA Rt [min]	1-AA Rt [min]
ASP	13,77	14,26	12,39	13,56
DEG	13,29	13,62	12,71	14,16
DET	13,22	13,42	12,44	13,90
GLA	14,34	14,44	11,88	13,34
GLU	14,39	14,43	11,89	13,34
HUM	13,68	13,89	11,67	13,21
LIS	13,92	14,17	12,06	13,50

Figure S-2 Impact of ammonia
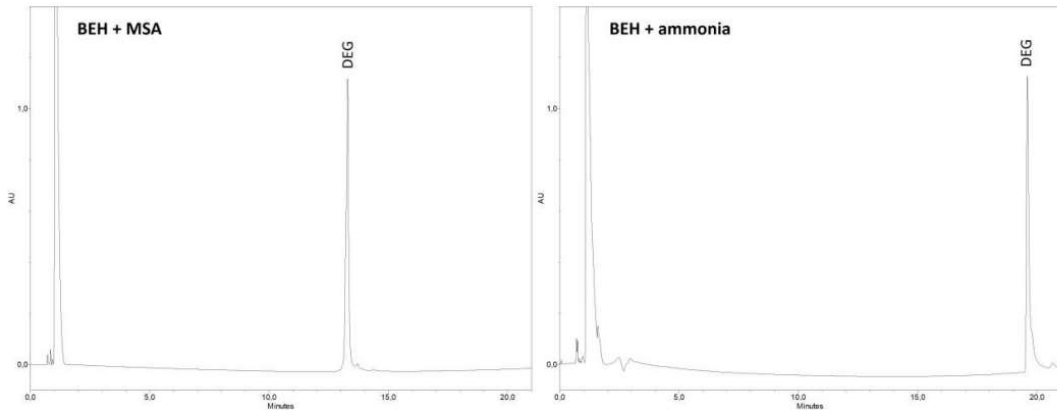
Table S-2 Design-of-Experiments

Experiment Design Matrix - DoE I

Run No.	Oven Temperature (°C)	ACN in MeOH (%)
1	40	0
2	40	20
3	40	40
4	40	60
5	50	0
6	50	20
7	50	40
8	50	60
9	60	0
10	60	20
11	60	40
12	60	60

Experiment Design Matrix II - DoE I

Run No.	Gradient Time (min)	Modifier at start gradient step (%)
1	15	55
2	25	55
3	25	60
4	15	60
5	25	65
6	15	65
7	15	70
8	25	70
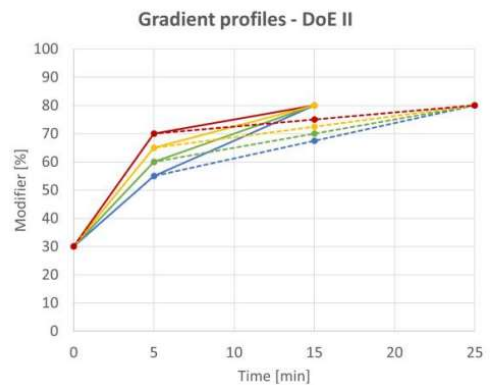
3.4 Manuscript IV: Retention modeling of therapeutic peptides in sub-/supercritical fluid chromatography

Jonas Neumann, Sebastian Schmidtsdorff, Alexander H. Schmidt, Maria K. Parr

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, 1.7 µm), 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.

RESEARCH ARTICLE

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, $1.7\,\mu$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

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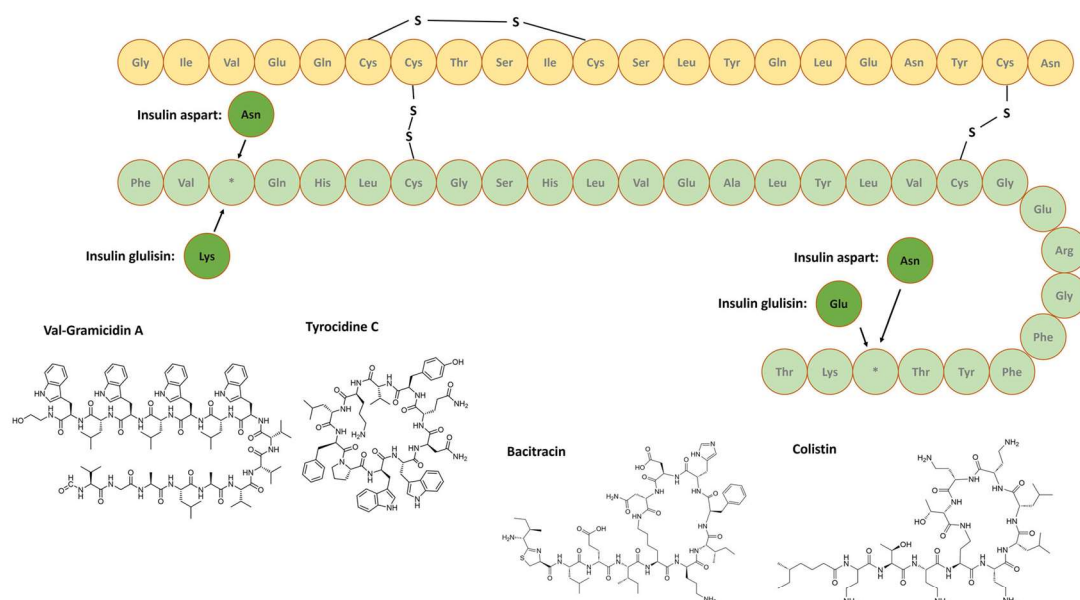
FIGURE 1 Amino acid sequences of the model compounds. For tyrothricin, which is a mixture of various compounds, a linear gramicidin, and a cyclic tyrocidine are exemplarily shown. For the insulins, the common amino acid sequence is shown with differing positions indicated.

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.

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 UPC^2 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 UPC^2 photodiode array detector (UV), an Acquity UPC^2 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 make-up 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,
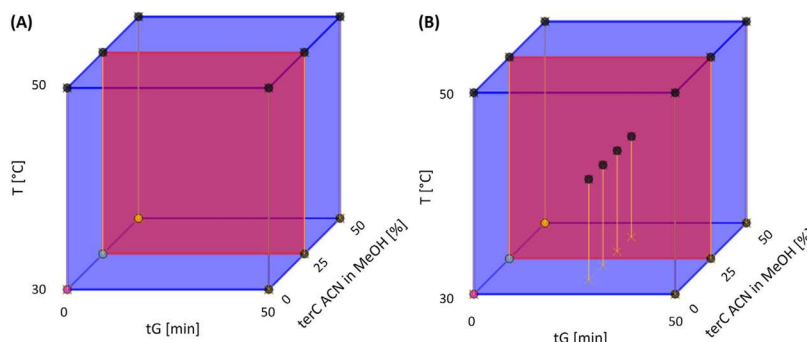
FIGURE 2   Input runs (n = 2 × 2 × 3 = 12) required to create the 3D prediction model indicated as dots (A). The chosen verification runs were performed to demonstrate the suitability of the prediction model (B).

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

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 × 2 × 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:

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

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

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.

	Input runs	WP I	WP II	WP III	WP IV	WP V
Stationary phase	Viridis BEH, 3.0 × 100 mm, 1.7 µm					
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] / B [%]	0 / 30; 20/40 / 95	0 / 30; 55 / 70	0 / 50; 55 / 95	0 / 30; 3 / 55; 55 / 70	0 / 30; 55 / 70	0 / 30; 55 / 70
Average error [%]		1.0	5.5	6.0	1.3	2.0
R^2 predicted vs. experimental RT		1.0000	0.9999	0.9996	0.9999	0.9999

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 ≤ 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
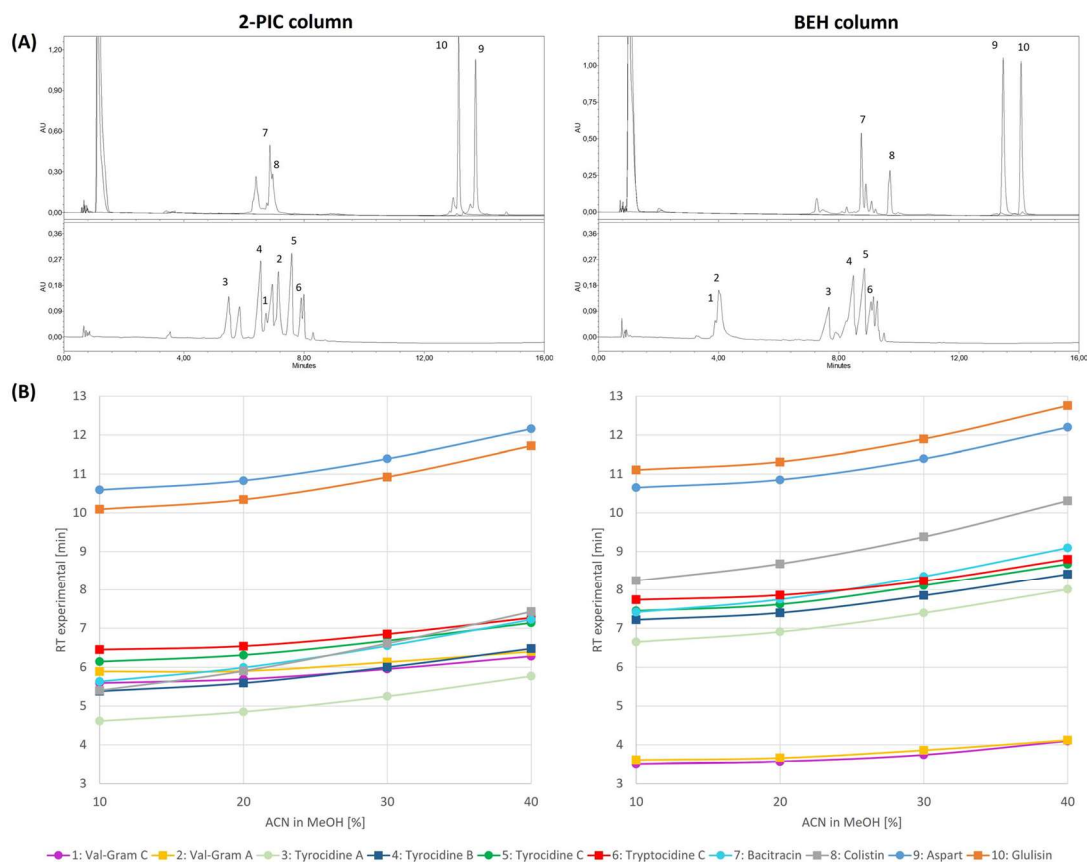
FIGURE 3 Exemplary ultraviolet (UV) overlay chromatograms of colistin, bacitracin, insulin aspart and insulin glulisine (top), and tyrothricin (bottom) derived from the base run combining 0% acetonitrile (ACN), 20 min gradient time, and 50°C column temperature. Representative peaks used for the calculations 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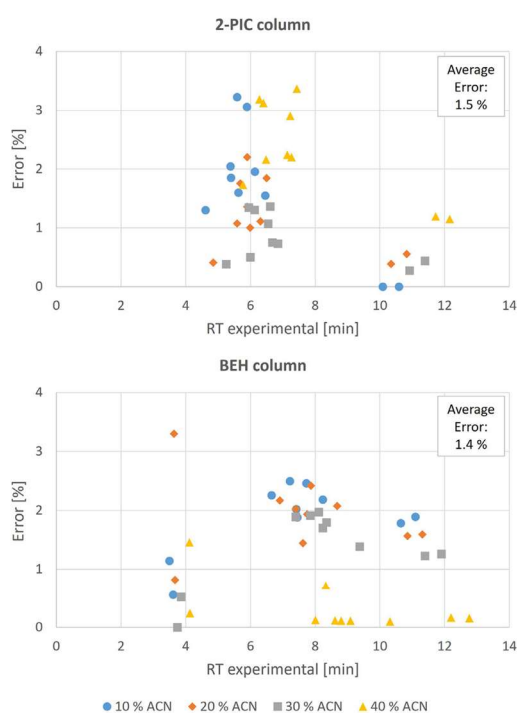 be adjusted. As a challenge to the prediction model, all these parameters were varied in different WPs. The gradient slope was varied by changing the start and end %B separately (WP I + II). A gradient step (WP III) was inserted, and the flow rate was increased (WP IV + V). The conditions were also adjusted 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 R^2 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 WP V 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
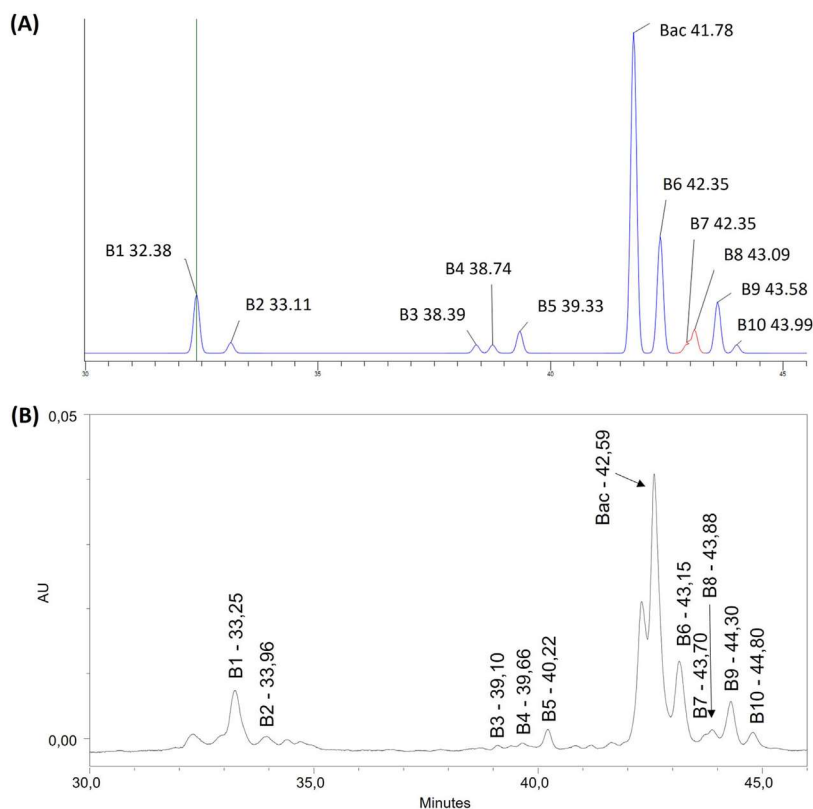
FIGURE 5 Predicted (A) and experimental (B) chromatograms for the working point V. The characteristic peaks used as the input data are marked.

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

Overall, the suitability and benefits of the software-based 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
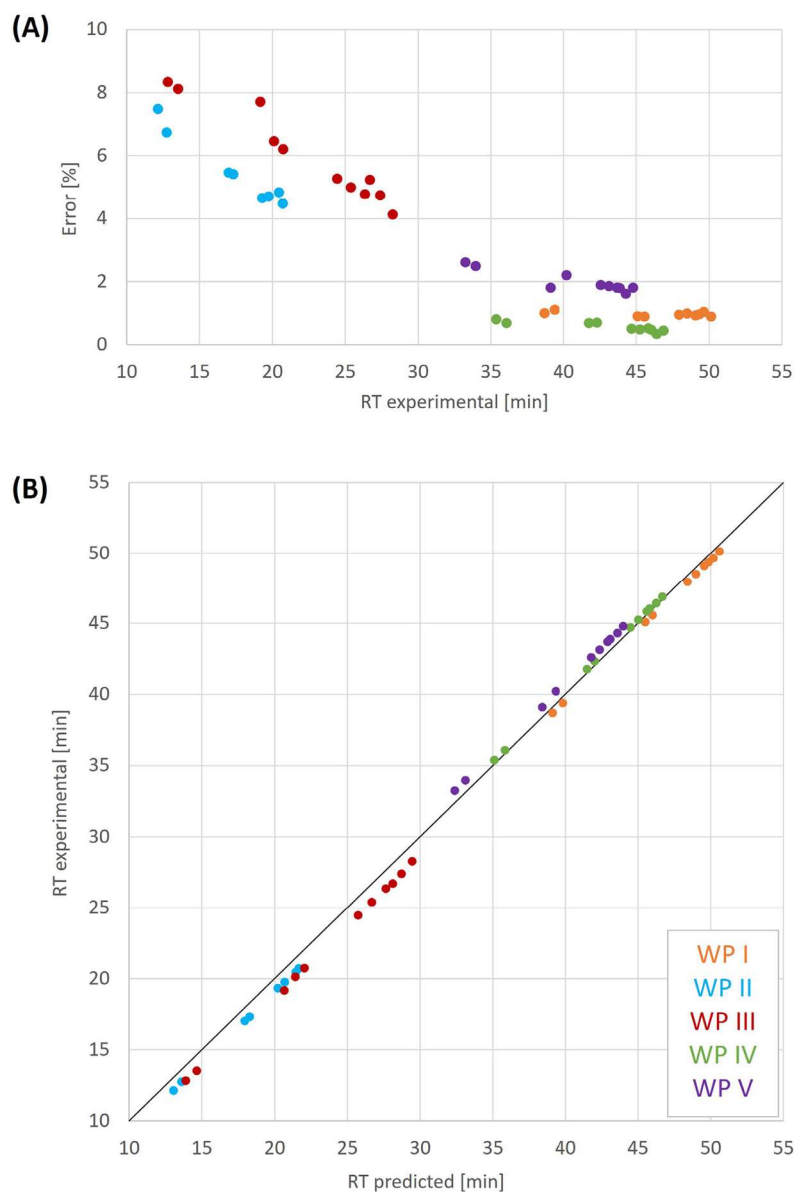
FIGURE 6 Graph showing the experimental retention times versus the percental error (A) and predicted versus the experimental retention times of bacitracin and byproducts for the chosen working points (B).

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
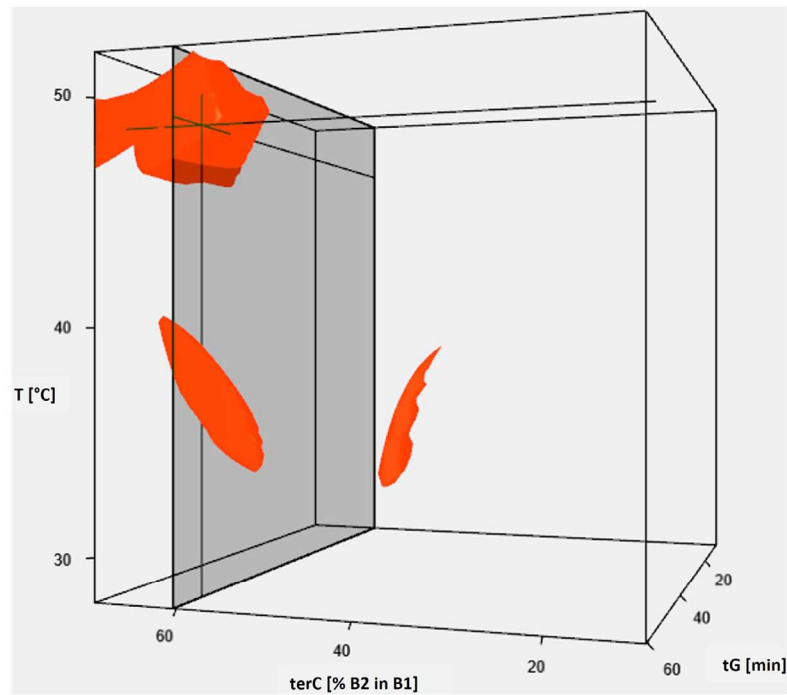
FIGURE 7 Three-dimensional design space for working point V with a critical resolution ≥ 1.0 indicated in red for bacitracin and its main impurities.
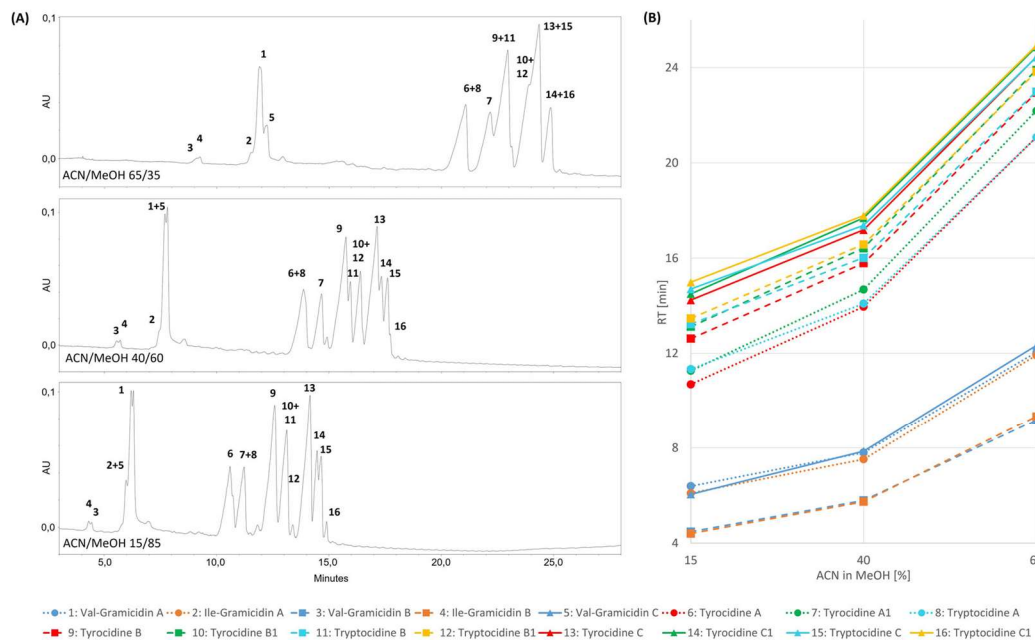
FIGURE 8 Overlay chromatogram of the input runs (A) and a plot showing the shift of the retention time order of tyrothricin compounds with variation in ternary composition (terC) while T (30°C) and gradient time (tG) (40 min) are kept constant (B). Please note that interpolated lines are just used to 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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4   Declaration of own contribution

The following list displays the author's contribution to the publications listed under "Manuscripts".

Manuscript I

- Conceptualization in cooperation with co-authors
- Methodology
- Investigation
- Formal analysis
- Data curation and visualization
- Preparation of the manuscript (original draft & revision)

Manuscript II

- Conceptualization in cooperation with co-authors
- Methodology
- Investigation
- Formal analysis
- Data curation and visualization
- Preparation of the manuscript (original draft & revision)

Manuscript III

- Conceptualization in cooperation with co-authors
- Methodology
- Investigation
- Formal analysis
- Data curation and visualization
- Preparation of the manuscript (original draft & revision)

Manuscript IV

- Conceptualization

- Methodology

- Investigation

- Formal analysis in cooperation with co-authors

- Data curation and visualization

- Preparation of the manuscript (original draft & revision)

5   Discussion

The data presented in the four manuscripts substantially supplements the state of knowledge regarding the potential of SFC for the separation of pharmaceutical peptides. In **Manuscript I**, a systematic column screening approach under DoE principles was employed to improve the separation of the complex composition of tyrothricin. The dependency between flow rate, efficiency, and UV response was investigated during the method optimization. The final SFC method holds advantages over RP-LC.

Manuscript II elaborated the benefits of binary mixtures of MeOH and ACN as the modifier in improving the resolution of bacitracin, colistin, daptomycin, and their impurities. When combined with an aromatic SP, even shifts in the elution order were observed. Summed up, the composition of the binary mixture is an effective chromatographic parameter leverageable to control selectivity during the method optimization. Thus, a generic method development workflow for the separation of peptides was proposed based on these observations.

The scope of analytes was extended to larger peptides in **Manuscript III** by transferring the knowledge of the previous manuscripts to insulin and its analogs. A crown ether as an alternative additive that also allows changing the elution order of the analytes in dependence to its concentration was investigated.

Finally, the binary modifier composition of MeOH and ACN was utilized as an effective input parameter to a chromatographic optimization software. The suitability of the linear solvent strength model (LSSM) as is for SFC separations within certain limits was shown in **Manuscript IV**. In addition, shifts in the retention time order were achieved via variations in the binary composition when combined with an SP rich in hydrogen bonding interactions, which complements the data of **Manuscript II**.

These individual studies shall now be contrasted to today's guidelines relevant to pharmaceutical analysis. Due to the adoption of the ICH Q14 guideline "Analytical procedure development" [41], tremendous regulatory changes to the analytical method development framework are expected. The manuscripts provide valuable data enabling a practical application of this concept. The combination of therapeutic

peptides as modern pharmaceuticals that are suffering from challenging analytics and SFC as a state-of-the-art technique is a vivid example, which benefits from this new guidance.

No dedicated guidance to analytical procedures was available until the adoption of the USP chapter <1220> "Analytical procedure lifecycle" [51] in 2022 and the recent ICH Q14 guideline in 2023. Previously, the development of analytical methods was derived from the ICH Q8 and Q9 guidelines, which introduced the quality by design (QbD) concept to the production of pharmaceuticals [137]. The consequent combination of RA, sound science principles, and knowledge management builds the foundation of these guidelines. As described in the ICH Q14 guideline (**Figure 6**), "prior knowledge" is the primary input into the concept, which "is explicitly or implicitly used for informing decisions during analytical procedure development and lifecycle management [41]. Prior knowledge can be internal knowledge […], external knowledge such as reference to scientific and technical publications or established scientific principles." It drives the processes of "technology selection" and "risk assessment". The USP chapter <1220> likewise emphasizes that "relevant prior knowledge can assist with the procedure development activities, which can include […] information in the scientific literature" [51]. The idea of prior knowledge management can be interpreted concerning the analyte, meaning physicochemical characteristics (pKa, isoelectric point, logP, etc.) or the knowledge about impurities and degradation pathways. However, the targeted analytical technique can and shall be considered as well.

As an additional input to the "technology selection", the "analytical target profile" (ATP) is combined with the prior knowledge to perform the RA. It defines the intended purpose and requirements of the future method, such as selectivity, working range, accuracy, precision, and other attributes.

This concept is implemented "in a phase-appropriate manner" [41]. That implies that the guidance's eternity should be applied to approved products or those entering the market, while a limited extent is suitable in early research and development (R&D) steps. In the early development phases, the maximization of knowledge regarding the presence of impurities is the driving force behind the analytical development. This aids in optimizing the synthesis and purification routes of the DS as well as the galenic development and manufacturing of the DP. Once formulation and manufacturing

processes are finalized and the impurity profile of the DS or DP is known, reproducibility and robustness of the analytical method, allowing a routine application for quality control, are the highest priorities of the analytical procedure development.
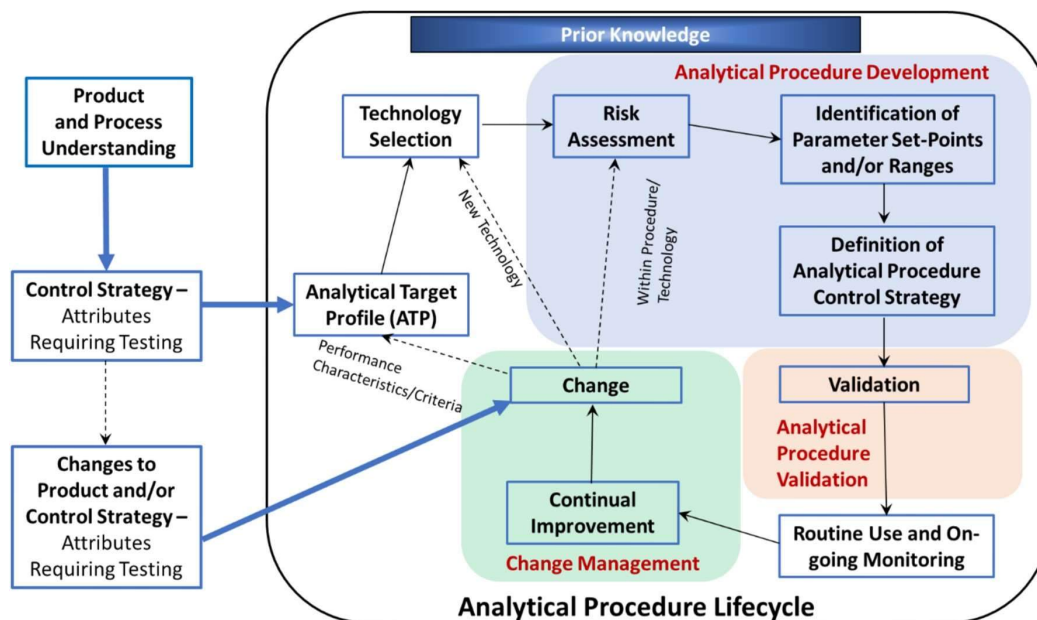
Figure 6: The analytical procedure lifecycle according to the ICH Q14 guideline. Reprinted from [41].

The new guidances from the ICH and USP provide a general concept that can be utilized to streamline analytical workflows. While the full extent of the guidances requires a defined impurity profile and a finalized DP, early drug development steps can also benefit from the proposed concept. Herein, efficient analytical approaches, which respect prior knowledge and apply state-of-the-art techniques, are demanded [138], considering that most drug candidates are dismissed and just a fraction enters clinical phases.

When evaluating SFC as a potential candidate during the "technology selection", it indeed can be rated as a state-of-the-art technique and an alternative to RP-LC for analyzing small molecules [75, 98, 139, 140]. However, the knowledge about peptide separations with SFC is limited, as summarized in **Section 2.5**. The early studies showed the general applicability of SFC for peptides in terms of sufficient retention and elution or that highly diverse peptides can be separated. Only a few of the more recent studies investigated SFC's potential for the determination of impurities, which means the separation of highly similar compounds [68, 69, 90, 111]. **Manuscripts I, II**,

III, and **IV** expand the overall knowledge on this topic and demonstrate that SFC can provide a degree of separation of the main compound and its related substances required for purity determinations or characterization purposes.

In **Manuscript I**, advances of SFC over RP-LC were found for tyrothricin's complex composition due to a better separation of the cyclic peptide fraction. Even isomers coeluting in RP-LC were resolved. This study needs to be assessed in the context of the other studies, which compared both techniques and found neither to be superior in all cases or showed the inferior performance of SFC to RP-LC in terms of the number of separated peaks [68, 69, 111]. In addition, the results of **Manuscript I** regarding the dependence of flow rate and sensitivity needs to be considered for impurities in lower concentrations. The two main studies [68, 69] comparing SFC and RP-LC for the separation of peptides applied much higher flow rates for SFC and detected fewer peaks. It needs to be scrutinized if these flow rates were optimal due to their impact on sensitivity. As a result, the authors might have underestimated the potential of SFC. The same is true for the binary composition of the modifier. Both studies did not consider this parameter. In fact, binary mixtures of the modifier were found to enhance the separation of the studied compounds distinctly in **Manuscripts II**, **III**, and **IV**. Additionally, the elution order of some compounds was changed with the composition of the binary mixture, highlighting its relevance for the optimization of the selectivity. Overall, these findings are relevant to verify that SFC is a state-of-the-art technique for peptide separations, which in some instances is superior to RP-LC and thus needs to be considered during the "technology selection" step, either for a new product or during the "change" process, when a "new technology" is introduced.

The specificity of an analytical method is a major characteristic to be demonstrated during method validation according to the ICH Q2 guideline [35]. This is possible through an orthogonal procedure comparison: "Specificity/selectivity can be verified by demonstrating that the measured result of an analyte is comparable to the measured result of a second, well-characterized analytical procedure that ideally applies a different measurement principle." As presented in **Manuscript I** and earlier works [68, 69, 111], SFC is an ideal candidate to fulfill these requirements. Also, the "stability indicating properties" of an analytical method are explicitly named in the recent revision of the ICH Q2 guideline. Stress tests are vital during the development of

analytical methods, "if impurities, related substances or degradation products cannot be prepared or isolated" Then "specificity can be proven by comparing the test results of samples containing typical impurities, related substances or degradation products with an orthogonal procedure" [35]. As shown by Losacco et al. [111], the parallel analysis of stressed peptide samples with SFC and RP-LC results in orthogonal separations and can be utilized to fulfill the demands of the ICH Q2(R2) guideline. It is also beneficial during R&D steps, where knowledge about the number and identity of impurities, intermediates, and degradants is of central importance. This underlines the relevance of SFC as a notable analytical option for peptides.

After selecting a suitable technology, an RA is performed. Method development and optimization steps highly benefit from RA, which identifies and prioritizes relevant parameters. The ICH Q14 guideline describes this procedure as: "RA is typically performed early in analytical procedure development and is repeated as more information becomes available. RA [...] can be supported by prior knowledge" and is necessary to "identify and prioritise analytical parameters to be investigated experimentally" [41]. The USP chapter <1220> likewise accounts for this: "Once potential variables have been identified, the risk associated with each variable can be estimated and ranked [...]. This Assessment is driven by prior knowledge and scientific expertise" [51]. Different RA tools are established, for instance, the Ishikawa (fishbone) model [141], CNX-analysis [142], or a heat map, as proposed in the USP chapter <1220>.

The fishbone model frequently finds application in identifying and gathering parameters in a brainstorming manner as the first step of the RA. Typically, no classification of the identified impact is carried out initially [143] but is consecutively performed through a numeric scale [135] or the transfer to a heat map or CNX-analysis [136]. In addition, it is helpful to categorize identified factors as either qualitative or quantitative. Qualitative factors are those that can be set as either "A" or "B", and quantitative factors can vary from "X" to "Y". In a CNX-analysis, parameters are categorized as controlled (C), noise (N), or experimentation (E). Those that do not change are considered controlled. Insignificant or difficult-to-control variables are classified with "N" and are not further evaluated. Parameters to be assessed experimentally are labeled "X". In contrast, a heat map uses colors from green to red

to rate a risk or an impact from low to high. As an alternative to these RA tools, a ranking can be applied as utilized in **Manuscript I**. Either way, a classification of the identified parameters is essential as it is the driving input for planning the subsequent experimentation.

An RA also helps to identify parameters where no or limited knowledge is available, especially in the case of SFC as a rather new technology. Experimentation is then required to investigate the effect of these parameters. For example, a lack of understanding of the impact of ternary eluent composition was determined before performing the study presented in **Manuscript II**. Based on the scientific data for small molecule separation with SFC or transferred from RP-LC peptide applications [132, 134, 148], it was considered a parameter that could be valuable to improve the separation. Thus, it was evaluated in **Manuscripts II**, **III**, and **IV**.

To generate an RA generally applicable to the SFC separation of peptides, the expected impact of the chromatographic parameters on peak resolution must be assessed. For the purity analysis of a peptide, a range of compounds must be separated. These must be retained and eluted in an adequate time. For compounds of high chemical similarity, the efficiency calculated as peak width also plays a vital role. According to the Purnell equation [144], chromatographic separations are calculated as the peak resolution (R_s). It is defined as the product of efficiency (1), retention (2), and selectivity (3) calculated via the plate number (N), retention factor (k) and separation of two peaks (α):

$$
R_s = \underbrace{\left(\frac{\sqrt{N}}{4}\right)}_{1} \times \underbrace{\left(\frac{k}{k-1}\right)}_{2} \times \underbrace{\left(\frac{\alpha}{\alpha-1}\right)}_{3}
$$

A heat map (**Table 2**) is used to estimate the impact of the SFC parameters for the separation of peptides, considering prior knowledge in the form of scientific publications as summarized in section 2.5 complemented by **Manuscripts I**, **II**, **III**, and **IV**. In some cases, data on small molecule or RR-LC separations is respected when data is unavailable for the SFC separation of peptides. The impact of each parameter on peak resolution is rated from minor (green) over medium (yellow) to strong (red).

Table 2: *Risk assessment of SFC parameters for peptide separations applicable pre (initial RA) and post (final RA) method development.*

	Chromatographic parameter	Initial RA	References	Comment	Final RA	Comment
Stationary phase	Ligand chemistry	Qual	[68, 90, 111, 122, 123, 125-131, 133, 135, 136], M I - IV	Main factor determining selectivity, retention and efficiency.	C	
	Temperature	Quant	[69, 90, 121, 122, 128, 129], M I, III, IV		N/X	Classification based on the knowledge gained during method development.
	Dimensions	Qual	[125, 126], M II		C	
	Batch-to-batch reproducibility	Qual	[145, 146]	No SFC data available. Evaluated based on RP-LC data.	N/X	Typically tested as a robustness parameter. Specific lot(s) might be defined.
Mobile phase	Additive type	Qual	[68, 90, 105, 111, 122, 125-127, 129-131, 133, 136, 147], M I - III	Additives determine the charged state of peptides. IP reagents are essential for basic peptides.	C	
	Modifier type	Qual	[122, 123, 129, 131], M II - IV	MeOH is dominantly used. Other alcohols or ACN are also applicable.	C	
	Binary modifier composition	Quant	[132, 134, 148], M II - IV	Little knowledge available. High Impact was evaluated in M I - IV.	N/X	Classification based on the knowledge gained during method development.
	Water concentration	Quant	[105, 128, 147], M I	Presence of water is essential for an acceptable peak shape.	N/X	Classification based on the knowledge gained during method development.
	Gradient time and slope	Quant	[90, 128, 131], M I - IV		C	
	Gradient start and end % B	Quant	[69, 90, 105, 122, 129, 147], M I - IV		C	
	Additive concentration	Quant	[105, 111, 121, 125, 127, 128, 147]		N/X	Classification based on the knowledge gained during method development.
	Backpressure	Quant	[69, 121, 122, 125, 129, 149], M I		C	
	Flow rate	Quant	[90, 128], M I, IV		C	
Sample	Sample solvent composition	Quant	[133]		N/X	Classification based on the knowledge gained during method development.
	Column load	Quant	[133]		C	

Qual: qualitative, *Quant:* quantitative   **M:** Manuscript(s)   **C:** controlled, **N:** noise, **X:** experimentation

Based on the RA, the experiments to be performed under design of experiments (DoE) principles are planned. A combination of screening and optimization experiments is typically executed [150, 151]. Generally, the goal of screening experiments is the identification of factors that have a significant effect on the goal defined in the ATP. Often, two-level designs are used to study a larger number of factors. Plackett-Burman or fractional-factorial models are popular options to maximize the number of factors examined while minimizing the experimental effort [152]. However, chromatography follows certain well-established principles. The factors having the highest impact can be estimated from the available scientific literature as evaluated in the RA in **Table 2**. These factors are investigated experimentally according to their expected effect on the resolution, which ranges from strong to minor impact. As shown in **Manuscripts II**, **III**, and **IV** and earlier studies [90, 111], the combination of SPs, additives, and type of organic solvent is most relevant. The right combination determines if the analyte can be eluted, emphasizing the relevance of these strong impact and qualitative parameters. Nevertheless, the need for streamlining such column screening steps was identified by several chromatographers to accelerate method development activities [153-155].

The adequate visualization of the screening data that allows a straightforward interpretation is obligatory. While studying two or more quantitative factors enables the visualization as a contour plot or response surface (**Manuscripts I** and **III**), this is not possible for qualitative parameters. Therefore, a color-coded table, as used in **Manuscripts II**, is a valuable option. Alternatively, Derringer's desirability functions are a versatile tool to evaluate a screening experiment [90, 154, 156]. These enable the data visualization of multiple responses dependent on several qualitative factors. Several responses, such as the number of peaks, peak shape, peak height, signal-to-noise ratio, etc., can be evaluated simultaneously. Normalizing all responses then allows the determination of the overall best combination of the studied factors [152].

Optimization experiments are performed after setting the best combination of SP, modifier composition, and additive. As shown in **Manuscripts I**, **II**, **III**, and **IV**, the separation of the peptides requires long and flat gradients. Thus, the parameters that primarily determine the retention time window of the analyte must be defined first. Consecutively, the other lower-impact parameters are studied and optimized.

Otherwise, it is complicated to evaluate the impact of parameters that result in relatively small improvements in the resolution (e.g. the backpressure). These can be studied via a set of optimization designs, such as Box-Behnke or full-factorials [152]. Often, several DoEs are required to achieve a sufficient separation, and software-assisted experimentation is demanded due to the complexity of the data generated. An empirical full-factorial approach was used in **Manuscripts I** and **III**.

Alternatively, a mechanistic approach based on the LSSM can be adopted from RP-LC principles. Established software can be applied to high-modifier SFC separations of peptides, as demonstrated in **Manuscript IV**. Apart from the input parameters (binary modifier composition, gradient time, and column temperature), others (gradient points, flow rate, column dimensions, etc.) are predictable due to the LSSM principles and can be optimized in-silico. This expands the possibilities of software-assisted optimizations and further reduces the experimental efforts during method optimization steps.

In **Manuscript II**, a generic method development protocol was proposed. It is now updated by the knowledge gained in **Manuscripts III** and **IV**, supplemented by the RA and the above-discussed aspects, as displayed in **Figure 7**. Considering regulatory demands, describing these workflows in a manner that connects an action to a scientific fundament is relevant. This approach provides scientific justification in regulated environments and streamlines the analytical development process in a time-effective manner.

Consecutive cycles of screening and optimization experiments are performed until the requirements of the ATP are fulfilled. If this is not possible, a return to technology selection and RA is required. Once the ATP is met, the chromatographic parameters studied and optimized during the method development must be (re)assessed in a final RA. A CNX-analysis (**Table 2**) is appropriate at this stage. This RA is more focused on the robustness of the method. Most high-impact factors identified in the initial RA are qualitative (SP, modifier type, additive type, etc.) and can now be categorized as controlled (C). Based on the knowledge gained during the development, the remaining parameters causing the highest impact on the separation are classified to be experimentally (X) investigated during robustness testing while leaving the others as noise (N).
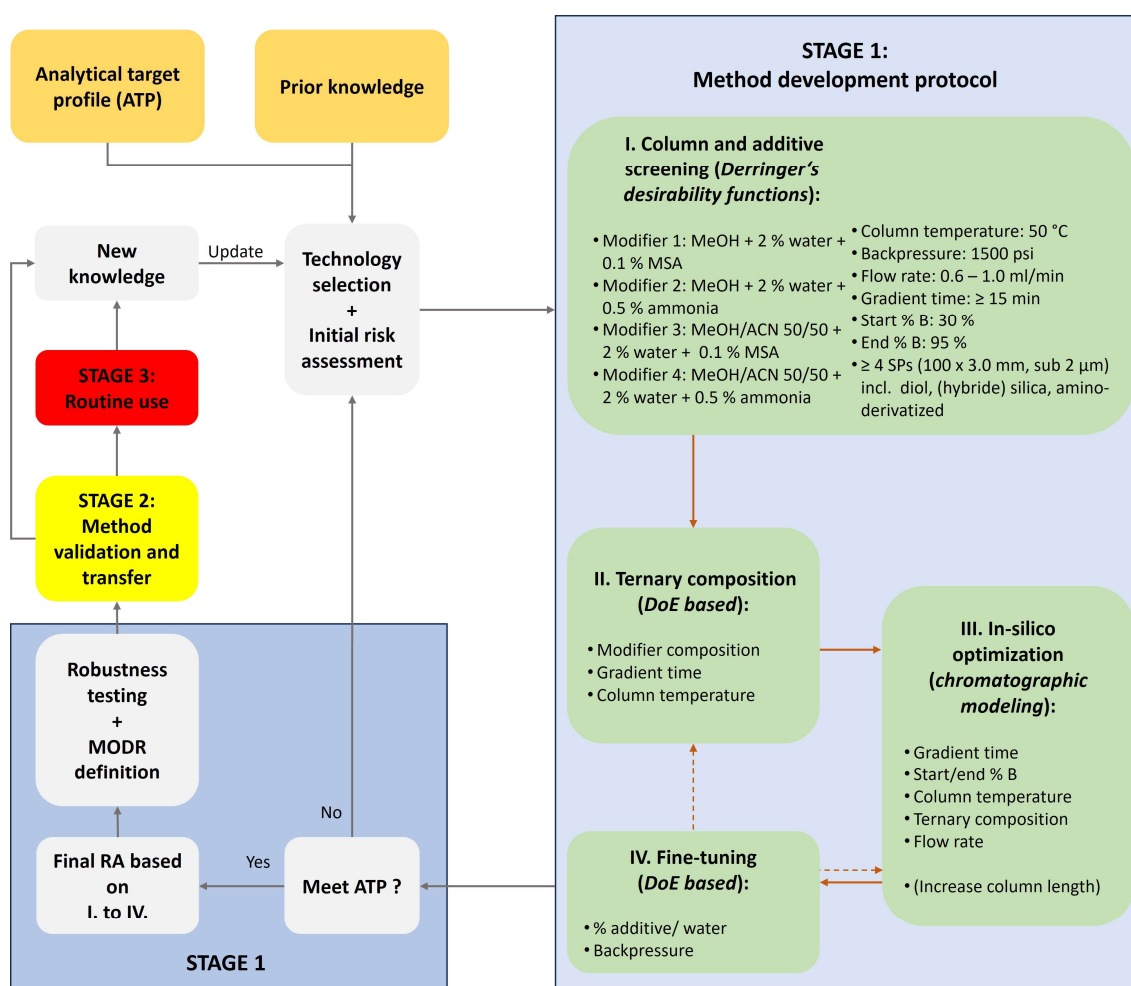
Figure 7: *A generic SFC method development protocol for therapeutic peptides after considering relevant prior knowledge from scientific publications in the form of a risk assessment. Dotted lines indicate optional steps.*

The ranges of these parameters shall at least include inaccuracies caused by the analyst (e.g. manual preparation of the modifier) or the instrument (e.g. column oven specification: ± 2 °C) and optimally hold a certain capacity for future adjustments of the method. With the adoption of the ICH Q14 and the revision of the Q2 guideline, robustness testing is considered the last step of the method development that shall be performed before entering the method validation stage. The data of the robustness testing finally defines the method operable design region (MODR). The MODR specifies the combination of the parameter ranges applicable to fulfill the ATP during the routine application of the analytical method.

The consistent integration of knowledge management to all stages of the analytical procedure should be utilized to improve a specific procedure. Likewise, new knowledge about a technique is respected in future actions and used to regularly update the RA and connected workflows. Scientific publications are respected equivalently.

The procedure presented shows how SFC can be considered and integrated as a "new technology", according to the ICH Q14 guideline, for the separation of peptides. The outlined approach, which reflects the available knowledge in the scope of an RA to define a generic workflow for a particular analytical technique for a specific compound class, can be used as a blueprint and is transferable to any other analytical technique or compound class.

6   Outlook

Apart from regulatory aspects and the current state of scientific knowledge, technological aspects and potential improvements need to be discussed to assess the capabilities of SFC for peptide separations. Considering the Purnell equation [144], reducing the peak width increases the resolution of the analytes. As **Manuscripts I** and **IV** show, higher flow rates result in sharper peaks and an increase in peak resolution. A shortcoming of the current generation of SFC pumps is the lower pressure limit (Agilent 1260 Infinity II SFC: 600 bar, Shimadzu Nexera UC: 660 bar, Waters Acquity UPC²: 410 bar) [157] compared to state-of-art ultra high-performance liquid chromatography (UHPLC) instrumentation with pressure limits up to 1500 bar. This is even more relevant when working with water-rich modifiers running a gradient up to 100 % to elute polar biomolecules.

Several factors cause extra-column band broadening in SFC instruments and impair efficiency [73, 157, 158]. Today's SFC instruments evolved minimally in this aspect since the 1990s. Broad flow rate ranges (up to 5 ml/min) require larger capillary diameters, resulting in excessive extra-column dispersion effects. As discussed by the authors, the benefits decreasing the diameter are compensated by an increased backpressure and a lowering of the applicable flow rate. Furthermore, excessive volumina of the UV detector cells (Agilent 1260 Infinity II SFC: 13 µl, Shimadzu Nexera UC: 13 µl, Waters Acquity UPC²: 8.4 µL) contribute to the extra-column dispersion. In contrast, the Waters Acquity H-Class UHPLC system has a 0.58 µl flow cell in the standard setup. Thus, smaller pressure-resistant flow cells optimized for today's high-modifier SFC conditions are needed.

Nowadays, column dimensions of 100 x 3.0 mm with sub-2 µm particles are the standard SFC format [73, 158]. Smaller diameter columns might be helpful to reduce band broadening. Reducing the inner diameter increases the pressure, which requires adequate pump and backpressure systems. Alternatively, superficial porous particles provide lower backpressure. However, the commercially available range, especially for modern SFC-dedicated chemistries, is limited compared to fully porous particles.

Several effects add up in peptide analysis where long and shallow gradients are necessary. As shown in **Manuscripts I**, **II**, **III**, and **IV**, these result in broader peaks and a distinct loss in sensitivity. Higher flow rates can help to improve efficiency but also come with a sensitivity loss when concentration-dependent detectors (UV or MS) are used, as indicated in **Manuscript I**.

Fluorescence detection holds excellent potential to compensate for the loss in sensitivity due to the intrinsic fluorescence of peptides [159-161]. However, the available fluorescence flow cell designs are pressure-sensitive and would burst under SFC backpressure conditions. Currently, none of the major manufacturers of SFC instruments (Agilent, Shimadzu, and Waters) provides SFC-compatible fluorescence detectors [162]. Peptide applications surely would benefit from advances in this area.

Apart from the pressure limitations discussed, additional modifications to the pump are desirable. The current pump designs utilize a binary pump system, with pump A providing CO_2 and pump B the modifier. In fact, the advances of the dual gradient UC mode were just demonstrated, requiring a dynamic mixing of the modifier [89, 97]. Thus, exchanging the isocratic B pump for a quaternary pump would be highly beneficial. Additionally, such a pump would enhance the mixing accuracy and robustness of binary modifier compositions as those used in **Manuscripts II**, **III**, and **IV**.

Non-specific adsorption effects on stainless steel parts of the flow path gained growing attention in the last years [163]. These effects lead to analyte and efficiency loss and predominantly affect anionic and phosphorylated compounds [164]. Hence, several modifications of the surface materials were introduced. Stainless steel was exchanged for titanium or MP35N (nickel–cobalt alloy). Alternatively, PEEK or ethylene-bridged siloxane polymer (hybrid surface) coatings were applied [165]. Several manufacturers released dedicated brands to bioanalysis based on such modifications: Bio-inert (Agilent), Nexera XS inert (Shimadzu), and Premier (Waters). Also, modified column hardware is becoming more and more available, for example Accura (YMC), Premier (Waters), and Shim-pack Scepter (Shimadzu). Dedicated instrument series are currently only available for LC systems. Nevertheless, an introduction to SFC systems is desirable when working with biomolecules.

Apart from instrumental advances, further scientific work is required. Considering the RA in **Section 5**, which summarizes the available literature, two of the most critical

parameters impacting the selectivity are the type of SP and the additives. Regularly, studies on new particle derivatizations are published. However, reliable commercial availability and high batch-to-batch reproducibility are mandatory for pharmaceutical applications and need to catch up with the scientific advances. Polar HILIC-type and modern SFC-dedicated SPs are especially beneficial for peptide separations. As an exemplary addition to these SPs, the amino-modified PEI (polyethylene imine) and Arginine chemistries by Dr. Maisch, Regis Technologies, and Thermo Fisher Scientific are named. Also, SPs used for chiral separations, which combine amino, hydrogen bonding, and aromatic functionalities, might hold some exciting candidates, as already demonstrated for small molecules [166]. Potential examples include teicoplanin-type or cinchona alkaloid-based modifications. In addition, the growing commercial range of mixed-mode SPs, such as those developed by HELIX Chromatography, SIELC Technologies, and Thermo Fisher Scientific, are noteworthy.

An efficient approach to column chracterizations for peptide separations via RP-LC was proposed by Field et al. [146, 167-170]. This concept needs to be adapted for SFC and diverse SPs combined with relevant additives (MSA and ammonium hydroxide), and compositions of MeOH and ACN plus water must be covered. In addition, the combination of two or more SPs also holds the potential to improve peptide separations. Apart from improving efficiency due to coupling identical columns, as demonstrated in **Manuscript II**, differing chemistries can be combined to optimize the selectivity, as was shown for small molecule separations [171-173].

Most additives currently used in SFC were adapted from the RP-LC mode. Obviously, solubility under SFC conditions is a precondition. In SFC, basic analytes pose the same problems of excessive peak tailing as in RP-LC. Thus, IP reagents were studied successfully early on [109, 174]. Following this concept, chaotropic additives might likewise be helpful. Recently, sodium perchlorate ($NaClO_4$) was studied as an SFC additive for the separation of nucleosides and nucleotides without causing solubility issues [86]. Ammonium hexafluorophosphate (NH_4F_6P) is another promising option that showed benefits in RP-LC peptide separations [169, 170]. In addition, natural deep eutectic solvents (NADES) form a rich reservoir of potential additive candidates [175]. These are prepared by mixing choline or other quaternary ammonium salts with a hydrogen bond donor. The resulting liquid then is SFC compatible and improves the

separation of polar analytes [176, 177]. Alternate green organic solvents, such as propylene carbonate [178], also need to be evaluated for potential selectivity benefits in SFC.

In summary, instrumental advances, including innovative SP chemistries and scientific progress in column characterization, additives, and solvents, are required to further evolve SFC for peptide separations.

7   Conclusion

This work highlights the notable potential of SFC as a viable alternative to RP-LC for the analysis of peptides. It extends the existing knowledge in this domain by providing valuable insights into the separation of predominantly cyclic peptides within a mass range of 1000 to 6000 Da.

The purity determination or fingerprinting of peptides requires the separation of highly similar compounds. Advances of SFC over RP-LC were demonstrated for the complex mixture of tyrothricin that contains several isomers. Major improvements in the resolution of the fingerprints of additional model compounds were achieved due to the application of binary modifier compositions, which offers an alternative to the prevalent use of methanol as the sole solvent. Furthermore, the suitability of SFC for larger peptides, insulin and its analogs, was shown. Finally, the feasibility of a well-established chromatographic modeling software for the in-silico retention time prediction and method optimization of peptide separations with SFC was demonstrated.

These findings were incorporated into the analytical procedure lifecycle concept introduced by the ICH Q14 guideline and USP chapter <1220>. The presented manuscripts and earlier studies were evaluated in a general risk assessment of the SFC parameters impacting the separation of peptides in SFC. This enabled the definition of a generic method development protocol that streamlines optimization approaches. The proposed strategy can be easily transferred to any other analytical technique or compound class.

In conclusion, SFC needs to be considered for the analysis of therapeutic peptides, particularly for isomeric or cyclic compounds. Potential enhancements of the instruments may elevate SFC to a level comparable to current UHPLC systems. Ongoing research in stationary phase chemistry and additives also holds promise for further improvements. However, it is crucial to emphasize that while SFC presents a valuable alternative, it will not replace RP-LC, but both techniques must be considered complementary. Their combination, especially during R&D and early (pre)clinical

phases, is valuable to generate comprehensive knowledge about a drug, ensuring its quality, efficiency, and safety.

8   References

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12   Appendix

Stationary phases adapted from [99] and indicated as numbers in **Figure 5**:

Number	Name	Manufacturer	Ligand
1	Kinetex F5 (2)	Phenomenex	Pentafluorophenyl
2	Titan PFP	Sigma-Aldrich	Pentafluorophenyl
3	Kinetex PFP	Phenomenex	Pentafluorophenyl
4	Accucore PFP	Thermo	Pentafluorophenyl
5	Ascentis Express F5	Sigma-Aldrich	Pentafluorophenyl
6	Nucleoshell PFP	Macherey-Nagel	Pentafluorophenyl
7	Acquity UPC² CSH Fluorophenyl	Waters	Pentafluorophenyl
8	Accucore HILIC	Thermo	Bare silica
9	Cortecs HILIC Waters	Waters	Bare silica
10	Titan Silica	Sigma-Aldrich	Bare silica
11	Shim-pack UC Sil	Shimadzu	Bare silica
12	Hypersil Gold Silica	Thermo	Bare silica
13	Kinetex HILIC	Phenomenex	Bare silica
14	Acquity UPC² BEH	Waters	Bare hybrid silica
15	Accucore Urea-HILIC	Thermo	Propylurea
16	Accucore 150-Amide-HILIC	Thermo	Polyamide
17	Shim-pack UC Amide	Shimadzu	Amide
18	Shim-pack UC Diol	Shimadzu	Propanediol
19	Acquity UPC² Torus DIOL	Waters	Propanediol
20	Ascentis Express OH5	Sigma-Aldrich	Pentahydroxyl
21	Shim-pack UC CN	Shimadzu	Cyanopropyl
22	Hypersil Gold CN	Thermo	Cyanopropyl-bonded silica
23	Shim-pack UC Triazol	Shimadzu	3-Amino-triazole
24	Nucleoshell HILIC	Macherey-Nagel	Sulfobetaine
25	Syncronis HILIC	Thermo	Sulfobetaine
26	Acquity UPC² Torus DEA	Waters	Diethylamine
27	Shim-pack UC NH2	Shimadzu	Amino
28	Shim-pack UC Hyp	Shimadzu	3-Hydroxphenyl
29	Acquity UPC² BEH 2-EP	Waters	2-ethylpyridine
30	Shim-pack UC Py	Shimadzu	Pyridinyl
31	Acquity UPC² Torus 2-PIC	Waters	2-Picolyl-amine
32	Acquity UPC² Torus 1-AA	Waters	1-Amino-anthracene
33	Shim-pack UC PBr	Shimadzu	Pentabromobenzyl
34	Shim-pack UC PyE	Shimadzu	Pyrenylethyl
35	Shim-pack UC Phenyl	Shimadzu	Phenyl
36	Accucore Phenyl-X	Thermo	Phenyl-alkyl
37	Synergi Polar RP	Phenomenex	Phenylxoxypropyl
38	Kinetex Biphenyl	Phenomenex	Biphenyl
39	Ascentis Express Biphenyl	Sigma-Aldrich	Biphenyl
40	Speedcore Diphenyl	Fortis Technologies	Diphenyl
41	Ascentis Express Phenyl-hexyl	Sigma-Aldrich	Phenyl-hexyl
42	Accucore Phenyl-hexyl	Thermo	Phenyl-hexyl
43	Acquity UPC² HSS C18 SB	Waters	Octadecyl, non endcapped
44	Shim-pack UC GIS II	Shimadzu	Octadecyl, endcapped
45	Kinetex XB-C18	Phenomenex	Octadecyl, endcapped, di-isobutyl sidechain
46	Nucleodur C18 Gravity-SB	Macherey-Nagel	Octadecyl, endcapped, di-isobutyl sidechain
47	Shim-pack UC RP	Shimadzu	Octadecyl, embedded group
48	Acquity UPLC BEH Shield RP18	Waters	Octadecyl, embedded group
49	Cosmocore Cholester	Nacalai Tesque	Cholesteryl
50	Shim-pack UC Choles	Shimadzu	Cholesteryl