Development of Pseudo-Linear Gradient Elution for High-Throughput Resin Selectivity Screening in RoboColumn® Format

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Rapid development of chromatographic processes relies on effective high-throughput screening (HTS) methods. This article describes the development of pseudo-linear gradient elution for resin selectivity screening using RoboColumns®. It gives guidelines for the implementation of this HTS method on a Tecan Freedom EVO® robotic platform, addressing fundamental aspects of scale down and liquid handling. The creation of a flexible script for buffer preparation and column operation plus efficient data processing provided the basis for this work. Based on the concept of discretization, linear gradient elution was transformed into multistep gradients. The impact of column size, flow rate, multistep gradient design, and fractionation scheme on separation efficiency was systematically investigated, using a ternary model protein mixture. We identified key parameters and defined optimal settings for effective column performance. For proof of concept, we examined the selectivity of several cation exchange resins using various buffer conditions. The final protocol enabled a clear differentiation of resin selectivity on miniature chromatography column (MCC) scale. Distinct differences in separation behavior of individual resins and the influence of buffer conditions could be demonstrated. Results obtained with the robotic platform were representative and consistent with data generated on a conventional chromatography system. A study on antibody monomer/high molecular weight separation comparing MCC and lab scale under higher loading conditions provided evidence of the applicability of the miniaturized approach to practically relevant feedstocks with challenging separation tasks as well as of the predictive quality for larger scale. A comparison of varying degrees of robotic method complexity with corresponding effort (analysis time and labware consumption) and output quality highlights tradeoffs to select a method appropriate for a given separation challenge or analytical constraints. © 2016 The Authors Biotechnology Progress published by Wiley Periodicals, Inc. on behalf of American Institute of Chemical Engineers

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Introduction

High-throughput technologies have become an integral part in the development of chromatographic purification steps. With miniature chromatography columns (MCCs), a resin-screening tool exists which applies the packed bed principle of conventional chromatography but on a microliter scale.1

Parallel operation of MCCs with an automated liquid handling system (LHS) enables rapid experimentation allowing for a more comprehensive exploration of the experimental space within short time. Besides saving time, a lower demand for feed material is involved in this approach compared to sequential testing in lab scale column format. These advantages over the traditional concept are reasons for the rapid adoption by pharmaceutical research and development departments/groups. The effective collaboration between the key supplier of miniature columns - Atoll GmbH, Weinhardt, Germany - and a provider for automated liquid handling systems - Tecan Group, Maennedorf, Switzerland - contributed to the quick dissemination of this technology as well.

Additional Supporting Information may be found in the online version of this article.
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The range of possible applications for MCCs is broad and mostly includes sample preparation, dynamic binding capacity determination, establishing cleaning regimens, and optimization of step elution.\textsuperscript{2,5} Academic groups have proven early on that also complex separations are possible with this format employing pseudo-linear gradients,\textsuperscript{6-8} and industry is recently following suit.\textsuperscript{9} Common high-throughput technologies were extended towards the characterization of a multicolumn chromatographic purification, and the technique was found to make an appropriate scale-down tool for the formal characterization of biotherapeutic processes.\textsuperscript{10}

Process developers demand that screening results collected from gradient elution testing with MCCs on a LHS provide a meaningful basis for selection of resins or buffer conditions. In practical terms, data created with the MCC format should be comparable and predictive for lab scale. The adoption of a MCC-based screening platform on a LHS represents a challenging task, which requires the users to gain multidisciplinary knowledge in the area of automation, liquid handling, data processing, and application of chromatography scale-down theories. A particular challenge is that only limited information and technical guidance exist, which experimental strategy should be followed when working with MCCs on a LHS.

The adsorption of proteins onto chromatographic resins has been investigated in numerous studies. Results showed that the dynamic binding behavior of miniature column resembles that of large scale quite well when residence time is kept constant.\textsuperscript{4,8} However, the predictive accuracy of binding capacity data of MCC experiments can vary depending on the specific application conditions, namely the combination of resin particle size, hydrodynamic conditions, and protein properties.\textsuperscript{11}

More parameters need to be considered when developing a method for mimicking a chromatographic separation using MCCs on a LHS. The mechanisms underlying the separation of proteins in ion exchange chromatography have been thoroughly investigated and described by means of mathematical models.\textsuperscript{12,13} Findings from scientific research and observations of effects in linear gradient elution are fundamental for the understanding of scale down principles. When transferring a linear gradient separation to columns with very short bed heights, both the gradient slope and the flow rate have to be properly adjusted to compensate for the loss of column efficiency and hence preserve resolution.\textsuperscript{14-16}

Conventional robotic liquid handlers can be configured to accommodate small scale parallel chromatography experiments, but there are limitations as compared to a traditional lab scale setup. These systems employ dispenser pumps with limited dosage capacity which are not suitable for forming continuous buffer gradients. Hence, linear elution gradients need to be translated into a sequence of multiple elution steps which closely resembles a continuous change of elution buffer composition, e.g. with regard to salt concentration, pH, etc. A balance between the following aspects needs to be found: (a) a high approximation towards linear gradient by using finely graduated steps, (b) the effort of preparing infinitely large buffers sets, and (c) low expenses in terms of preparation time and microtiter plate (MTP) consumption. Inline absorption measurement of the column effluent is not possible with a LHS. As a consequence, the eluate must be fractionated. The eluate fractions need to be analyzed offline (by photometry e.g.) to collect data in order to reconstruct the chromatographic elution profile. The information density, respectively, the resolution of the chromatogram, is clearly dependent on the fractionation scheme, respectively, the number of data points.

When developing a chromatography method for MCC operation on a LHS, these technical limitations have to be considered and compromises need to be found with regard to quality of gradient formation and data collection.

The heuristics as well as a general description of different methods for microscale chromatography (microliter batch incubation, micropipette chromatography tips, miniature columns) with its pros and cons were given in a review by Chhatre et al.\textsuperscript{17}

The applicability of the Tecan Freedom EVO 200 LHS platform to multistep gradient elution with RoboColumns\textsuperscript{5} has been demonstrated by Wiendahl et al.,\textsuperscript{8} who employed a number of small step gradients to mimic linear gradients for ion exchange separation. Elution experiments on 200 µL columns were carried out with the LHS. Comparative linear gradient elution data at the same column size were generated with a standard AKTAP\textsuperscript{TM} chromatography system. The separation of three different feed streams was investigated with various resins and the chromatograms obtained with the LHS and the AKTAP\textsuperscript{TM} system compared. For all three separation examples, a good match of the elution curves and shape of elution peaks was achieved on both systems, leading to the conclusion that the concept of discretization for mimicking linear gradients is feasible with the LHS. The article gives valuable information on designing of a multistep gradient and the fractionation scheme. Total gradient volume, gradient slope, fraction size, and flow rates for LHS elution experiments are specified. However, for each separation example on the LHS, different linear flow rates (ranging from 11 to 132 cm/h) as well as different step gradient elution parameters were applied. Scaling factors for linear flow rate adaptation between lab and LHS scale differed significantly (by a factor of 1.5–5). No explanations were given, though, on which basis the decisions for the actual settings for RoboColumn operation on a LHS were made.

Treier et al.\textsuperscript{6} presented and discussed a scale down methodology based on a two-step monoclonal antibody (mAb) purification process, consisting of a protein A capture followed by cation exchange (CEX) purification in 200 µL MCC format. When switching between lab and MCCs, the residence time was kept constant for all chromatographic steps by adjusting the flow rate accordingly. At LHS scale, a linear flow rate of 60 cm/h was used. Only one simple single-step gradient was employed for elution, but the authors addressed fundamental aspects of liquid handling with robotic systems and gave helpful recommendations for improving the quality of pipetting and absorptive determination of fraction volumes, which is required for accurate mass balancing.

Welsh et al.\textsuperscript{10} used high-throughput strategies as a general guide for scaling and assessing operating space. For purification development of mAbs, they investigated CEX resins as the second step after Protein A capture chromatography. Employing 200 µL RoboColumns\textsuperscript{5} on a LHS with pseudo-linear gradient elution, the optimal conditions for a difficult separation of a mAb monomer from aggregates were determined. Gradient elution was constituted empirically by choosing a few multistep gradients with an increasing number of steps and evaluating the resulting separation
performance. Around 30 steps were considered as an ideal balance between data quality (comparing well enough to a linear gradient) and the number of fractions for analysis. Good agreement was seen with head-to-head comparison of the miniature and lab scale column techniques, concerning impurity removal as well as mAb yield. The investigation of the impact of parameter variation on separation was limited, though, as parameters were either kept constant (flow rate, fraction size, column size) or were varied over a limited range only (gradient slope by a factor of 2.5).

Elution gradient optimization is one of the main tasks of chromatography process development. Susanto et al.\textsuperscript{7} and Osberghaus et al.\textsuperscript{3} proved that this optimization can be achieved on MCC scale utilizing the multistep gradient approach. In their work, the authors presented new concepts of process optimization based on high-throughput data generated in conjunction with mechanistic modelling and generic algorithms, respectively. Within the practical work, the peak resolution in the separation of a three-component protein mixture on 200 µL scale was improved by changing the shape of the elution gradient. The results clearly illustrate the enormous potential of the multistep gradient elution method.

Published literature so far discloses only little about the background or rationale of parameter settings of multistep gradient elution protocols and liquid handling. No comprehensive investigation of the impact of varying operating conditions on the separation performance of miniature columns used with LHS has been provided. Also, in most of the application work 50–200 µL columns with very short bed heights of 2.5–10 mm were used. Little attention has been paid to alternative MCC scales with 450 or 600 µL bed volume (22 or 30 mm bed length), even though there may be good arguments to utilize these column formats. Several studies refer to the lack of fast sensitive and selective analytical methods.\textsuperscript{6,18–20} From an analytical viewpoint, the use of larger miniature columns would help to ease this problem because fraction size and absolute amount of analyte are directly related to column scale. Moreover, gradient elution experiments would benefit from improved separation efficiency gained from the increase in column length.

In this work, we have extensively examined the impact of various parameters for the application of MCCs on a LHS. We identified key parameters and defined optimal settings in order to establish a protocol for basic selectivity screening that provides sufficient resolution and practicability.

Prior to using the LHS for chromatography applications, we verified pipetting accuracy and precision and investigated potential backmixing with system fluid during liquid handling operations.

We designed multistep gradients with varying gradient slopes by changing the step length and molar step height and def"ned fractionation schemes allowing successive offline analysis. Different gradient protocols were applied in the separation of a three-component mixture of model proteins on 200 and 600 µL MCCs packed with CEX resins using different elution flow rates. We constructed chromatograms from absorbance data and calculated peak resolution values from fitted peak curves. Resolution data obtained from MCC runs were compared against reference data collected from lab scale trials in order to illustrate the performance differences between the respective formats. The predictive quality of MCC results with regard to peak elution conductivity at different scales and conditions was assessed based on a correlation analysis between gradient slope and protein retention.

As a result of the method development, we selected one protocol and demonstrated its suitability for selectivity differentiation between four different cation exchange resins and two different pH conditions at the 600 µL MCC scale. The high-throughput method was used to reveal maximum selectivity along with minimized time and feed volume requirements as compared to traditional sequential experiments at lab scale.

### Theoretical Considerations

#### Scale down prediction

Linear gradient elution (LGE) is associated with many parameters including gradient slope, flow velocity, column length, and mobile phase properties. This complex parameter space can make it challenging to optimize a chromatography process. Mathematical models can help to predict the separation and help to gain a better understanding of the separation mechanism in ion exchange chromatography.

Yamamoto et al.\textsuperscript{15} developed a straightforward method for predicting the separation behavior in LGE. The method is based on the numerical solution of an extended ion exchange equilibrium model which considers the peak sharpening effect and the distribution coefficient as a function of the linear salt gradient. Column efficiency (HETP\textsubscript{LGE} from gradient elution), normalized gradient slope ($G$), column length ($Z$), and a dimensional constant ($L_d$) are combined into a dimensionless parameter named “O-factor” which is proportional to the resolution ($R_s$) and serves as a predictor for the separation performance:

$$O = \frac{Z L_s}{G \cdot HETP\textsubscript{LGE}} \propto R_s \quad (1)$$

From their studies, the following conclusions were drawn:

- In order to achieve the same resolution between two columns with different dimensions (e.g., columns length), the O-factors must be equal.
- The same resolution of two proteins can be obtained with various combinations of operating variables and column dimensions.
- If column length decreases, the gradient slope must be reduced or HETP increased, the latter by reducing the flow rate.

#### Aspects of liquid handling

MCC operation with a liquid handling system requires an air-free pumping system to produce a constant, pressure-driven flow. For this purpose, systems with piston pumps (syringes) are used. The pumps are connected via tubes to pipetting needles (stainless steel fixed tips), enabling a flexible connection to the inlet of the MCC and hence allowing the application of sample or buffer to the column. The fluid path is filled with system liquid which acts like a hydraulic plunger. A small airgap (20 µL) between system liquid and sample serves to prevent backmixing of the sample with system liquid. However, the problem of unwanted backmixing (dilution) introduced by the LHS is well known and is related to liquid transfer parameters (leading airgap, aspirate, dispense speed, excess volumes) as well as sample properties.\textsuperscript{6,21} A known cause for dilution effects are thin films of the system liquid
that are retained on the inside wall of the tips or drops of system liquid remaining on the outer wall of the needle tip after washing. Sample diffusion and mixing with system liquid across the airgap also contributes to sample dilution. To minimize this risk, the airgap must be renewed after each pipetting step.

Pumping a liquid sample into a MCC represents an atypical pipetting operation. While the dispense speed of common pipetting modes is high (e.g. standard water class from Tecan uses a dispense speed of 600 µL/s), the flow rate at which sample or buffer is applied to MCCs can be extremely low (e.g. 2.7 µL/s corresponding to a flow velocity of 50 cm/h). Consequently, the hold time of a sample in the needle is prolonged. Secondly, while there is no back pressure in normal dispense mode, the MCC creates pressure resistance to a significant extent. Both factors increase the risk of system liquid dilution, or may damage the system trailing airgap separating sample and system liquid. Grönberg et al. 22 and Welsh et al. 10 reported system liquid aggregates. Chromatographic Media and Column Formats. The following ion exchange chromatography (IEX) media from Merck KGaA were tested: Eshmuno\(^S\) CEX prototype, Eshmuno\(^S\) CPX, Eshmuno\(^S\) S, Fractogel\(^R\) EMD SE Hicap (M) and Fractogel\(^R\) EMD COO\(^-\) (M) resins. Properties of the strong CEX resins stated by the vendor are given in Table 4-1. For LHS experiments, the prepacked MediaScout\(^\circ\) RoboColumn\(^\circ\) format (Atoll GmbH) was employed with either 0.2 mL (1 cm bed height) or 0.6 mL (3 cm bed height) column volume (CV). Experiments on lab scale were either performed with prepacked MediaScout\(^\circ\) MiniChrom columns (Atoll GmbH) with 5 and 1 mL CV (100 mm x 8 mm inner diameter (ID) and 20 mm x 8 mm ID, respectively) or reusable Superformance\(^\circ\) columns with 5 mm ID from Götec-Labortechnik GmbH (Bickenbach, Germany). Minimum requirements for self-packed columns were peak symmetries of 0.8–1.4 and greater than 1,500 plates/m.

Analytical HPLC. TSK G3000SWXL size-exclusion column (5 µm, 300 mm × 7.8 mm ID, Cat# 08541) was from Tosoh Bioscience GmbH (Stuttgart, Germany), guard column cartridge GFC-3000 (4 mm × 3 mm ID, Cat# AJO-4488) was from Phenomenex (Torrance, USA).

### Experimental

#### Materials

**Chemicals and Proteins.** Di-sodium hydrogen phosphate dihydrate (Na\(_2\)HPO\(_4\)·2 H\(_2\)O, Cat# 1.06580), 1 M hydrochloric acid (HCl, Cat# 1.09057), sodium chloride (NaCl, Cat# 1.06404), sodium dihydrogen phosphate (Na\(_2\)HPO\(_4\)·H\(_2\)O, Cat# 1.06346), 1 M sodium hydroxide solution (NaOH, Cat# 1.09137), sodium hydrochloride solution (NaClO, Cat# 1.05614), potassium nitrate (KNO\(_3\), Cat# 1.05063), acetic acid (C\(_2\)H\(_4\)O\(_2\), Cat# 1.00063), 5 M hydrochloric acid (HCl, Cat# 1.09911), sodium azide (Na\(_3\)N, Cat# 1.06688), 5 M sodium hydroxide solution (NaOH, Cat# 1.09913), sodium sulfate (Na\(_2\)SO\(_4\), Cat# 1.06649), tris(hydroxymethyl)-aminomethane (H\(_2\)N(CH\(_2\)OH)\(_3\), Cat# 1.08382) were purchased from Merck KGaA, Darmstadt, Germany.

Orange G was purchased from Sigma-Aldrich (Cat# 03756-25G). For LGE experiments, a set of 20 mM sodium phosphate buffers with 0, 0.5, and 1 M NaCl at pH 5.0, 6.0, and 7.0 was prepared and filtrated with 0.22 µm Express Plus\(^\circ\) PES (Merck KGaA). As model feed for LGE experiments a mixture of three proteins, dissolved at equal amounts in equilibration buffer (20 mM sodium phosphate buffer) at corresponding pH values, was used. Model proteins used were \(\alpha\)-chymotrypsinogen A (bovine pancreas) from Sigma-Aldrich (St. Louis, USA), cytochrome C (equine heart), and lysozyme (egg white) from Merck KGaA.

Mab feed material obtained from protein A capture followed by pH and conductivity adjustment was supplied in-house (pH was 5.0, conductivity was 5 mS/cm at 25°C, 3.8% aggregates).

### Liquid handling system and software

Liquid handling studies were carried out using a Freedom EVO\(^\circ\) 200 LHS from Tecan (Crailsheim, Germany). The LHS was equipped with one robotic manipulator arm (ROMA) and one liquid handling arm (LIHA) with eight independently controllable pipetting needles. Liquid pipetting was executed with stainless steel standard tips. Absorbance of elution fractions was measured with an Infinite M200 microplate reader from Tecan. Experimental LHS protocols including automated photometry were developed using EVOware\(^\circ\) 2.5 and Magellan (version 7.1) from Tecan. Additionally, a TE-Link (Tecan) was installed for transferring elution plates under the column array carrier to collect fractions. Plates were stored in hotels. Deep well plates (DWP), used for storing elution buffers, and ultraviolet (UV) microtiter plates (MTPs) containing elution fractions were from Greiner (Frickenhausen, Germany). All MTPs were covered with a lid to minimize evaporation. Small fractions with less than 100 µL volume were collected in half-area UV plates. The size of dilutor syringes was 1 mL, enabling volume pipetting in the range of 1–1000 µL. Liquid volume application greater than 1 mL was conducted via iterative loops.
Methods

**LHS Buffer Mixing Test Method.** The quality of elution buffer preparation by the LHS and potential system liquid dilution effects when injecting samples into the MCC during the gradient elution were investigated using a color dye test. High salt elution buffers were spiked with dye to enable verification of the mixing ratio of stock elution buffers by absorbance measurement. High and low salt buffer stock solutions were pipetted in individual steps into deep well plates (yielding a final volume of at least 950 μL), covering the whole volume range of the protocol for elution buffer preparation, and mixed according to a prescribed sequence. TECAN’s standard liquid class settings for water were used for pipetting.

Different protocols for mixing were tested:

Protocol 1: Mixing with the LHS directly after addition of the second stock buffer using the mixing command defined within the liquid class: A volume of 750 μL sample was aspirated and dispensed. This step was repeated two times.

Protocol 2: Manual mixing: DWPs were covered with an adhesive seal foil and shaken overhead.

Finally, samples were transferred into a UV-MTP and analyzed by photometry including path length correction. The resulting absorbance data were converted into salt concentration values. Precision and accuracy data of the pipetting across eight channels were evaluated. The coefficient of variation (%CV) was reported as percentage and calculated as the standard deviation (STD) divided by the mean salt concentration $C$ of buffer mixtures prepared with eight tips:

$$\%CV = \left( \frac{\text{STD}}{C} \right) \times 100\%$$

$$\text{STD} = \sqrt{\frac{\sum_{i=1}^{n} (C_i - \bar{C})^2}{(n-1)}}$$

$$\bar{C} = \frac{\sum_{i=1}^{n} C_i}{n}$$

The deviation %Dev was reported as percentage and defined as the absolute mean salt concentration divided by the nominal (set) salt concentration $C_{\text{nominal}}$:

$$\%\text{Dev} = \left( \frac{C - C_{\text{nominal}}}{C_{\text{nominal}}} \right) \times 100\%$$

**RoboColumn® Sample Injection Test Method.** Potential sample dilution during injection into the column was investigated under standard operating conditions except that test solutions were used. The test solutions were 50 mM KNO₃ in water or 250 mM KNO₃ plus 750 mM sodium chloride in water, respectively. The experiment was performed with 600 μL Eshmuno® CEX prototype RoboColumns® at flow rates of 20 and 300 cm/h. Columns were equilibrated with test solution prior to the test. For the actual test, KNO₃ solution was applied to the columns in cycles and the effluent collected in fractions. In each cycle, a volume of 900 μL solution was aspirated and dispensed in 300 μL steps, each step being defined as one fraction.

KNO₃ concentration in each fraction was analyzed by photometric measurement at 300 nm and the observed values compared against the KNO₃ feed concentration to reveal possible concentration changes because of dilution of the sample with system liquid.

For the sample injection test, the pipetting parameters were set according to TECAN’s standard liquid class for water. Aspiration settings were as follows: 20 μL system trailing airgap, 10 μL excess volume, 80 μL/s aspiration speed. Dispense speeds of 1.1 and 16.7 μL/s were tested.

For routine MCC operation an optimized liquid class was used. In addition to the sample volume being finally dispensed into the RoboColumn®, a small extra volume of sample (50 μL) was aspirated first with a leading airgap (20 μL), separating the two sample volume segments and a system trailing airgap (10 μL) plus leading airgap (20 μL) separating the system liquid and the small extra sample volume. The sample excess volume was set to 20 μL.

**Scale Down Experiments with an ÄKTAPurifier™.** Elution experiments were carried out with Eshmuno® CEX prototype resin packed into 5 and 1 mL MediaScout® MiniChrom columns. Columns were operated with an ÄKTAPurifier™ 10 liquid chromatography system run by Unicorn™ 5.1 from GE Healthcare, Sweden. Equilibration buffer was 20 mM phosphate pH 6.0. Elution buffer was 20 mM phosphate + 1 M NaCl, pH 6.0. The total protein concentration of the ternary protein feed was 5 mg/mL. Column load was set to 0.2 mg total protein per mL packed resin volume. Following sample application, linear gradient elution was performed applying 0–100% elution buffer at varying gradient volumes and elution flow rates. Flow rate during load and re-equilibration was constant at 150 cm/h.

**LHS Elution Buffer Preparation Method.** In order to mimic linear gradient elution operation, a finely designed multistep gradient was applied to the MCCs using the LIHA. The gradient, as shown in Figure 4-1 was defined by

- the number of elution steps $N$
- the difference of elution salt concentration between two successive elution steps, hereinafter referred to as molar step height [mM]
- the volume of elution buffer applied onto the column per each step, hereinafter referred to as step volume [μL] and the normalized step volume which relates to the packed resin volume [CV]
- total gradient volume $V_g$ [mL] and the normalized gradient volume $V_{g,\text{Norm}}$ which accounts for the packed column volume [CV]

Prior to the chromatographic experiment, a set of gradient elution buffers with increasing salt concentration was prepared from three stock solutions: Buffer A, the equilibration buffer without NaCl; buffer B, the final elution buffer with 1
or 0.5 M NaCl; and buffer C, a 1:1 mix of buffer A and B. Stock solutions were pipetted into DWPs and stored in hotels without lids at room temperature. The reason for using three stock buffers was not to fall below a minimal pipetting volume of 20 μL. The desired buffer composition of each individual step and the corresponding volumes were calculated in Microsoft Excel (Microsoft, Redmond, WA, USA) and compiled into a pipetting worklist, which was then imported into the robotic script. Using all tips of the LIHA in parallel allows for preparing eight elution buffers at once, optionally each with a different buffer salt, pH, or salt gradient.

**LHS Gradient Elution Method Development Protocol.** RoboColumns® were equilibrated with 5 CV of 20 mM sodium phosphate buffer at corresponding pH (5.0 and 6.0, respectively) followed by injection of a ternary model protein feed. The total protein concentrations of the ternary protein feed were 2.5 mg/mL and 5 mg/mL for the 200 μL and 600 μL MCC format, respectively. Column load was 10 mg total protein per mL packed resin. Feed volume was 800 μL for the 200 μL MCC format and 1200 μL for the 600 μL MCC columns. A postloading wash step with equilibration buffer was included using 4 CV for 200 μL and 2.6 CV for 600 μL MCC columns, respectively. Protein separations were conducted by applying different multistep gradient elution protocols, which are described in detail below (see Results and Discussion part). Except for elution, flow velocity was constant at 100 cm/h. Residence time during sample injection was short (0.6 min with 200 μL MCCs or 1.8 min with 600 μL MCCs) but sufficient for the purpose of this study. The protein load was rather low compared to the maximum protein binding capacities of the resins and no breakthrough of sample during the load or wash was observed. Flow-through, wash and eluate fractions were collected in UV-MTPs and fractions analyzed by photometry.

**UV Absorption Measurement in UV Microtitre Plates.** Fraction collection plates from the LGE experiments were analyzed by photometry to determine the absorption values at 280 nm (total protein) and 528 nm (cytochrome C). The peak length was determined by difference measurement at 975 and 900 nm. A path length correction factor for water of K = 0.173 was used. The difference of raw absorption data Araw and buffer blank Abuffer was normalized to a path length of 10 mm. Path length corrected absorption values Accorr were calculated as follows:

$$A_{corr} = \frac{(A_{raw} - A_{buffer})}{K_{well}}$$

**Chromatogram Evaluation and Peak Resolution Calculations.** The huge volume of LHS raw data collected from UV measurements was processed by means of a self-programmed Visual Basic® based Microsoft Excel® macro application. A user interface enabled a convenient import of UV result files, subsequent automatic data conversion, and creation of chromatograms. Evaluation of a complete data set from eight parallel column runs was carried out within less than five minutes. Elution volumes were calculated based on the nominal fraction size. To allow a better assessment of the chromatographic data and to enable the calculation of peak resolution values, peak data were fitted and overlapping peaks were deconvoluted. In this work, a GEMG5 function which combines the EMG (Exponentially Modified Gaussian) and GMG (Half-Gaussian Modified Gaussian) model and the modified extreme value function (Eval4 Area Frtd, for fitting of fronting peaks) was applied to describe peak shapes using PeakFIT V4.11 (Systat Software, London, UK). An overview of these peak functions can be found in the PeakFitTM v4 user guide (ISBN 81-88341-07-X).

PeakFit uses the following equation to calculate the chromatographic resolution where V1 and V2 are the elution volumes of the first and the second peak and W1 and W2 represent the full width at the peak base:

$$R_s = \frac{|V_{R1} - V_{R2}|}{2(W_1 + W_2)}$$  \hspace{1cm} (7)

ÄKTATM chromatograms were evaluated using the UnicornTM software for calculating resolution (Rs) as described in the following equation in which Ws represents the peak width at half height and VR the peak elution volume. The subscript numbers indicate the sequence of elution peaks:

$$R_s = \frac{|V_{R1} - V_{R2}|}{2 \left( \frac{w_{R1}}{2^{355}} + \frac{w_{R2}}{2^{354}} \right)}$$  \hspace{1cm} (8)

In order to compare the separation quality between lab scale and MCC trials, ÄKTATM peak data were fitted with PeakFit and Rs calculated according to Eq. (7).

For the sake of visual comparability, ÄKTATM chromatograms from different column scales were transformed to account for dissimilar peak heights and elution volumes. The absorbance values Abs were normalized to the height of the first peak Absp1 and the elution volume Ṽe was scaled based on the difference in elution volume between the first and second peak Vp1 and Vp2.

$$Abs_{norm} = \frac{Abs}{Abs_{p1}}$$

$$V_{E \_scale} = \frac{V_{E}}{V_{p2} - V_{p1}}$$

Normalized chromatograms were superimposed at the maximum of the first peak allowing for a visual assessment of the resolution of the first two peaks based on peak width.

**Calculation of Gradient Slope.** The gradient slope g [M/mL] is defined by the difference of salt concentration at the end, It, and at the beginning, Ih, of the linear or pseudo-linear gradient divided by the total volume of the gradient, Vg [mL]:

$$g = \frac{I_t - I_b}{V_g}$$

The normalized gradient slope GA [mM/cm] accounts for the cross sectional area of the column A [cm²] and is calculated by

$$GA = g \times A \times 1000$$

A comparison of gradient elution results based on the same GA values and identical linear flow velocities reveals the impact of column length on peak resolution.

The dependency of the elution salt concentration at peak maximum on the gradient steepness was evaluated based on the normalized gradient slope GH which is defined by the following equation according to Yamamoto et al. 15.
GH is the gradient slope normalized with respect to the column stationary phase volume (resin). \( V_0 \) is the column void volume and \( V_i \) is the total bed volume. The volumetric phase ratio \( H \) is defined by \( H = \frac{V_i - V_0}{V_0} \). In case the same resin type was used, the volumetric phase ratio was assumed to be constant for different column scales.

GH vs. \( I_R \) plots can be used for predicting \( I_R \) in linear gradient elution of various column dimensions and gradient slopes. For nonoverloading conditions and strong binding of the proteins at the initial salt concentration \( I_0 \) of the gradient, GH–\( I_R \) curves are not affected by flow velocity.\(^{14} \)

Size Exclusion HPLC. Analytical size-exclusion chromatography (SEC) was conducted using a G3000SWXL column (Toosoh Bioscience GmbH) with a GFC-3000 guard cartridge (Phenomenex). The mobile phase was 25 mM NaHPO\(_4\) \( \cdot \) H\(_2\)O buffer solution (pH adjusted to 7.0 with 5 M NaOH) containing 15 mM NaSO\(_4\) and 0.05% NaN\(_3\). The column was operated isocratically at a constant flow rate (1 mL/min) using a LaChrom HPLC system (Merck-Hitachi, Darmstadt, Germany). Directly after sample collection, mAb samples at pH 5.0 were neutralized by adding 132 \( \mu \)L 1 M Tris buffer pH 7.5 to 1500 \( \mu \)L sample. The sample volume applied to the HPLC column was 20 \( \mu \)L. The concentration of mAb in unknown samples was determined using a standard calibration curve generated with the purified antibody.

Mab Purification Method. Fractogel\textsuperscript{R} EMD COO\textsuperscript{-} (M) packed into a 200 x 5 mm ID Superformance\textsuperscript{R} column and, respectively, a 600 \( \mu \)L RoboColumn\textsuperscript{R} (30 x 5 mm ID) was evaluated for isolating mAb monomer from aggregates (3.8%).

Column equilibration and post-loading wash was carried out with 5 CV running buffer (24 mM NaCl in 50 mM Na-acetate buffer pH 5.0). The linear gradient on lab scale was run from 24 to 500 mM in 50 mM Na-acetate buffer pH 5.0 within 20 CV (corresponds to GA 1.19 mM/cm); for the MCC experiment, a pseudo-linear gradient over 96 CV (corresponds to GA 1.62 mM/cm) formed by 96 steps with a step volume set to 600 \( \mu \)L was applied. At both lab scale and MCC format, the column loading was conducted at 6 min residence time (r.t.) and with a protein load of 40 mg mAb/mL CV. Flow rate for wash, equilibration, and gradient elution was set to 200 cm/h (6 min r.t.) for the lab-scale run; for the MCC run, a flow rate of 100 cm/h (1.8 min) was applied. Column eluate was fractionated and mAb peak fractions analyzed by means of size exclusion HPLC.

### Results and Discussion

**Liquid handling**

Prior to performing chromatography separation experiments on MCC scale with the Tecan LHS, the pipetting performance of the LHS was tested and the quality of automated gradient elution buffer preparation verified. Precise and accurate elution gradient formation is an essential requirement for obtaining consistent separation results. Unlike conventional dual-pump chromatography systems with continuous gradient mixing, the accuracy and precision of buffer mixing with a liquid handler is strongly affected by the properties of the sample fluid (viscosity, vapor pressure) and thus requires appropriate choice of pipetting parameters.\(^{24} \)

Major differences in the accuracy and precision between the protocols tested for buffer preparation have been found. Table 5-1 summarizes the statistic values of each individual buffer mix and shows a graphical comparison of the set and actual concentrations of elution buffer B. Protocol 1 was divided into two main parts, the transfer of stock buffers one after another from reservoirs into a DWP (with an intermediate tip wash after dispense of the first buffer) and two aspirate/dispense cycles executed with Tecan’s standard mixing command directly after dispense of the second stock buffer. Large deviations between the set and the actual concentrations of buffer mixtures over the entire mixing range were observed. The precision was conspicuously high and actual concentrations were much below the target value indicating severe system liquid dilution effects. The system dilution effect during buffer preparation was caused by a malfunction of Tecan’s standard mixing command, which misses necessary tip wash commands for renewal of the system trailing airgap. The use of an additional wash command before each mix step, however, would inevitably result in an extremely time-consuming buffer preparation procedure.

Hence, in the second protocol, the automated mixing step was skipped and replaced by manual mixing, which takes less than 1 min per plate. A significant improvement was observed when changing the mixing method. The mean accuracy and precision values of protocol 2 were below 1% over the whole pipetting protocol. If desired, manual mixing can be automated with appropriate instrument configuration.

Potential system liquid dilution effects can occur when sample is injected into a microchromatography column. An additional target of this study was to investigate potential sample dilution effects and distortion of the elution gradient during MCC operation. Figure 5-1 depicts results for the pipetting of high salt solution (250 mM KNO\(_3\) + 750 mM NaCl) into a 600 \( \mu \)L MCCs with a single tip. It was found, that the cleanliness of the stainless steel needles plays a significant role for the performance of the fixed pipetting tips.
Sample dilution with system liquid was observed when the tips had not been properly cleaned before use. When pipetting with clean tips, the salt concentration in different volume segments of test solution dispensed within cycles remained unchanged at both dispense speeds (Supporting Information Figure S1). The data collected did not indicate significant system liquid dilution effects under tested conditions. Similar tests carried out with a low salt solution (50 mM KNO₃ in water) confirmed this finding (data not shown). These observations lead to the assumptions that the inner surface of the tips needs to be free from any residuals, and that an alteration of the inner contact surface of the tips impairs the formation of a stable system trailing airgap separating the system liquid and the sample during pipetting. Hence, in this study the tips were cleaned before each run with bleach (flushing and incubation with 2-5% NaClO for 30 min). In contrast to the recommendation given by Grönberg et al.,22 the outcome of the present study does not give reason to use large excess volumes for the injection of sample into MCCs.

**Lab scale LGE experiments**

Elution trials on lab scale were performed in order to prove the scale down principles explained in Scale down prediction section and to create reference data for comparison with MCC experiments conducted on the LHS. The first separation run on 5 mL scale was realized with an elution flow rate of 240 cm/h and a gradient length of 32 CV yielding baseline separation between all three peaks (Figure 5-2).

With the aim of reducing the elution buffer consumption on the 5 mL scale, the gradient length was restricted to eight CV. By lowering the flow velocity from 240 to 60 cm/h, the quality of separation was maintained as confirmed by the peak resolution values listed in Table 5-2. For the third run, a 1 mL column with a shorter bed height was employed and the elution flow velocity was kept low at 60 cm/h. Even with such a very short bed height, a similar separation performance could be achieved. However, a disproportionally long gradient was required in order to compensate for the loss of separation distance (less theoretical plates because of reduced bed height) resulting in a prolonged separation time.

**Establishing the multistep gradient elution protocol using a LHS**

The goal of this work was a structured and rationale approach to investigate the impact of operational variables on the separation performance of MCCs during pseudo-linear gradient run with a LHS. The separation of a ternary protein mixture on Eshmuno°CEX prototype resin was chosen as test model. This separation example has been well characterized on lab-scale format and existing data provide a reference (Lab scale LGE experiments section) to assess the quality of protein separation obtained on the MCC scale. In addition, other studies used similar CEX test systems for elution experiments with MCCs that also can serve as comparison.3,7,20 Elution buffers were prepared according to protocol 2 (Liquid Handling section). The initial trials were carried out with 200 μL MCCs (1 cm bed height) since this has been by far the most frequently used format according to literature. In the second part, the 600 μL MCC format (3 cm bed height) representing the largest RoboColumn size available was evaluated. In order to verify method robustness, duplicate experiments were performed by parallel testing of two columns.

**Pseudo-Linear Gradient Elution Trials in 200 μL MCC Format.** The first MCC run employed a gradient from 0 to 1 M NaCl formed by 48 steps corresponding to elution salt increments of 20.8 mM NaCl per step. Elution step volume was set to 450 μL. These settings translated into a total gradient volume of 108 CV (21.6 mL), i.e. normalized gradient slope of 9.1 mM/cm. Column effluent was collected in 225 μL fractions (two fractions per elution step), generating 96 data points throughout the gradient. The flow rate was set to 300 cm/h in order to allow a short run time, which is a basic requirement for powerful screening. The overall method run
time for parallel testing of up to eight columns was 2.8 h, comprising the preparation of elution buffers with the LHS, gradient elution process, and absorbance measurement. Under these conditions, partial separation of the individual components from the ternary protein mixture was achieved (Figure 5-3, Exp. 1-1, Exp. 1-2). The results from replicate runs were almost identical which indicates consistent quality of the MCCs and high reproducibility of the multistep elution protocol. The resolution values were both 0.86 for Rs1/2 and for Rs2/3. Table 5-3 lists all experimental details and results of the MCC trials. Selected chromatograms are depicted in Figure 5-3. Chromatograms for all runs can be found in the Supporting Information Document (Figure S2).

In the second experiment, gradient parameters were varied with the aim to improve peak resolution. The gradient steepness was reduced by a factor of two (from 9.1 to 4.5 mM/cm), but this resulted in a marginal improvement of the separation quality only (see Figure 5-3 and Table 5-3). Apparently, the attempt to realize a shallower gradient by increasing the step length but maintaining the number of elution steps was not viable. Although the data point density was 1.5 times higher compared to run 1, this did not result in a better peak shape. Instead, distinct scattering around the peak maximum was observed. To counteract this effect, the gradient was refined in the following run.

In the third trial, the step length was limited to one CV and the step height lowered by a factor of 4 from 20.8 to 5.2 mM. The gradient slope was 5.1 mM/cm and was almost equal to the previous run. The combined adjustment of flatter gradient slope and refinement of the multistep gradient led to a noticeable improvement. Observed resolution values were 1.09 for Rs1/2 and 1.06 for Rs2/3. However, method run time leaped by a factor of 2.2–7.8 h.

Subsequent reduction of flow velocity from 300 to 100 cm/h enhanced resolution slightly (see experiments 4 and 3 in Table 5-3). The overall method run time further increased to 9 h.

Run 5 was conducted at 100 cm/h but with a steeper gradient slope of 10.2 mM/cm in order to shorten the method time. The elution gradient was divided into 10.4 mM salt increments and 96 fractions. Total method run time was 4.6 h. Rs values of 1.06 for Rs1/2 and of 1.15 for Rs2/3 were calculated.

For run 6, gradient parameters of the previous experiment were kept constant, but the flow velocity was lowered from 100 to 50 cm/h. This caused a significant gain in resolution for peaks 2 and 3 but not for peaks 1 and 2.

These few results already indicated that the potential for additional improvement was quite limited. The parameter settings used in experiment 5 provided a good balance between separation quality and run time. Any further gain of resolution by decreasing flow velocity or gradient slope, refinement of steps or fractionation would be at the expense of longer method run time.

The duration of the LGE block is mainly determined by ROMA, LIHA, and transfer slide operations for plate handling, buffer aspiration and fraction collection, rather than by

| Exp. no. | Column bed height [cm] | Column vol. [ml] | Flow velocity [cm/h] | Norm. gradient slope GA [mM/cm] | Gradient vol. Vg [mL] | Norm. gradient vol. VgNorm [CV] | Rs1/2 | Rs2/3 | Rs1/2 | Rs2/3 | Time* [h] |
|----------|------------------------|-----------------|----------------------|-------------------------------|----------------------|-------------------------------|-------|-------|-------|-------|-----------|
| 1        | 10                     | 5.02            | 240                  | 3.1                           | 161                  | 32                            | 1.0   | 3.10  | 3.10  | 2.41  | 1.3       |
| 2        | 10                     | 5.02            | 60                   | 12.5                          | 40                   | 8                             | 1.0   | 3.20  | 3.20  | 2.37  | 1.3       |
| 3        | 2                      | 1.00            | 60                   | 3.1                           | 161                  | 160                           | 1.0   | 3.09  | 3.09  | 2.35  | 1.3       |

*Duration of gradient elution phase.

**Figure 5-3. Selected chromatograms of LGE runs with 200 μL MCCs using a LHS.**

Table 5-2. Lab Scale Elution Conditions and Peak Resolution Values Obtained on Different Columns Scales
Taking runs 4 and 3 as examples, elution flow rates were 100 and 300 cm/h, respectively. Under otherwise identical conditions, the threefold increase in elution flow velocity led to a minor shortening of the LGE period from 4.8 to 3.6 h only. This is linked to break times caused by afore-mentioned steps, which account in total for 3 h in case of runs 3 and 4. This implies that the effective residence time during LGE is longer than the theoretical value calculated from linear flow velocity and column bed height. The use of 600 µL MCCs offers two advantages: higher column efficiency (larger number of theoretical plates) because of longer bed height as well as greater flexibility with regard to potential offline analytics since larger sample quantity, i.e., larger volume of analyte, is generated in a single MCC run. Figure 5-4 shows selected chromatograms of the 600 µL column experiments (the full set of chromatograms is shown in Supporting Information Figure S3). In MCC run 9, the identical method to the 200 µL MCC run 5 was applied. Baseline separation was still not achieved. The obtained results indicate a small positive effect on peak separation gained from the increase in column length.

Graph A in Figure 5-5 depicts peak resolution as a function of elution flow velocity. The protocol for gradient formation and step elution was the same for all runs. The graph includes results obtained from separations at 25 cm/h (run 7), 50 cm/h (run 8), 100 cm/h (run 9), and 300 cm/h (run 10). The linear regression line indicates an inversely proportional relationship between elution flow velocity and peak resolution, confirming the expected behavior. With the elution flow velocity increasing from 100 to 300 cm/h, R_{s1/2} dropped from 1.32 to 0.90 and R_{s2/3} from 1.39 to 1.03. Lowering the velocity from 100 to 25 cm/h improved the resolution values to 1.47 for R_{s1/2} and 1.89 for R_{s2/3}.

In order to achieve high resolution with the 600 µL format, elution flow velocity should not exceed 100 cm/h, which corresponds to a minimum residence times of 1.8 min. Typically, in normal scale column operation, residence times of a similar order of magnitude are applied, ranging between 3 and 4 min.

The influence of gradient slope on peak separation was studied, varying the elution step length based on the elution protocol of run 9. Although flattening of the gradient improved the separation performance as expected (Figure 5-5, graph B), this effect was small under the conditions tested. With a three-fold reduction of gradient slope from 10.2 mM/cm (run 9) to 3.4 mM/cm (run 11), R_{s2/3} increased only slightly from 1.39 to 1.69. Resolution of the first and second peak remained almost the same, as seen from R_{s1/2} values of 1.32.

### Table 5-3. Part 1. Parameter Settings of the Elution Protocols of MCC Experiments

| Experiment   | Column bed height [cm] | Column vol. [mL] | Column diam. [cm] | Flow velocity [cm/h] | Norm. gradient slope GA [mM/cm] | Gradient vol. [mL] | Molar step height [mM NaCl] | Step length [CV] | Step length [µL] | No. of elution steps | Frac. size [µL] | Data points |
|--------------|------------------------|------------------|------------------|----------------------|-------------------------------|------------------|-----------------------------|-----------------|-----------------|---------------------|----------------|-------------|
| MCC run 1    | 1                      | 0.2              | 0.5              | 300                  | 9.1                           | 21.6             | 108                         | 1.0             | 20.8            | 2.29                | 450             | 48          |
| MCC run 2    | 1                      | 0.2              | 0.5              | 300                  | 4.5                           | 43.2             | 216                         | 1.0             | 20.8            | 4.59                | 900             | 48          |
| MCC run 3    | 1                      | 0.2              | 0.5              | 300                  | 5.1                           | 38.4             | 192                         | 1.0             | 5.2             | 1.02                | 200             | 192         |
| MCC run 4    | 1                      | 0.2              | 0.5              | 100                  | 5.1                           | 38.4             | 192                         | 1.0             | 5.2             | 1.02                | 200             | 192         |
| MCC run 5    | 1                      | 0.2              | 0.5              | 100                  | 10.2                          | 19.2             | 96                           | 1.0             | 10.4            | 1.02                | 200             | 96          |
| MCC run 6    | 1                      | 0.2              | 0.5              | 50                   | 10.2                          | 19.2             | 96                           | 1.0             | 10.4            | 1.02                | 200             | 96          |
| MCC run 7    | 3                      | 0.6              | 0.5              | 25                   | 10.2                          | 19.2             | 32                           | 1.0             | 10.4            | 0.34                | 200             | 96          |
| MCC run 8    | 3                      | 0.6              | 0.5              | 50                   | 10.2                          | 19.2             | 32                           | 1.0             | 10.4            | 0.34                | 200             | 96          |
| MCC run 9    | 3                      | 0.6              | 0.5              | 100                  | 10.2                          | 19.2             | 32                           | 1.0             | 10.4            | 0.34                | 200             | 96          |
| MCC run 10   | 3                      | 0.6              | 0.5              | 300                  | 10.2                          | 19.2             | 32                           | 1.0             | 10.4            | 0.34                | 200             | 96          |
| MCC run 11   | 3                      | 0.6              | 0.5              | 100                  | 3.4                           | 57.6             | 96                           | 1.0             | 10.4            | 1.02                | 600             | 96          |
| MCC run 12   | 3                      | 0.6              | 0.5              | 100                  | 27.3                          | 7.2              | 12                           | 1.0             | 10.4            | 1.13                | 75              | 75          |

*Mean and % CV are for n = 2, except for single experiment MCC run 1 (n = 1).
and 1.29 for runs 9 and 11. The effect of gradient slope becomes more visible when comparing run 12 and run 11 which involved a greater decrease of gradient steepness by a factor of 8, changing GA from 27.3 to 3.4 mM/cm. An improvement of Rs1/2 from 1.11 to 1.29 and of Rs 1/2 from 1.34 to 1.69 was observed.

Within the tested range, variation of gradient design, fraction scheme, and column length had only minor effects on the separation performance. However, the gradient design is of high practical importance. Keeping the data point density (number of fractions) constant, shallower gradients result in larger fraction volumes, which are less affected by volume fluctuation (see Supporting Information Figure S4). Because of the dropwise column effluent flow, fraction volumes consistently vary by approximately ±25 µL (estimated volume of one drop). For small fraction volumes, this systematic error corresponds to large relative deviations between the nominal and the actual fraction volume rendering correct mass balancing a challenge.

With the 600 µL MCC format, the best separation of the model protein mixture using the multistep elution method on the LHS was accomplished. Run 8 presents the best compromise between high resolution and operational feasibility (adequate method run time and number of data points).

**Figure 5-4. Selected chromatograms of LGE runs with 600 µL MCCs using a LHS.**

**Figure 5-5. Impact of linear elution flow velocity (graph A) and gradient slope (graph B) on peak resolution using a 600 µL MCC column operated in a pseudo-linear gradient elution mode with a LHS. RS1/2 refers to the resolution of chymotrypsinogen A (1) and cytochrome C (2), RS2/3 to the resolution of cytochrome C (2) and lysozyme (3).**
and conductivity flow cells. Results from lab-scale elution experiments conducted at very low and high loading (ranging from 0.2 to 60 mg total protein/mL CV) showed that the conductivity profile of the gradient and the position of the peak moment in the gradient were unchanged (data not shown).

Finally, the observed elution salt concentrations at the maxima of elution peaks for LHS and lab scale experiments, listed in Supporting Information Table S1, were evaluated by means of a GH–IR plot (Figure 5-7).

Since the shape of GH–IR curves is independent of column scale and flow rate, the IR values in linear elution can be predicted for any column scale. The GH–IR plot provides useful information about charge characteristics of proteins and the interaction of protein and resin for various salt concentrations. It was shown that the dependency of $I_R$ on the GH–I curve became stronger and the curve shifted to higher $I_R$ with steeper slope, as the net charge of the protein increases.\textsuperscript{14}

So far, the GH–IR relationship and the underlying chromatographic models have been applied to linear gradient elution only. Figure 5-7 shows the logarithm of the normalized gradient slope GH versus $I_R$ for each model protein. Data points from lab scale experiments (open symbols) were fitted by linear regression, each fit representing a characteristic GH–IR curve of one model protein. For comparison, the elution data obtained from the MCC LHS trials (filled symbols) and the corresponding linear fits were included in the diagram. The GH–IR plots generated from MCC trial data are almost parallel to the GH–IR curves created from lab scale. The curves are slightly shifted towards higher $I_R$ values (by a factor of approximately 1.05), but the relative position of the GH–IR plots of individual proteins was maintained. The congruency of GH–IR curves proves that selectivity data generated with the MCC multistep elution method are suitable to predict the outcome of lab scale runs. Given these results, GH–IR data from pseudo-linear gradient elution allow a good estimation of ionic strength values at the maximum of elution peaks for larger column formats and varying elution conditions. Higher accuracy of prediction would require an accurate qualification of the column packings and more elution steps for better approximation of the step profile towards linearity. For this purpose, innovative liquid handling concepts for gradient mixing on a LHS based on a binary dispenser pump system can be utilized.\textsuperscript{25}

Comparison of LHS and ÅKTA™ Approaches. The use of a LHS in combination with the pseudo-linear gradient elution approach offers great potential for enhancement of resin selectivity screening. Figure 5-8 compares the time need for screening experiments conducted with a LHS or an ÅKTA™ system on example of linear gradient elution performed in 3 cm bed height column formats. In both cases, separation was performed at 100 cm/h linear flow rate (throughout load,
wash, and elution) and a gradient slope of 3.4 mM/cm and loaded with 800 mL model protein feed to 10 mg total protein/mL packed resin. Separation on the LHS was performed according to MCC protocol 11 (Table 5-3). A detailed breakdown of the method into individual steps reveals the extra time need of the LHS method for offline elution buffer mixing, offline UV reading, plate handling and holding times caused by pump flow interruptions. The longer method run time, though, is overcompensated by the ability of parallel operation, allowing testing of up to eight columns within 8 h compared to the LHS. Performing the same number of column runs sequentially with an AKTA™ would take more than 24 h.

Despite the considerably different operational mode and design of the Tecan system, very similar separation efficiency as compared to the AKTA™ run was obtained (Figure 5-9). While peak resolution on lab scale was marginally better (Table 5-4), conductivity values at the peak moment were almost identical (Supporting Information Table S2). Both the LHS and the AKTA™ system were equally suited for the operation of miniature chromatography columns and delivered equivalent results. However, higher throughput and automated sample handling with the LHS represent a substantial advantage, thus rendering the LHS approach superior for resin screening.

**Resin selectivity screening in 600 µL MCC format**

In this part of our study, four cation exchange resins were screened with regard to their selectivity at different pH conditions. We applied the protocol of run 11 and defined these parameter settings as default conditions in favor of higher accuracy for mass balancing for future applications, even though 9 and 10 can attain a similar or higher resolution within shorter time.

The panel of resins represents a diverse selection of chromatographic materials with respect to their physical and chemical properties (Table 4-1). The strong cation exchangers belong to the Eshmuno® or Fractogel® EMD media families, which make use of different base matrix chemistries (polyvinylether in case of Eshmuno®, media, polymethacrylate in case of Fractogel® EMD media). Each material carries a specific type of grafted polymer surface modification (“tentacles”) featuring sulfonic acid residues as functional groups. While all Eshmuno® CEX resins tested bear a sulfoisobutyl modification, Fractogel® EMD SE Hicap (M) resin employs the more hydrophilic sulfoethyl group.

Each experiment was conducted in duplicate by running two columns in parallel, yielding a total number of 16 column runs, which were grouped into two blocks, each of it comprising eight parallel runs at the same buffer condition.

Results from duplicate runs showed a high degree of similarity and confirmed that the separation on MCC scale with the LHS is reproducible (Supporting Information Figure S5). Figure 5-10 depicts selected chromatograms of runs performed with different resins at various buffer conditions. The elution order of the proteins on the different resins was the same, but clear differences in selectivity and efficiency for the separation of the model proteins occurred.

With all Eshmuno® CEX resins tested, all three individual proteins of the model feed could be resolved at both pH conditions (5.0 and 6.0). Very similar elution profiles were observed for the Eshmuno® CEX prototype and the Eshmuno® CPX resin (compare Figures 5-10, A and B). Despite this similarity, proteins were much more strongly retained on the prototype resin, as the shift of the peak maxima towards higher elution volumes suggests. The difference in binding strength is consistent with a higher ligand density for the Eshmuno® CEX prototype resin compared to the Eshmuno® CPX resin. When buffer pH was changed from pH 6.0 to 5.0, proteins eluted at higher ionic strength but the elution order of the proteins remained the same. The effect of pH on elution selectivity becomes best visible when looking at cytochrome C (middle peak) as the target molecule to be purified. While at pH 6.0 this peak was well separated from both chymotrypsinogen (first peak) and lysozyme (third peak), the resolution of cytochrome C and lysozyme was impaired at pH 5.0.

Amongst the Eshmuno® CEX resins tested, Eshmuno® S resin showed the weakest binding strength. Even though the ligand density is higher compared to Eshmuno® CPX resin and the Eshmuno® CEX prototype resin, the model proteins eluted at significantly lower salt concentration. This example illustrates that the strength of the binding of molecules to a resin does not exclusively depend on the density of

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**Table 5-4. Peak Resolution Obtained from AKTA™ and LHS Gradient Elution Experiments Performed with Similar Column Scales Under Identical Separation Conditions**

|         | Resolution (PeakFit) | Rs1/2 | Rs2/3 |
|---------|----------------------|-------|-------|
| AKTA™   | Mean (n=2)           | 1.5   | 1.9   |
|         | % CV                 | 3.4   | 3.7   |
| LHS     | Mean (n=2)           | 1.3   | 1.7   |
|         | % CV                 | 1.7   | 7.1   |

Figure 5-9. Separation of a ternary protein mixture with Eshmuno® CEX prototype resin in a 30 x 5 mm ID RoboColumn™ on a LHS (black lines) and 30 x 5 mm ID Superformance™ column on an AktaTM system (red lines). UV trace at 280 nm and salt gradient are shown. With similar miniature column formats, the same separation performance and chromatographic results were obtained on the LHS and the AKTA™ when the same linear flow rate and elution gradient were applied.
functional groups. The chemical structure of the graft polymer, the base matrix and ligand type also need to be considered.

Eshmuno® S resin exhibited a different pH optimum for the separation of the test proteins. Elution at pH 5.0 provided a better separation of cytochrome C from the other two components than at pH 6.0 (Figure 5-10, C). For Eshmuno® CPX and Eshmuno® CEX prototype resins, instead, pH 6.0 was best suited for this separation task.

Examination of Fractogel® EMD SE Hicap (M) resin revealed a distinctively lower selectivity for the separation of the three model proteins. Baseline separation of the components was not achieved. At pH 5.0, lysozyme co-eluted with cytochrome C as one peak after chymotrypsinogen A resulting in two elution peaks only. At pH 6.0 a partial separation of the three proteins was possible. Compared with the Eshmuno® CEX media candidates, Fractogel® EMD SE Hicap (M) resin showed a reduced binding strength, especially for lysozyme, a protein with a pronounced hydrophobic nature. These observations suggest, that functional groups (ligands) in classical ion exchange resins (mixed-mode resins are not considered here) which also bear some hydrophobic side features, can increase separation performance considerably with regard to both capacity and selectivity, when proteins with proportionate hydrophobic character are involved.

The results presented in this study give an idea as to how much the selectivity of different resins may vary and to which extent process conditions such as elution buffer pH can influence the separation performance. In order to find the optimal chromatography resin and process conditions, it is beneficial to screen a high number of resin candidates and to explore the operational space extensively.

This application example proves that the pseudo-linear gradient elution developed by us provides a high quality of information with respect to resin selectivity. The entire screening task comprising the testing of four resins at two pH values was automatically processed within 16 h excluding the time required for the preparation of stock buffer solutions (ca. 1 h).
While previous experiments for method development presented in this work were conducted with a well characterized model protein feed for purpose of simplification of analytics, this part of the study focusses on demonstrating the feasibility of the pseudo-linear gradient LHS method for a more complex and industry relevant separation task, the removal of high molecular weight species from monoclonal antibody monomer.

The separation of closely related mAb forms by cation exchange chromatography in bind-and-elute mode requires resins with high selectivity and high separation efficiency. Hence, purification protocols designed for production scale typically utilize columns with long packed beds between 20 and 30 cm and shallow gradients to cope with this situation and achieve sufficient resolution. In this example, mAb aggregate removal on Fractogel® EMD COO⁻ (M) on a 200 mm x 5 mm ID lab scale column operated on an ÄKTA™ system and a 30 mm x 5 mm ID MCC (600 µL) on a LHS was investigated. Transfer of this application to miniature column scale on a LHS without significant loss of resolution required appropriate settings for the pseudo-linear gradient elution protocol. The scale-down theory (Scale down prediction section) offers different strategies to compensate reduced separation capabilities of MCC columns by employing flow-rate and gradient adjustments. However, for sake of mass balance accuracy and limited chromatographic capabilities of the LHS, the use of short (steep) gradients in combination with very low flow rates and small fraction volumes does not represent a preferred option.

For this reason, shallow gradient slope as applied on lab scale (GA 1.19 mM/cm) was adopted to the MCC format, with only slight adjustment in order to match the parameters of the existing LHS method defined for basic resin selectivity screening tasks. The actual gradient slope of the MCC separation was 1.62 mM/cm. At both scales, mAb was loaded at 6 min r.t. to comparable levels (MCC: 40.6 mg mAb/mL CV; Superformance® column: 37 mg mAb/mL CV). While the lab scale experiment used 6 min r.t. (200 cm/h) for wash, equilibration and elution, on MCC format these steps were performed at 1.8 min r.t. (100 cm/h) which was described before as the minimum residence time required for high resolution for the 600 µL format (Pseudo-linear gradient elution trials in 600 µL MCC format section).

The elution profiles illustrate, that the mAb aggregate and monomer separation obtained with the MCC (Figure 5-11) was representative of the lab scale. On both scales, elution peaks were uniformly shaped, showing aggregates partially separated at the descending flank of the monomer peak. The calculated yields and aggregate levels in the final mAb pools reveal similar separation efficiency (Figure 5-12). Also closed mass balancing was possible on both scales, achieving mAb recoveries greater 95%.

As such, the performance attributes of both scales confirm the validity of information created with MCCs on a LHS and the suitability of the scale-down approach applied.

The transfer of gradient elution from lab to MCC scale by keeping the GA values similar, inherently translates into smaller GH values (gradient slope normalized to packed resin volume) at MCC scale. As a result, the eluate pool characteristics mAb concentration and relative pool volume.
(which is the pool volume related to the column volume) differ significantly between scales.

However, keeping the GH value constant between different scales does not represent a feasible alternative as this concept would result in very short (steep) gradients on very short bed height columns. In turn, this situation would call for extremely low flow rates and small fraction volumes in order to maintain resolution and a sufficiently high data point density, pushing the LHS to its limits.

Conclusions

The development of a pseudo-linear gradient elution method for resin selectivity screening using MCCs on a robotic LHS has been described in detail. The current state of approaches in the literature was reviewed and relevant information on this technology was incorporated, enabling a straightforward implementation of this method. Sufficient understanding and knowledge of the liquid handling characteristics of the LHS was the most important issue to remove obstacles associated with the operation of MCCs with a LHS.

Based on a detailed investigation of the pipetting performance and potential system liquid dilution effects, an efficient procedure for reproducible and precise elution buffer mixing was defined.

The theory on the principles of transferring gradient elution to different column scales, while maintaining peak resolution, was first applied on lab scale. Highly comparable separations of a well characterized ternary model protein solution, was first applied on lab scale. Highly comparable separations of a well characterized ternary model protein feed on columns with different bed length varying from 10 to 2 cm were achieved by making appropriate gradient slope and flow rate adjustments guided by the concept of Yamamoto. Findings from lab scale experiments were successfully utilized for creating a pseudo-linear gradient elution method for MCC operation on a LHS. The separation performance of 200 µL MCCs (1 cm bed height) and 600 µL MCCs (3 cm bed height) was shown for different gradient designs and fractionation schemes as well as elution flow velocities ranging from 25 to 300 cm/h. Gradient slopes varying by up to a factor of 8 in the range of 3.4–27.4 mM/cm were evaluated. The approach of using shallow gradients for improving peak resolution was verified. However, even with steep gradients a sufficient resolution of peaks was achieved offering the flexibility to perform resin screening at reduced method run time and buffer consumption.

Direct comparison of separations performed with 200 and 600 µL MCCs revealed little impact of column length on resolution. The decision for the right MCC format, though, should not be made on resolution requirements only, but also needs to take the analytical constraints into account. The larger quantity of sample material obtained from the 600 µL MCC format facilitates a comprehensive characterization by several analytical methods.

The analysis of elution salt concentration at the moment of protein peaks by means of a GH–I_R plot further confirmed the suitability of the pseudo-linear gradient elution method for predicting the salt concentration window for protein elution at different gradient conditions. Close resemblance between data derived from miniature column screening on an AKTA™ system and MCCs on a LHS was observed.

For the purpose of a basic resin selectivity screening which is applicable to (unknown) samples with components covering a range of binding strengths and which ensures undistorted, authentic peak patterns, a multistep gradient from 0 to 1 M salt concentration with 96 elution steps, with a minimum step length of 200 µL, and with collection of at least one fraction per elution step, provided sufficient resolution and adequate data point density.

The extensive data of this work give evidence for the reproducibility, consistency, and validity of the established high-throughput screening method. Great capacity for the enhancement of chromatography process development was demonstrated on the basis of a selectivity screening of four cation exchange resins at different pH conditions using a model protein feed. The suitability of the miniaturized approach with regard to the prediction of results on larger scale was further demonstrated using a practically relevant feedstock with a complex purification task: Comparative yield and purity data were achieved for an antibody monomer/high molecular weight separation on MCC scale with a LHS and on lab scale using an AKTA system. The specifics as well as the degree of complexity and refinement of the method should be defined as required by a given separation challenge or other process constraints.

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