Investigation of robustness for supercritical fluid chromatography separation of peptides: Isocratic vs gradient mode

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A B S T R A C T
We investigated and compared the robustness of supercritical fluid chromatography (SFC) separations of the peptide gramicidin, using either isocratic or gradient elution. This was done using design of experiments in a design space of co-solvent fraction, water mass fraction in co-solvent, pressure, and temperature. The density of the eluent (CO₂-MeOH-H₂O) was experimentally determined using a Coriolis mass flow meter to calculate the volumetric flow rate required by the design. For both retention models, the most important factor was the total co-solvent fraction and water mass fraction in co-solvent. Comparing the elution modes, we found that gradient elution was more than three times more robust than isocratic elution. We also observed a relationship between the sensitivity to changes and the gradient steepness and used this to draw general conclusions beyond the studied experimental system.

To test the robustness in a practical context, both the isocratic and gradient separations were transferred to another laboratory. The gradient elution was highly reproducible between laboratories, whereas the isocratic system was not. Using measurements of the actual operational conditions (not the set system conditions), the isocratic deviation was quantitatively explained using the retention model. The findings indicate the benefits of using gradient elution in SFC as well as the importance of measuring the actual operational conditions to be able to explain observed differences between laboratories when conducting method transfer.

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1. Introduction

The separation of therapeutic peptides has long been an important application area for chromatography, particularly reversed-phase liquid chromatography (RPLC) [1]. With growing interest in supercritical fluid chromatography (SFC) for analyzing and purifying small molecules (i.e. molecular weights < 1 kDa) [2,3], several authors from both academia and industry have also started to investigate how SFC could be used to analyze and purify peptides [4–13]. While the quality-by-design (QbD) paradigm is firmly established in liquid chromatography [14], it is not similarly established in SFC, probably because SFC is less robust than liquid chromatography [3]. Some studies have investigated the robustness of SFC separation methods in the context of method transfer and by investigating the robustness in a design space [15].

The small but growing body of studies treating the SFC separation of peptides [4–13] has investigated a limited number of peptides, for example, gramicidin D [6,12,13], leucine-enkephalin [4–6,10], methionine-enkephalin [4–6,10], angiotensin I [4], angiotensin II [4–6], cyclosporin analogs [7], betamethylphenylalanine [11], oxytocin [10], bradykinin [4,10], Pro-Leu-Gly amide [4], sauvagine [4], leupeptide [4], urotensin II [4], sulfomycin [8], cyclic peptides [16], and custom acidic and basic linear uncapped peptides [9].

Most studies have used traditional liquid chromatographic stationary phases such as silica [7,9], diol [8,9], C18 [4,9], 2-ethylpyridine [4,5,9], cyano [6], and various chiral phases [11].
Table 1

Properties of the gramicidin isoforms partially separated in the study.

<table>
<thead>
<tr>
<th>Gramicidin species</th>
<th>Mw [g mol⁻¹]</th>
<th>X</th>
<th>Y</th>
<th>Specified purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>formyl-X-Gly-Ala-Leu-Ala-Val-Trp-Leu-Trp-Leu-Trp-ethanolalamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val-A</td>
<td>1881</td>
<td>Val</td>
<td>Trp</td>
<td>80–85%</td>
</tr>
<tr>
<td>Ile-A</td>
<td>1895</td>
<td>Ile</td>
<td>Trp</td>
<td>Unknown</td>
</tr>
<tr>
<td>Val-B</td>
<td>1842</td>
<td>Val</td>
<td>Phe</td>
<td>6–7%</td>
</tr>
<tr>
<td>Ile-B</td>
<td>1856</td>
<td>Ile</td>
<td>Phe</td>
<td>Unknown</td>
</tr>
<tr>
<td>Val-C</td>
<td>1858</td>
<td>Val</td>
<td>Tyr</td>
<td>5–14%</td>
</tr>
<tr>
<td>Ile-C</td>
<td>1872</td>
<td>Ile</td>
<td>Tyr</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

* Specified by vendor.  ** Not specified by vendor.

as well as polymer-based phases such as divinylbenzene [10] or poly(styrene–divinylbenzene) [12,13]. Eluents are typically CO₂ modified with acetonitrile/water [5,8], acetonitrile [4,11], methanol [4,6–9,11], ethanol [7,11], and isopropyl alcohol [7,11] to which acidic or basic additives such as trifluoroacetic acid (TFA) [4,5,9], 2,2,2-trifluoroethanol (TFE) [6], ammonium acetate [4,6], acetic acid [6], and isopropyl amine [6,8] are added. Several studies have investigated the modification of co-solvents with water, and found its addition necessary to achieve resolution or to improve peak shape [4,6,9]. Most studies have used gradient elution, but some have also investigated the isocratic elution mode [11].

Due to the small chemical space investigated, it is difficult to draw general conclusions as to the feasibility of using SFC for peptide analysis and purification. However, several of the mentioned studies did investigate the effects of the stationary phase, eluent, and other operational conditions, such as back pressure and temperature [7]. Clearly, a mechanistic understanding of peptide separation in SFC is lacking compared with our understanding of the much more mature RPLC technique [17–19].

Robustness is “a measure of … an analytical procedure’s capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage” [20]. It is well known that deliberate variations in operating conditions in SFC can greatly affect separation [21,22] which is an advantage of SFC as compared to LC for improving selectivity; however, this also affects the robustness. However, it is less known and understood that unintentional variations can also have a major impact, for example, when operating SFC in highly compressible regions or when general retention mechanisms are poorly understood. Studying the robustness of separations conducted in the high co-solvent regime of SFC, technically in subcritical conditions [23] can give valuable insight into areas typically not studied in SFC where the eluent is more LC like because the fluid compressibility decreases with increasing co-solvent in the eluent. Most studies indicate that working with a large fraction of co-solvent is necessary to elute peptides.

Bayaz et al. [24] systematically studied the effects of different instrumental and operating conditions on the precision of retention times for a large set of solutes eluted on C18 using acetonitrile/buffer/water. They concluded, for example, that isocratic elution was more sensitive than was gradient elution when studying the effects of variation in the mobile phase composition. No similar investigation has been done for SFC.

The aim of this study is to investigate and compare the robustness of peptide separations conducted under isocratic and gradient conditions in SFC. As a model compound, we studied the linear uncharged pentapeptide gramicidin separated on a pH-stable hybrid silica column using an eluent containing CO₂, water, and methanol. The robustness was investigated by evaluating the variation in the retention factor using design of experiments (DoE) by perturbing the most important operational conditions, i.e. varying the total or initial gradient fraction of co-solvent, water mass fraction in co-solvent, pressure, and temperature. Secondly, simulations based on the experimental data, but with different sensitivities to the perturbations, were performed in order to reveal how the robustness would vary for hypothetical solutes in gradient separations of different gradient slopes. Finally, the practical consequences of the observed differences in robustness between gradient and isocratic separations were quantified by transferring the isocratic and gradient methods to a different laboratory.

2. Material and methods

2.1. Chemicals

The mobile phase consisted of CO₂ (99.99%) from AGA Gas AB (Lidingö, Sweden), HPLC-grade methanol from VWR (Radnor, PA, USA), and water with conductivity of 18.2 MΩ cm from a Milli-Q Plus 185 water purification system from Merck Millipore (Darmstadt, Germany). Gramicidin (CAS# 1405-97-6) from Bacillus aneurinolyticus was obtained from Sigma-Aldrich (St. Louis, MO, USA). This linear peptide has the sequence formyl-X-Gly-Ala-Leu-Ala-Val-Trp-Leu-Y-Leu-Trp-Leu-Trp-ethanolalamine, where X can be either Val or Ile and Y either Trp (Gramicidin A), Phe (Gramicidin B), or Tyr (Gramicidin C) [25], and is therefore referred to as comprising the X–Y isoforms of gramicidin (Table 1). All Gramicidin samples were dissolved in neat MeOH to a concentration of 1 mg mL⁻¹.

2.2. Instrumentation

In this study, two different analytical SFC systems, of the same model and manufacturer were used, but at two different locations: Karlstad University (denoted Laboratory 1) and at AstraZeneca in Gothenburg (Laboratory 2). The Laboratory 1 system was a Waters UPC² (Waters Corporation, Milford, MA, USA) equipped with a PDA detector. The Laboratory 2 system was also a Waters UPC² but connected via a passive splitter (UPC² MS splitter) to a PDA detector and a Waters single-quadrupole mass spectrometer (Waters SQD2) using electrospray ionization in positive mode. Both selected ion monitoring and scan mode (800–1000 m/z; 833 Da s⁻¹) were used, respectively. In Laboratory 1 the UPC² instrument had a single stack configuration from bottom to top of pump, auto sampler, convergence manager (back pressure regulator), column manager and PDA detector. In Laboratory 2, the UPC² in Laboratory 2 had a two-stack configuration with pump, auto sampler, convergence manager in the first stack and make-up pump, column manager and PDA detector in the second stack. The inner diameter of the stainless steel and PEEK tubing from injector to column to PDA to convergence manager was 0.18 mm at both laboratories except from the splitter to convergence manager at Laboratory 2 were the inner diameter was 0.25 mm. The PDA flow cell volume was 8.4 µL at both laboratories.

The mobile phase flow to the mass spectrometer was split with a passive splitter and diluted with a 0.2 mL min⁻¹ mixture of 95/5% (v/v) methanol/10 mM ammonium formate. The extra column volume was measured from the retention time of an injection of 1 mg mL⁻¹ gramicidin with a zero-dead-volume union in place of the column in both systems. The difference was compensated for in comparisons between the two systems. Pressure was measured using two model EJX530A absolute pressure transmitters (Yokogawa Electric Corporation, Tokyo, Japan) connected to the column inlet and outlet using a tee. A data logger from Pico Technology (St. Neots, UK) was used to record the pressure.

The total and co-solvent mass flows were measured directly after the mobile phase mixer and between the co-solvent pump
and the vent valve, respectively, using a mini CORI-FLOW M12 low-flow Coriolis mass flow meter (Bronkhorst High-Tech B.V., Ruurlo, Netherlands), hereafter denoted “CFM.” The columns were a 2.5-μm Kromasil SFC-2.5-XT (100 × 3.0 mm) (AkzoNobel, Bohus, Sweden) and a 1.7-μm Waters 2-picolyamine (100 × 3.0 mm) (Waters Corporation).

2.3. Procedure

2.3.1. Design of experiments

A three-level, four-factor, central composite face-centered experimental design with three center points was used to investigate how the logarithmic value of the retention factor of the Val-A isomer of gramicidin varies with total co-solvent fraction (MeOH + water), water mass fraction in co-solvent, pressure, and temperature for the isocratic and gradient elutions, respectively. Only the Kromasil SFC-2.5-XT column was investigated. The log transform of the retention factor was used because the retention generally has a logarithmic relationship with the fraction of co-solvent used in the separation [26]. The central composite face-centered experimental design model was selected in order to achieve good predictive power in the design space [27] as well as to investigate potential quadratic terms and interaction terms between factors. In the isocratic elution experiments, the total co-solvent fraction was identical to the isocratic composition, and in the gradient elution experiments, the total co-solvent fraction indicated the condition at the start (and end) of the gradient. The set design was as follows: the total co-solvent fraction during the isocratic experiments was 30, 33, and 35 v/v%, and the co-solvent gradient was 28.3–61.3, 30.0–65.0, and 31.7–68.7 v/v% in 5 min when the retention times were found to be reasonable. After each change of eluent composition, the system was equilibrated for at least one hour. The water mass fraction in co-solvent was 1.2, 5, and 8.7 w/w%. The set, back pressure was 110, 130, and 150 bar. The temperature was 30, 45, and 60 °C. Due to the nature of mixing a binary co-solvent [28,29] with CO₂, the set volumetric fraction of co-solvent was not used but rather the measured mass fraction [30]. The design was rescaled for the actual and measured values of the co-solvent fraction, water content, and pressure. 2 μL injections of 1 mg mL⁻¹ gramicidin in neat MeOH were made at least in duplicate for each experimental condition. Chromatograms were recorded at 220 nm. Retention times were estimated from peak apex and normalized to retention volumes using the measured mass flow and density (see section 2.3.2). The void time was obtained from the initial baseline disturbance and was normalized to void volume. The average of all void volumes was used in calculating each retention factor. Multiple linear regressions of the log₁₀-transformed retention factors were performed using MODDE 11 (Umetrics, Umeå, Sweden) with a 95% confidence level and non-significant factors were manually removed.

2.3.2. Characterizing the experimental conditions

As factors for the experimental design, the total co-solvent mass fraction, water mass fraction in co-solvent, column temperature, and average column pressure were used. The co-solvent fractions were measured using the CFM. The arithmetic mean of the column inlet and outlet pressures for each isocratic and gradient condition was used as the pressure factor. The instrument set temperature was used as input to the experimental design, as several of our studies have indicated that our instrument set temperature is very accurate [22,30]. The mass fraction of water in MeOH, taken from the gravimetric preparation of co-solvents, was used as input to the experimental design.

To calculate the volumetric flow rate, the density of the eluent is required. However, to our knowledge it is impossible to accurately calculate the density of the ternary CO₂–MeOH–H₂O fluid used here. Therefore, direct density measurement using the CFM was evaluated and performed. The main challenge was that the pressure and temperature inside the Coriolis flow cell must be identical to those inside the column. This was achieved by removing the column and setting the back-pressure regulator so that the column average pressures were achieved in the CFM. The temperature was adjusted by simultaneously increasing the flow rate and the set column oven temperature until the desired temperature in the CFM was obtained and stabilized. Tubing from the UPC² to the CFM was insulated to minimize heat loss.

To plot contour plots and calculate densities other than the experimental measured data points, see Supplementary Data Table S1; the experimental data were fitted to a second-order multivariate polynomial with interaction terms using MODDE 11.

The accuracy of these density measurements was first evaluated by comparing theoretical and measured densities using pure CO₂ at three set back pressures (110, 130, and 150 bar) and three temperatures (30, 45, and 60 °C) at 3 mL min⁻¹. The theoretical density was calculated using NIST Reference Fluid Thermodynamic and Transport properties Database version 9.1 (REFROP) [31] with the measured arithmetic mean pressure and measured temperature as inputs, see Supplementary Data Table S2.

All pressure and density measurements were conducted separately to minimize extra column volumes.

2.3.3. Method transfer experiments

The same 2.5-μm Kromasil SFC-2.5-XT used for the DOE in Laboratory 1 was installed in Laboratory 2 and the same set method conditions were used as when running the experimental designs center-point experiments in the isocratic and gradient elutions. The total mass flow, co-solvent mass flow and average column pressure were determined at both sites.

3. Results and discussion

The retention behavior in SFC of the main isomer of gramicidin, Val-A, was investigated in the isocratic and gradient elution modes using a mixture of MeOH and water as co-solvents at different temperatures and pressures. The goal was to use a quantitative model of the retention factor to compare the robustness of the separation system in either elution mode within the defined design space. Experimental data were also extrapolated to give general insight into the robustness of the isocratic and gradient elution separation systems. To calculate the retention volume in the experimental space, the eluent density was determined using the CFM. Finally, the separation system was transferred to a different laboratory to evaluate the practical implications of transferring a more or less robust separation system.

3.1. Retention characteristics of gramicidin

The goal of the screening was to find a satisfactory separation system and to find suitable boundaries for the experimental design. Initial screening of the chromatographic behavior of gramicidin and its isomers was done on hybrid silica and 2-picolyamine stationary phases using MeOH/water as the co-solvent. Fig. 1 presents the chromatogram from a 2-μL injection of 1.0 mg mL⁻¹ gramicidin separated on the hybrid silica (Fig. 1a) and 2-picolyamine (Fig. 1b) columns. From mass spectrometric data, the retention order on the hybrid silica phase was found to be Ile-B, Val-B, Ile-C/Ile-A, and Val-C/Val-A (Fig. 1c) and on the 2-picolyamine phase to be Ile-B/Val-B, Ile-C/Val-C, Ile-A, and Val-A (Fig. 1d). The 2-picolyamine stationary phase managed to resolve each aromatic isofrom but not the aliphatic forms, except for Ile-A and Val-A. The hybrid silica stationary phase, on the other hand, managed to resolve the aliphatic isoforms but was less able to differentiate between the aromatic
forms. These results are as expected considering the nature of the hybrid silica and the 2-picolylamine ligand. Further stability experiments using the 2-picolylamine column revealed a non-reversible retention drift when varying the amount of water, so this column was not used in further studies (data not shown).

Adding water to the methanol co-solvent [32,33], was found to significantly affect the retention and peak shape in the case of gramicidin (Fig. 2). To investigate whether adding water to the eluent resulted in a continuous or discontinuous change in retention and/or peak shape, the first injections were performed with neat methanol on new columns using the isocratic (Fig. 2a, b) and gradient (Fig. 2c, d) elution modes. Following the neat methanol experiments, injections were done at 1.2, 5, and 8.7 w/w% water added to the co-solvent. While the solubility of water in neat CO2 in supercritical conditions is generally below a molar fraction of 0.01 [34], it is significantly higher when the water is added together with methanol [35]. By increasing the water content of the eluent, the apparent tailing of the main peak decreases in semi-analytical conditions (Fig. 2a, c) and is considerably reduced in semi-overloaded conditions in both the isocratic and gradient elutions (Fig. 2b, d).

To conclude, we found the retention on the hybrid silica column to be reproducible and able to separate aliphatic forms of gramicidin. We also found that water reduced the retention factor and considerably reduced the peak tailing, especially in semi-overloaded conditions. Adding water to the eluent in this range did not induce any discontinuous or unexpected behaviors in the retention or peak shape.

3.2. Measurement of density to estimate volumetric flow

To evaluate the estimation of density using the CFM, the density of neat CO2 was measured over the range of 30–60 °C and 134–175 bar, in which the CO2 density varies from 530 to 871 kg m⁻³ (Supplementary Data Table S2). Comparing the measured and calculated (REFPROP) densities showed that the relative difference never exceeded 0.4%. This indicates that CFM should be able to accurately measure the eluent density.

Because little is known of the properties of the CO2-MeOH-H2O eluents used here, the density was measured at all experimental conditions (Supplementary Data Table S1). These data were then fitted to a second-order multi-polynomial equation with interaction terms to interpolate densities in other conditions. It was possible to find an acceptable correlation ($R^2 = 0.79$ at the 95% confidence level) between the factors and the measured density.

Fig. 3a–c plots the density variation as a function of temperature and pressure for a co-solvent fraction of 31.5 w/w% with 1.3 (a), 5 (b), and 8.7 (c) w/w% water in the co-solvent. As can be seen, the density varies only slightly with pressure and temperature, and adding water to the eluent only slightly increases the density of the mobile phase. This means that, from a density perspective, the system is rather insensitive to changes in temperature, pressure, and the fraction of water added to the eluent. Little is known of the system investigated here, so we can compare the results using a calculated CO2-MeOH mixture with a high MeOH fraction in the eluent. The density of a CO2-MeOH fluid at the center point (68.5/31.5 w/w% co-solvent fraction, 5 w/w% H2O, 45 °C, and 163.3 bar) was measured to be 844 ± 8 kg m⁻³, while it was calculated to be 843 kg m⁻³ using REFPROP, indicating that water has little effect on the density of the eluent.

From the correlation of pressure to density at constant temperature and constant fractions of co-solvent and water, it was also possible to determine how density varied inside the column during a separation. Fig. 3d plots the density variation along the column assuming a linear pressure drop at the center point in the experimental design. The density along the column varied by approximately 1.5% from column inlet to column outlet (Fig. 3d), meaning that it is reasonable to use the average density to determine the average volumetric flow rate.
Fig. 2. Analytical (a, c) and semi-preparative (b, d) injections of gramicidin at 0, 1.2, 5.0, and 8.7 w/w% water in MeOH co-solvent: (a, b) isocratic elution conditions at the center point of the DOE experiments, 35 v/v% co-solvent, 130 bar BPR, and 45 °C; (c, d) gradient elution conditions at the center point of the DOE experiments, 30–65 v/v% co-solvent in 5 min, 130 bar BPR, 45 °C. Injections were 2 μL, 1 mg mL⁻¹ (a, c); and 2 μL 20 mg mL⁻¹ (b, d).

Fig. 3. Density variation in the experimental design space: isopycnic plots for 1.3, 5, and 8.7 w/w% water in MeOH at the isocratic center point of 31.5 w/w% over the studied pressure and temperature range (a–c). Plot (d) shows the density profile along the column as a function of a linear pressure drop in the isocratic center point.
The density drop over the column could be further reduced by operating the system at much lower flow rates, as recommended earlier [36], but both column efficiency and separation time will suffer for this slight improvement.

In this investigation, the average volumetric flow rate varied between 1.01 and 1.16 mL min\(^{-1}\) over the entire experimental design (Supplementary Data Table S1), clearly indicating the importance of normalizing retention factors before using them in an experimental design, as not doing so would underestimate the retention times by up to 16% and skew the retention model. This has previously been suggested by us and several other authors [29,37].

### 3.3. Robustness of separation system

We chose a face-centered central composite design for the purpose of quantitatively describing the variation in retention volume in order to estimate the robustness of the separation system. All linear factors were found to be significant as well as some quadratic and interaction terms. Their coefficients are presented in Table 2.

The model determined in the DoE describes how the elution volume varies with changes in total co-solvent (w/w%), water in co-solvent (w%), pressure, and temperature within the experimental design space. Using this model, it is possible to quantify the sensitivity of the separation system, in this case the retention volume of Val-A, to perturbations in the co-solvent fraction, water mass fraction in co-solvent, pressure, and temperature in either the isocratic or gradient elution mode. The model coefficients (Table 2) indicate that the isocratic separation system is 2.5, 3.2, 2.5, and 1.4 times more sensitive to changes in the total co-solvent fraction, water mass fraction in co-solvent, pressure, and temperature than in the gradient elution system.

One way of visually representing the robustness of the isocratic and gradient elution systems is presented in Fig. 4a for isocratic conditions and Fig. 4b for gradient conditions. The plot represents a contour surface indicating the relative error, \( E_R \), of the retention factor, \( k \), at any position in the design relative to the retention factor at the center point, \( k_{\text{ref}} \).

\[
E_R = 100 \times \frac{k - k_{\text{ref}}}{k_{\text{ref}}} \tag{1}
\]

The plot was generated to investigate how perturbations in the two most important factors, total co-solvent fraction and water mass fraction in co-solvent, affect the retention. Naturally, the complete robustness of the separation system is related to changes in any factors inside or outside the experimental design. Starting at the isocratic center point (Fig. 4a, circle/cross), it is apparent that if the total co-solvent fraction is kept constant, a perturbation of up to approximately ±5% in the water mass fraction (observe the relative changes, in this case 4.75–5.25 w/w MeOH/H\(_2\)O) in the co-solvent would be allowed if the method specifies that the retention factor can vary by ±2%. If the tolerance is increased to ±10%, a perturbation of up to approximately -25/+30% in the water mass fraction would be possible. Similar observations can be made for perturbations of total co-solvent fraction with a constant water mass fraction. The system is least robust when both total co-solvent fraction and water mass fraction are simultaneously perturbed in the same direction, because both factors affect the retention volume in the same direction. From the diagonal shape of the contour surface, it is also apparent that if the factors are simultaneously perturbed in opposite directions, it is possible to perturb the system unknowingly, i.e., maintaining a near constant retention factor while having changed the operational conditions. However, a large perturbation in the total co-solvent fraction and water mass fraction would also alter the system pressure and further change the retention factor, making the interpretation slightly more complicated.

Focusing on the gradient center point (Fig. 4b, circle/cross), it is apparent that if the total co-solvent fraction is kept constant, a perturbation of up to approximately ±17% in the water mass fraction would be allowed if the method specifies that the retention factor can vary by ±2%. If the tolerance is increased to ±10%, a perturbation of up to approximately -80/+90% in the water mass fraction would be possible. The contour surface has the same characteristics as in isocratic elution, meaning that a perturbation of both factors in the same direction or opposite directions would maximize or minimize the response of the system, respectively. The most important conclusion is that the gradient system is less sensitive to co-solvent or water perturbations than is the isocratic system.

There are several possible origins of perturbations in the co-solvent and water levels, the most likely to occur and simultaneously, the most easily mitigated is inaccuracy in the eluent preparation. Because MeOH is very hygroscopic, another source of perturbation is the accumulation of water over time due to absorption from the air. Changes in total co-solvent fraction are much more difficult to identify, as they could result from different pump performance or pump leakage, which also could be affected by different system pressures. This matter is discussed further in section 3.5.

### 3.4. Simulated robustness of modified separation system

The robustness of the studied separation system is a function of the sensitivity of the Val-A isof orm of gramicidin to total co-solvent fraction, water mass fraction, pressure, and temperature on the silica-based stationary phase. This is described, after removing all non-significant terms, by the following second-degree polynomial:

\[
\log_{10}(k) = \alpha_1 P + \alpha_2 C_{\text{tot}} + \alpha_3 T + \alpha_4 C_{H_2O} + \alpha_5 T^2 + \alpha_6 PT + \alpha_7 T C_{H_2O} + \beta \tag{2}
\]

where the coefficients \( \alpha_1 \) to \( \alpha_7 \) and constant \( \beta \) are listed in Table 2. \( C_{H_2O} \) (w/w) is the water mass fraction in the co-solvent, \( C_{\text{tot}} \) (w/w) is the total fraction of co-solvent in the eluent, \( T(\text{°C}) \) is the temperature, and \( P(\text{bar}) \) is the pressure. If the pressure and temperature are kept constant and we just consider the water and total co-solvent, the model can be reduced to:

\[
\log_{10}(k) = \alpha_2 C_{\text{tot}} + (\alpha_4 + \alpha_7 T) C_{H_2O} + \gamma \tag{3}
\]

where \( \gamma \) is a constant. Using this simplified model, two additional separation systems were investigated: first, in which the sensitivity to total co-solvent and water was reduced to half that of the

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**Table 2**

<table>
<thead>
<tr>
<th>Response</th>
<th>( C_{\text{tot/init}} )</th>
<th>( C_{H_2O} )</th>
<th>( P )</th>
<th>( T )</th>
<th>( T^2 )</th>
<th>( C_{H_2O}T )</th>
<th>( PT )</th>
<th>Constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \log(k_{\text{valA}}) ) isocratic( ^a )</td>
<td>-17.0 ± 2.50</td>
<td>-11.9 ± 2.03</td>
<td>-4.91 ± 2.69</td>
<td>3.50 ± 2.06</td>
<td>5.40 ± 3.63</td>
<td>NS</td>
<td>NS</td>
<td>85.7 ± 3.02</td>
</tr>
<tr>
<td>( \log(k_{\text{valA}}) ) gradient( ^b )</td>
<td>-6.69 ± 0.81</td>
<td>-3.75 ± 0.66</td>
<td>-2.00 ± 0.94</td>
<td>2.28 ± 0.66</td>
<td>1.45 ± 1.18</td>
<td>1.76 ± 0.692</td>
<td>-1.41 ± 1.00</td>
<td>75.9 ± 0.975</td>
</tr>
</tbody>
</table>

\( ^a \ Q^2 = 0.920, \ R^2 = 0.957. \)

\( ^b \ Q^2 = 0.942, \ R^2 = 0.973. \)

\( ^b \) Not significant.
Fig. 4. Robustness plots of the separation system described by variation in the retention factor of the Val-A isoform, showing the two most important factors describing the system, i.e., total co-solvent fraction and water mass fraction in co-solvent. Plot (a) shows the variation in isocratic elution mode and (b) in gradient elution mode. The crossed dots indicate the center reference points in the isocratic and gradient elution modes where the relative error is zero.

Fig. 5. Simulated robustness plots of isocratic elution based on the experimental system. Plot (b) is a robustness plot using the simplified regression model (Eq. 3); plots (a) and (c) represent theoretical systems less and more sensitive to the co-solvent and water fraction, by a factor of 2.

initial model and, second, in which the sensitivity was twice that of the original model. The pressure and temperature were set to be the same as at the center point, and these contributions to the retention were handled by adjusting $\gamma$. The robustness plots for isocratic conditions are presented in Fig. 5a–c. It is obvious from the plots that the system becomes more robust as the sensitivity decreases (going from Fig. 5c to a). This is unsurprising and leads to the conclusion that, for robust separations, one should avoid isocratic separations if the system is very sensitive to changes in eluent composition.
To simulate the effect of different sensitivities of the experimental system in gradient elution, we evaluated the use of linear solvent strength theory.\textsuperscript{[26,38]}

\[
\log_{10}(k) = \log_{10}(k_0) - S \cdot C_{tot}
\]

where \(k_0\) is the retention factor using neat \(\text{CO}_2\) as eluent, \(S\) is the sensitivity coefficient, and \(C_{tot}\) is the total co-solvent fraction in eluent. The linear solvent strength theory (LSS) was developed for liquid chromatography, but have recently been reported to describe the retention of solutes in SFC for separations using high fractions of \(\text{MeOH}\) in the eluent.\textsuperscript{[29,38]}

Assuming that \(S\) is a linear function of water fraction in the eluent:

\[
\log_{10}(k) = \log_{10}(k_0) - (S_0 + S_t \cdot C_{H_2O}) \cdot C_{tot}
\]

In classical SFC gradient experiments, the co-solvent (mixture of water and \(\text{MeOH}\)) is mixed with the \(\text{CO}_2\). This will result in that both the \(\text{MeOH}\) and water content will vary with time in the eluent. This justifies to include the water term in the gradient equation. Observe that \(C_{H_2O}\) is the water mass fraction in the co-solvent and not the water fraction in the eluent. However, multiplying \(C_{tot}\) with \(C_{H_2O}\) will give the water fraction in the eluent. Parameters \(S_0\) and \(S_t\) and \(\log_{10}(k_0)\) were estimated for each isocratic system (i.e., less sensitive, normal, and more sensitive) and are summarized in Supplementary Data Table S3. Assuming a linear gradient, the retention time can be calculated as follows:

\[
t_R = \frac{t_0}{G} (G \cdot k_{\text{start}} + 1) + t_0,
\]

\[
G = S \cdot \Delta \phi \frac{f_0}{f_g}
\]

where \(k_{\text{start}}\) is the retention factor for the starting eluent composition, \(t_0\) is the hold-up time, \(t_g\) is the gradient time, \(G\) is the gradient steepness factor, and \(\Delta \phi\) is the change in total co-solvent during the gradient.

In gradient elution, both the sensitivity to changes in the co-solvent as well as the gradient slope are important. The sensitivity was handled in the same way as in the isocratic case: 50%, 100%, and 200% of the initial model sensitivity. The gradient slopes were 1% min\(^{-1}\), 7% min\(^{-1}\) (center point), and 13% min\(^{-1}\) changes in co-solvent fraction in the gradient run. The first shallow gradient represents a high-resolution separation and the last steep gradient represents a fast high-throughput screening gradient. The results are presented in Fig. 6: (a) in the top row, 1% min\(^{-1}\), (b) middle row, 7% min\(^{-1}\), and (c) bottom row, 13% min\(^{-1}\) gradient slopes. From the figure, it is apparent that the robustness of the separation increases with gradient steepness, going from top to bottom. The left column (I) of plots in Fig. 6 represents a separation that is less sensitive, middle column (II) normally sensitive, and right column (III) more sensitive to changes in total co-solvent fraction. The robustness increases as the sensitivity to changes in the co-solvent decreases. Finally, we can also observe the diagonal pattern (\(a_2 \rightarrow b_2 \rightarrow c_2\)) illustrating that the more sensitive the solute, the steeper the gradient slope needs to be in order to maintain similar robustness.

While there has been limited generalizable discussion of the retention characteristics of solutes in SFC, there has been much more in the case of RPLC. For alkyl silica stationary phases using acetonitrile, methanol, or tetrahydrofuran with water as the eluent, \(S\) has been empirically estimated at 0.25 \(\text{v/v}\) /\(\text{M}_{\text{H}_{2}O}\), where \(\text{M}_{\text{H}_{2}O}\) is the molecular mass of the solute. In the case of the Val-A isoform of gramicidin with a molecular mass of 1881 g/mol, an \(S\) value of approximately 11 would be expected in RPLC; instead, here we observe 15.7 (Supplementary Data Table S3). Since the linear solvent strength theory has mostly been applied to reversed-phase chromatography, its validity in SFC has not been thoroughly inves-
tigated. Glenne et al. recently investigated the retention of several small, uncharged solutes on a Kromasil diol column as a function of the fraction of MeOH in the eluent.\textsuperscript{[38]} They pointed out that both the solute adsorption to the stationary phase as well as the co-solvent adsorption need to be considered to fully understand the retention.\textsuperscript{[29,38]} Furthermore, they demonstrated that at a low fraction of co-solvent in the eluent, below the maximum of the MeOH excess adsorption isotherm (13 v/v% in that study), the LSS model does not hold.\textsuperscript{[29,38]} However, at a higher co-solvent fraction (as used in this study), they found that the LSS model describes the solute retention well. This observation by Glenne et al. could explain why our experimental design model lacked any significant quadratic co-solvent terms (see Eq. (2)). This also indicates that caution should be exercised in generalizing the trends presented here, if the separation is conducted using a small fraction of co-solvent in the eluent. One could also note that the maximum of the co-solvent excess adsorption isotherm, where the LSS model became acceptable in describing the retention trends, depends on the type of co-solvent (e.g., MeOH, EtOH, or MeCN) and stationary phase used in the separation.\textsuperscript{[39]} For water, we do not have any adsorption data and can therefore only speculate that the water adsorption to the stationary phase is strong. However, in this experimental design we did not observe any significant quadratic water terms see Table 2 and Eq. (2); even if this cannot be used as evidence there is no water adsorption under these conditions, this indicates that within this design space the effect of adding water to the eluent follows the LSS theory.

The conclusion that can be drawn from the \(S\)-values is that our SFC system is more sensitive to variations in the co-solvent fraction than the corresponding RPLC separation would have been. The theoretical \(S\) value could represent a hypothetical solute with a molecular mass of approximately 1000 g mol\(^{-1}\) in the less sensitive system and of approximately 15,000 g mol\(^{-1}\) in the more sensitive system. Further studies with a diverse set of solutes, stationary phases, and eluents would give valuable insight into both the general retention characteristics and robustness of SFC separations. If smaller molecules tend to be more sensitive to the strong eluent in SFC than in RPLC, using gradient elution even for separation problems that do not require gradient elution to achieve reasonable separation time or productivity might be beneficial from a robustness perspective.

To summarize, both gradient and isocratic elution become less robust if the separation system under investigation is more sensitive to perturbations in the parameters under investigation. Using gradient elution, the robustness increases with increasing gradient slope.

3.5. Practical implications for method transfer

To put the robustness testing in a practical context, method transfer was conducted for both an isocratic and a gradient separation of gramicidin. In this case, we used the center point of the experimental design. Both SFC systems were in their original factory configurations, except for the additional MS detector (and passive flow splitter) on the SFC in Laboratory 2. To control the different system configurations, gramicidin in solution was injected without a column in both systems. A small difference in injector-detector volume was determined, approximately 70 \(\mu\)L in Laboratory 1 and 80 \(\mu\)L in Laboratory 2. The same identical column and identical instrumental set conditions (e.g., back-pressure, temperature, gradient, and programs) were used in both laboratories.

The isocratic chromatograms from Laboratories 1 and 2 are presented in Fig. 7c. From the chromatograms, we can observe a rather large difference between the separations conducted at the two laboratories. To investigate the underlying reason for the longer retention in Laboratory 2 than Laboratory 1, the pressure and mass
Fig. 6. Simulated robustness plots based on the experimental gradient system. Top row represents separation conducted using a gradient slope of 1% min⁻¹, center row 7% min⁻¹, and bottom row 13% min⁻¹. The center column comprises robustness plots using the simplified regression model (Eqs. 5 and 6). The left- and right-hand columns comprise robustness plots representing theoretical systems less and more sensitive to the co-solvent and water, respectively, by a factor of 2.

Fig. 7. Method transfer from Karlstad University (Laboratory 1) to AstraZeneca Gothenburg (Laboratory 2) using the identical column and maintaining the set center-point conditions for the isocratic and gradient methods. Contour plot shows the retention factor within the total co-solvent pressure dimension. The cross and circle indicate the measured conditions in Laboratories 1 and 2, respectively.

flows were measured. In Laboratory 1, the pressure over the column was measured at 162 bar and the total co-solvent fraction was 31.3 w/w%, while in Laboratory 2 the same point was measured at 159 bar and 29.5 w/w% co-solvent. Using the isocratic separation model from the experimental design (see section 3.3) results in a predicted retention factor of 6.7 ± 0.7 for Laboratory 1 (Fig. 7a, cross) and 9.2 ± 0.7 for Laboratory 2 (Fig. 7a, circle). This model prediction corresponds very well with the experimentally observed retention factors of 7.3 and 9.3 at Laboratories 1 and 2, respectively.

Gradient separation was also conducted, and the resulting chromatograms are presented in Fig. 7d. The difference between the laboratories was very small in this case. To determine the robust-
ness of gradient elution, the results of the method were compared between the laboratories: The average pressure at the center point in the gradient was measured to be 108 bar in Laboratory 1 and 164 bar in Laboratory 2 (see Fig. 7b). The corresponding initial gradient co-solvent (methanol) fractions were 26.6 w/w% in Laboratory 1 and 26.7 w/w% in Laboratory 2, leading to predicted (apparent) retention factors of 5.6 ± 0.2 in both laboratories. The obtained experimental values were 5.7 and 5.6 in Laboratories 1 and 2, respectively.

The experimental results of the method transfer support the predictions of the robustness calculations, in which gradient elution is predicted to give a more robust separation system than does isocratic elution. Although the actual conditions were more similar in the gradient case, the main reason for the more successful method transfer is believed to be the impact of the G factor (Eq. (6)) in the separation system, with separation systems having high G factors likely being more robust to differences in system pressure and in the actual w/w% of co-solvent.

The observed difference in co-solvent fraction between Laboratories 1 and 2 could have several origins, for example, due to different leakage rates of the CO₂ pumps and/or check valves, different instrument configurations, or day-to-day variations at either laboratory [40–42]. The slightly lower pressure at Laboratory 2 could simply have resulted from reducing the total flow into the back-pressure regulator by tending part of it into the MS detector. It is worth noting that there are no instrument indications of these differences, as they were only quantified using CFM and pressure transducers not part of the system. This means that from a practical perspective, a typical user without access to pressure transducers or mass flow meters cannot properly detect or compensate for these system differences except in an empirical manner.

It should be noted that both systems in Laboratories 1 and 2 were operating within their specifications and a recommendation to users of modern analytical SFC systems should therefore be to always measure flow, pressure and composition by external devises, for example by using the methodologies in this study. The results could be used either to (I) characterize systems in detail (II) to calibrate several different instruments to perform the same performance or to (III) detect if preventive maintenance needs to be performed. To mitigate effect of different system plumbing, stack configurations, etc. the operational conditions could be matched between the laboratories as we have previously done for preparative scale-up [22]. However, using gradient elution allows for much more robust operation, reducing the need for careful qualification depending on the requirements of the analysis.

The method transfer results indicate that, given an identical effort in replicating two separation systems, the robustness of the gradient elution method will lead to more successful transfer and should be preferred in separating gramicidin using SFC.

4. Conclusions

The robustness of peptide separation conducted under isocratic and gradient conditions in SFC mode was investigated. As a model system, we studied the linear uncharged pentapeptide gramicidin D separated on a pH-stable hybrid silica column (Kromasil SFC-2.5-XT) using an eluent containing CO₂, water, and methanol. The system was first characterized using a chemometric DoE approach. The experimental space was then numerically expanded to gain more general insight into the system. Finally, a gradient and an isocratic separation were transferred to another laboratory to put the robustness testing in a practical context.

To conduct experimental design, the density of the eluent (CO₂-MeOH-H₂O) was experimentally determined, as no accurate equation-of-state model is available for this eluent and we needed to determine the average volumetric flow rate at each design point. We found that Coriolis mass flow meters could accurately measure the density. We also concluded that working with a high-mass fraction of methanol and water as co-solvents resulted in small variations in density over a large area of pressure and temperature, inherently making SFC more robust.

From the DoE, we found that the total fraction of co-solvent in the eluent and the water fraction in the co-solvent were the most important factors controlling the retention. The measured sensitivity was higher than the RPLC values for similar separations reported in the literature. We also found that in gradient elution, the separation is at least three times more robust to perturbations than in isocratic elution.

Inspired by the DoE model, we investigated systems that are more and less sensitive to changes in the eluent composite as well as gradient elution conducted at different gradient slopes. We found that both gradient and isocratic elutions become less robust for more sensitive systems. Using gradient elution, the robustness increases with increasing gradient slope.

Finally, the methods were transferred to another laboratory. The results of the isocratic method differed greatly between the laboratories, the main reasons for this being differences in pressure and in the total co-solvent fraction between the systems. This deviation could be explained using the DoE model. For the gradient separation, the transfer was successful. The results clearly indicate that gradient elution resulted in a considerably more robust separation system.

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Appendix A. Supplementary data

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