

Matthias Wiendahl¹
Pierre Schulze Wierling²
Jacob Nielsen¹
Dorthe Fomsgaard
Christensen¹
Janus Krarup¹
Arne Staby¹
Jürgen Hubbuch³

Research Article

High Throughput Screening for the Design and Optimization of Chromatographic Processes – Miniaturization, Automation and Parallelization of Breakthrough and Elution Studies

¹ Protein Separation, Novo Nordisk A/S, Gentofte, Denmark.

² Bayer Technology Services GmbH, Leverkusen, Germany.

³ Institut für Bio- und Lebensmitteltechnik, Universität Karlsruhe, Karlsruhe, Germany.

This study evaluates the applicability of the liquid handling workstation Tecan Evo Freedom 200 and 200 μ L Media Scout RoboColumns to dynamic chromatographic operations such as frontal analysis (breakthrough) and elution experiments in a high throughput screening mode. Breakthrough experiments were conducted using BSA as a model protein and the stationary phases Poros 50 D, Q Ceramic HyperD and DEAE Sepharose FF. The obtained dynamic capacities at 10 and 50% breakthrough matched well with reference data. Elution experiments were performed applying three protein mixtures: a) lipolase and BSA, b) human growth hormone and a process-related impurity, and c) an insulin analogue and a process-related impurity. The resins used resembled a variety of different ion exchange resins. In all cases, the resulting elution curves matched well with reference measurements performed on an ÄKTA explorer system at 1 mL or 2 mL scale.

Keywords: Chromatography, Human growth hormone, Insulin analogue, Process development, Proteins

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1 Introduction

The production of complex molecules with the help of biological techniques has become a supporting pillar in the pharmaceutical industry. Biopharmaceuticals, i.e., recombinant therapeutic proteins, monoclonal antibody-based products used for in vivo medical purposes, and medical products based on nucleic acids, comprise one in every four new pharmaceuticals [1]. For these complexes, high-value products, downstream processing – the purification and formulation of the product – is responsible for the biggest share within production costs.

Monoclonal antibody production, which is based on a platform strategy with protein A as the first step and the heart of the process [2], is an excellent example of highlighting the cur-

rent dilemma of process development: despite the tremendous costs of the protein A step, an even broader use of this capture step is anticipated as a result of (1) high yields and purities within a single step, (2) well-documented reference processes, and (3) an accelerated process development due to the dominating performance of the protein A step. The last point, in particular – fast process development – has become increasingly important during the past years. ‘Time to market’ demands have increased the pressure on process development and an overall streamlined process development leading to ever shorter timelines from drug candidate screens to the launched product. Recent evaluations claim that each day a blockbuster drug is not released to the market, the company loses \$3 million [3].

The necessity of being ‘first on the market’ combined with the need to cut production costs highlights the demand for strategies and techniques allowing fast process development while at the same time covering a large number of potential process parameters in order to reach an optimal overall process performance. It is obvious that early process development sets the standards for subsequent process economy and thus the fo-

Correspondence: Prof. Dr. J. Hubbuch (Juergen.Hubbuch@ciw.uni-karlsruhe.de), Institut für Bio- und Lebensmitteltechnik, Universität Karlsruhe, Engler-Bunte-Ring 1, D-76131 Karlsruhe, Germany.

cus needs to be on this development stage [4]. The requirements to do so lie in the generation of a broad data basis describing process-relevant parameters, good process understanding and the development of high performance analytics. These requirements are subject to process inherent restrictions that sample material is available only in very low quantities at this early stage. The challenge thus lies in the need to create a large number of process relevant data sets with as little sample material as possible within as short a time frame as possible. A potential solution to this is to develop parallel, automated and miniaturized experimental setups and strategies. A technique that combines all these three characteristics is high throughput screening (HTS) using modern liquid handling stations (LHS) [5].

Current approaches to develop industrial chromatographic processes use sequential, experience-based iterative data generation which is mostly restricted to a few resin types. Some initial attempts to introduce 'low throughput' screening of chromatographic resins for a given task can be found in the literature [6–8]. An increase in sample throughput and thus data generation can be achieved by automation, parallelization and miniaturization. While the use of batch adsorption studies in HTS mode already meets these demands and is becoming a widely used technique [9,10], packed bed techniques are hardly available. To a certain degree, automation of chromatographic packed bed processes can be achieved by using programmable chromatography workstations such as the ÄKTA product family (GE Healthcare). As these workstations operate in sequential mode, the time needed for screening is, however, relatively long. Parallelization of preparative chromatographic steps on the other hand is not very widespread [11, 12]. Automation and parallelization of chromatographic operations usually do not affect the chromatographic process itself whereas miniaturization is known to have a significant impact on chromatographic resolution in particular due to packing problems and wall effects [13].

Until today, high-throughput adsorptive applications operated in a flow-through regime are restricted to either vacuum or centrifugal forces as a driving mechanism for fluid flow [14,15]. One of the reasons why especially chromatographic operations have proven difficult to establish on robotic LHS is the need for a constant, pressure-driven flow across the column. The latter cannot be provided by either of the traditional techniques and a positive driven flow application has only recently been reported [16]. Typically, chromatographic systems use screw threads to physically connect pump, tubing and column. This is, however, not feasible when using automated liquid handling systems where the piston pumps are connected to pipetting needles which have to be open at one end. A physical attachment to any reactor or column would significantly counteract this flexibility, and thus any attempt to connect the needles to chromatographic columns must be nonpermanent. In the present paper this system is described in detail and applied to industrially relevant separation problems. This study thus prepares the stage for work on packed bed chromatographic high throughput screening approaches and process development strategies [17, 18].

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Ethanol, Tris(hydroxymethyl)aminomethane (Tris), Bis-Tris-propane (B6755) and 2-(*N*-Morpholino)-ethanesulfonic acid (MES, M8250) were purchased from Sigma-Aldrich (Steinheim, Germany). Ethylenediamine tetracetic acid (EDTA, purum) was purchased from Fluka Bio Chemika (Buchs). All other chemicals were purchased from Merck (Darmstadt, Germany).

2.1.2 Proteins

Bovine serum albumin was purchased from Roth, Germany (> 98 %, fraction V). Human growth hormone (hGH), MEAE-hGH, Lipolase, an insulin analogue precursor and a modified insulin analogue precursor were kindly provided by Novo Nordisk A/S, Bagsværd, Denmark.

2.1.3 Chromatographic Resins

DEAE Sepharose FF was kindly donated by GE Healthcare (Uppsala, Sweden). Poros 50 D was kindly donated by Applied Biosystems (Foster City, USA). Q Ceramic HyperD was kindly donated by Ciphergen (Cergy-Saint-Christophe, France).

2.1.4 Chromatographic Columns

HR 5/100 columns were purchased from GE Healthcare (Uppsala, Sweden). 200 μ L Media Scout RoboColumns (microcolumns) were purchased from Atoll (Weinheim, Germany).

2.1.5 Liquid Handling Station

A Tecan Evo Freedom 200 (Tecan Crailsheim, Germany) liquid handling station (LHS) was used to perform the liquid handling station experiments. It was equipped with two liquid handling arms (LiHa) and one centric gripper, a centrifuge (Rotanta 46 RSC, Hettich, Kirchlingern, Germany) and an InfiniTe M200 photometer (Tecan Crailsheim, Germany). The LHS was controlled by the software package Evoware 1.4. The software used to control the photometer was Magellan (Tecan, Crailsheim, Germany; Version 6.0). The platform was additionally equipped with a carousel (LPX 220, Liconic, Mauren, Liechtenstein) for the storage of plates and a TE-Link (Tecan Crailsheim, Germany) for plate transport and fraction collection. The LiHas were capable of processing volumes of 0.25 μ L to 250 μ L or 1 μ L to 1000 μ L, respectively, depending on the dilutor syringes in use. Each LiHa consisted of eight separately controllable channels equipped with eight fixed tips. The detection of liquid surfaces was performed by the integrated liq-

uid level detection (LLD) function allowing parallel detection of liquid levels by all eight needles in parallel. This function is based on capacity measurements.

2.1.6 LHS Compatible Chromatography Columns

The LHS compatible microcolumns (see Fig. 1) carry a bed volume of 200 μL and bed dimensions of 1.1 cm length \times 0.5 cm I.D. The column inlet consists of a conical duct which directs the pipetting needle to the inlet of a microcolumn. A tight connection and seal between the column and the pipetting needle is ensured by an appropriate O-ring. The bed is compressed by using the conical duct and sealing section as a stamp pressing a frit on the top of the bed. This setup allows a reproducible packing of the bed, a tight but flexible linkage system between the pipetting needle and the column as well as the possibility of applying constant pressure-driven flow in the column.

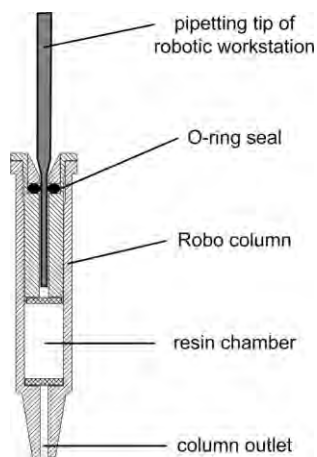


Figure 1. Schematic drawing of LHS compatible microcolumn and pipetting needle.

2.2 Methods

2.2.1 Preparation of Stock Solutions

Wet Lipolase crystals were dissolved in equilibration buffer to obtain a final concentration of 2 mg mL^{-1} . Insulin, hGH and MEAE-hGH were used as powders and were dissolved in the respective equilibration buffers.

2.2.2 Determination of Protein Concentration

The analysis of protein concentration by measuring UV absorption at 280 nm was performed in UV-MTP (360 μL , polystyrene, flat bottom). The volume present in single wells was either determined by a controlled dispensing step or measured by the liquid level detection (LLD) function integrated into the LHS. Absorbance measurements were performed by micro-

titer plate compatible photometer either 'online' using an InfiniTe M200 photometer (Tecan Crailsheim, Germany) or 'off-line' using a Cary 50-Bio UV-Vis photometer (Varian, Darmstadt, Germany).

2.2.3 Chromatography on LHS

The applied microcolumns were first introduced in a study on the elution of cell culture supernatant from different ion exchange resins [16]. A schematic drawing of such a microcolumn is presented in Fig. 1. Column inlets and outlets are not connected to tubes or capillaries thus enabling their use on a LHS. The chromatographic procedure consisting of equilibration, application, wash and elution was performed on a TECHrom module consisting of a 'Column Array Carrier' (CAC), in combination with a TECAN shuttle device (see Fig. 2). During the experiments presented in this work, 1000 μL and 250 μL syringes were used allowing an aspiration per stroke of 1000 μL or 250 μL , respectively. In order to aspirate larger volumes, e.g., for breakthrough curves, several strokes were performed sequentially. The range of flow rates which could be applied using this setup were 0.05 mL min^{-1} to 60 mL min^{-1} and 0.0125 mL min^{-1} to 15 mL min^{-1} for the 1000 μL and 250 μL syringes, respectively.

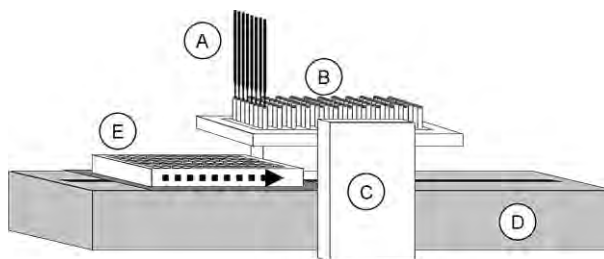


Figure 2. Tools used for the automation of parallel chromatography on the LHS. (A) pipetting tip of the LHS, (B) 96-column array provided by Atoll (Weingarten, Germany), (C) column array carrier (CAC) mounted on a (D) Te-Link module (TECAN Crailsheim, Germany), (E) microtiter plate in collect position.

Prior to any chromatographic experiments a column equilibration step was performed with the respective equilibration buffer. The equilibration step varied between 10 to 21 CV. Following this, a sample was applied by the respective needles. A detailed description of the volumes used for the different systems is given below.

Fraction collection was performed directly beneath the columns. Flowthrough exiting at the column outlet was collected in 96-well plates (see Fig. 2E) which moved automatically under the CAC by use of a plate shuttle (see Fig. 2D). The smallest fraction size to be collected within this setup was determined by the size of the droplets leaving the columns. The typical droplet size measured in this study was approximately 25 μL . This value can, however, not be seen as a constant parameter as it depends on column outlet geometry, flow rate and buffer composition, i.e., the interfacial tension of the fluid.

Depending on the number of droplets to be collected per fraction, different types of microtiter plates (UV-MTPs or half size UV-MTPs) were used in order to increase the liquid level height in the wells and thus to obtain a sufficient signal when the plate is read out in the photometer. During the application of the different feedstocks fractions of 100 μL (for samples with a loading concentration of 9 mg mL^{-1}) or 350 μL (for samples with a loading concentration of 1.5 mg mL^{-1}) were collected in a 150 μL half size 96-well UV microtiter plate or a 360 μL 96-well UV-MTP, respectively. After collecting 12 fractions for each column, the plate was covered with a lid and subsequently exchanged by a new plate. During this procedure sample application was halted.

Due to well-to-well deviations caused by variations in the number of droplets collected per fraction, LLD was performed in order to correct later analysis, i.e., absorption values were linearly correlated to the light path and to calculate the true fraction volumes. This procedure showed to be a necessary prerequisite to reduce experimental noise.

The complete procedure of using the microcolumns in connection with the pipetting capabilities of the LHS is shown in Fig. 3 for breakthrough and elution studies. While column equilibration and wash as well as feed application can be performed according to the known principles, gradient elution is more complex. Since each column is connected to a single pump via the pipetting needle, a linear gradient must be mimicked by a series of small steps. The buffer solutions required

for these steps must be prepared prior to an experimental run in order to minimize the time required to apply different steps during the gradient.

2.2.3.1 Breakthrough Studies

Breakthrough studies were conducted with a solution containing 9 mg mL^{-1} BSA in 25 mM Bis/Tris propane buffer, pH 8 and different ion exchange resins. For experiments with Poros 50 D experiments were repeated with concentrations of 1.5 mg mL^{-1} . The flow rates used are given in Tab. 1. Equilibration was performed with 15 CV of 25 mM Bis/Tris propane buffer, pH 8. The applied volume of 4 mL could be aspirated in one step into the tubing of the LHS and subsequently applied to the microcolumns.

2.2.3.2 Elution Studies

Separation of BSA and Lipolase

The separation of a BSA/Lipolase mixture was evaluated using DEAE Sepharose FF. Equilibration was conducted with 50 mM Bis/Tris propane buffer, pH 8 for 10 CV. The flow rate used was 1.66 CV min^{-1} equivalent to linear flow rates of 1.1 m h^{-1} for the LHS runs and 5.5 m h^{-1} for lab-scale experiments. The applied protein solution consisted of 2 mg mL^{-1} BSA and 2 mg mL^{-1} Lipolase in 50 mM Bis/Tris propane buffer, pH 8. Sample load in all scales was 20 mg protein per-mL of resin. Elution was carried out in a complex gradient: The

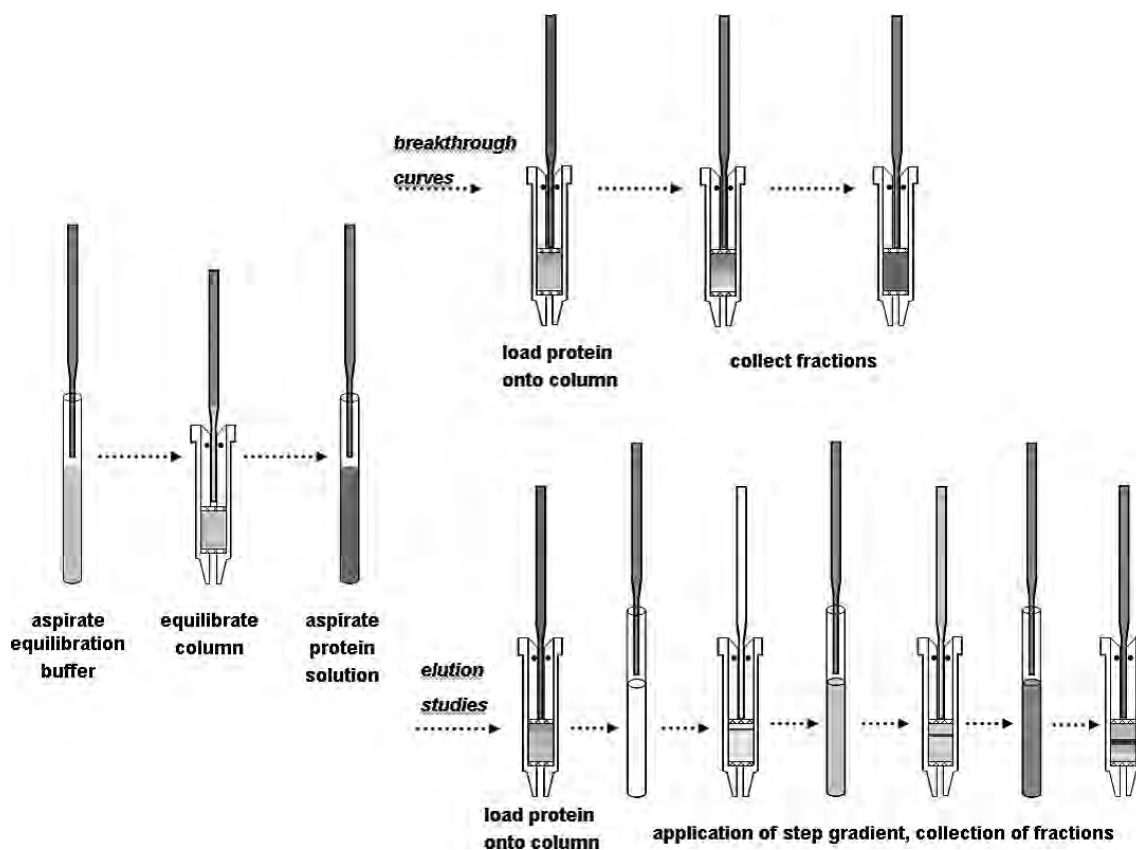


Figure 3. Schematic overview of chromatography experiments using the LHS.

Table 1. Capacities at 10% (Q10) and 50% (Q50) of breakthrough capacity; standard deviation of capacities, flow rates and comparison with published data of breakthrough experiments with BSA (9 mg mL⁻¹ in 25 mM BIS/Tris propane buffer, pH 8) on miniaturized columns filled with different resin materials.

Resin	Flow rate [CV min ⁻¹]	Capacity [mg mL _{resin} ⁻¹]	Standard deviation [-]	Capacity* [mg mL _{resin} ⁻¹]	Deviation from literature value* [%]	
DEAE Sepharose FF**	0.3	Q10	65.5	5.3	59	11
		Q50	88.9	6.0	70	27
	1	Q10	52.6	14.7	52	1
		Q50	79.2	10.2	66	20
Poros 50 D**	1	Q10	127.6	7.8	77	66
		Q50	139.7	4.2	96	45
	4	Q10	123.3	3.8	68	81
		Q50	137.8	3.5	92	50
Poros 50 D***	1	Q10	83.8	3.5	77	9
		Q50	92.6	1.5	96	-4
	4	Q10	85.3	2.2	68	25
		Q50	92.5	3.6	92	1
Q Ceramic HyperD**	1	Q10	79.9	1.2	85	-6
		Q50	86.1	2.2	87	-1
	6	Q10	71.0	4.1	79	-10
		Q50	77.2	4.1	81	-5

* from [13]

** c_0 (BSA) = 9 mg mL⁻¹*** c_0 (BSA) = 1.5 mg mL⁻¹

first gradient consisted of 50 mM Bis/Tris propane buffer, pH 8 containing 0 mM to 40 mM NaCl over a length of 91 CV. Following this, the gradient slope was increased covering the salt range 40 mM to 175 mM NaCl over a length of 15 CV. A final plateau using 175 mM NaCl over 5 CV completed the gradient. Eight identical runs were performed in parallel.

Separation of human growth hormone (hGH) and a precursor (MEAE-hGH)

For the separation of MEAE-hGH and hGH Q Ceramic HyperD and Poros 50 D were used as stationary phases. Columns were equilibrated with 10 mM MES, pH 6 for 21 CV. The applied protein solution consisted of 0.25 CV containing 0.5 mg mL⁻¹ hGH and 0.5 mg mL⁻¹ MEAE-hGH in desalted water. Elution was performed with 10 mM MES, pH 6 as base buffer and an increasing salt gradient ranging from 0 mM to 200 mM. Salts used for gradient generation were: a) sodium chloride, b) sodium phosphate and c) sodium sulfate. The average volume of collected fractions was 139 μ L. Columns were regenerated by 5 CV 1 M NaOH and 10 CV 100 mM MES, pH 6. For Poros 50 D and Q Ceramic HyperD, flow rates of 2 CV min⁻¹ equivalent to linear flow rates of 1.32 m h⁻¹ for LHS runs and 6.6 m h⁻¹ for lab-scale experiments were applied. Experiments were performed in duplicates.

Separation of insulin precursor and a related contaminant

For the separation of insulin precursor and process relevant contaminants a comparison of retention volumes and resolution for different system was analyzed. A representative example is given here using data on Porous 50 D. When examining the performance of Poros 50 D the equilibration buffer contained Tris and ethanol at neutral pH. The elution buffer additionally contained sodium acetate for conductivity-based desorption. Equilibration was performed using 6 CV equilibration buffer followed by a sample application of 1.66 CV. The wash step was performed with 2 CV on the LHS and 4 CV at lab scale. Elution was performed with a gradient from 2.5% to 80% elution buffer in equilibration buffer. Equilibration, sample application and elution were conducted at a flow rate of 0.25 CV min⁻¹ and 0.17 CV min⁻¹ equivalent to linear flow rates of 0.165 m h⁻¹ and 0.11 m h⁻¹ for LHS runs and 0.275 m h⁻¹ and 0.55 m h⁻¹ for lab-scale experiments. Four runs were performed in parallel.

2.2.4 Lab-scale Chromatography

In analogy to the chromatographic separations on the LHS, identical experiments were performed using an ÄKTA Explorer

system. HR 5/100 columns (GE Healthcare, Uppsala, Sweden) were packed with the respective resins to a volume of 1 mL for hGH and insulin experiments and 2 mL for the BSA/Lipolase experiment. All columns were packed according to supplier's instructions. Feedstock samples were applied via a 500 μL sample loop for the experiments where microcolumns were connected to the ÄKTA system and via a 2 mL sample loop for the experiments with the HR 5/100 columns. Linear gradients applied resembled the step gradients on the LHS in length, slope and starting concentrations.

3 Results and Discussion

While automation has been an issue for some while in chromatographic hardware development, experimentation was mostly restricted to sequential single column procedures. This restriction was closely linked to the necessary prerequisites of chromatographic parallelization, namely, a system with multiple pumps, two dimensional fraction collectors and the potential to analyze the obtained fractions. Modern LHS offer all the requirements necessary for such an approach: in general, eight independent pumping systems, the potential to handle a large amount of multiwell formats, operations such as shaking, incubation, vacuum filtration and centrifugation are automated and finally, a wide range of photometric devices can be incorporated. This integration of experimental procedure, sample preparation and complex analytical assays (i.e., enzymatic reactions, ELISA, etc.) offers a new level of automation when compared to existing automated chromatographic devices. The only missing parts to date were miniaturized columns allowing a tight but flexible linkage of the LHS pipetting needles and a module for operation of these column arrays on the LHS. Figs. 1, 2 and 3 present schematic drawings of the solution developed in this study. In the following, initial studies on the applicability of this technology to an evaluation of chromatographic operations are shown.

3.1 Chromatographic Breakthrough Studies

The breakthrough behavior of a target molecule for a given chromatographic system is a common indicator for the feasibility of this system concerning capacity and process time needed. In the absence of detailed chromatographic models, this procedure is currently the industrial method of choice when evaluating different systems. Dynamic breakthrough data can further be used for scale-up considerations and the design and optimization of the successive elution step.

To evaluate the feasibility of the proposed method, the breakthrough behavior of bovine serum albumin (BSA) on several ion-exchange resins was analyzed. Fig. 4 shows normalized breakthrough data when challenging DEAE Sepharose FF (A), Poros 50 D (B) and Q Ceramic HyperD (C) at high and low flow rates with a BSA solution of 9 mg/mL. Details are given in Tab. 1. Only for Poros 50 D a second measurement with an initial protein concentration of 1.5 mg mL⁻¹ was per-

formed (D). Error bars represent standard deviations arising from three to eight parallel experiments.

The data obtained show a slight decrease in absorption after every twelfth sample. This decrease originates from the exchange of fraction collection plates as described above. In general, experimental noise is within an acceptable range throughout the chromatographic procedure. Data scattering increases, however, significantly for high protein concentrations in the effluent stream. The reason for this behavior is the asymptotic shape of the UV calibration curve as the initial protein concentration is outside the linear range of the calibration curve. A reduction of scatter would thus be possible by diluting the respective samples in order to measure within the linear range of the calibration curve. This information, however, is of limited industrial relevance. When performing flowthrough experiments total capacity is not as important as dynamic capacity; consequently no dilution was performed for the current systems.

Qualitatively, a clear difference between absorptions measured at high and low flow rates can be stated for DEAE Sepharose FF. For Q Ceramic HyperD, the influence of flow rate was less pronounced, while for Poros 50 D hardly any difference was seen at all. Q Ceramic HyperD and Poros 50 D are rigid resins specifically designed for use at high flow rates tolerating 200 bar (Q Ceramic HyperD) or 100 bar (Poros 50 D), respectively. For these two resins, very little differences between dynamic capacities at low and high flow rates have been reported previously [19, 20].

Tab. 1 depicts dynamic capacities at 10% and 50% of the maximum capacity as well as standard deviations measured when comparing different experimental runs. The values are compared with literature data using packed beds of 2 mL and initial BSA concentrations of 1.5 mg mL⁻¹ [19]. Except for data obtained with DEAE Sepharose and Poros 50 D experiments with an initial concentration of 9 mg mL⁻¹, the deviations between LHS values and literature data are low. This underlines the suitability of the method to screen for dynamic capacities. The necessity of a plate exchange after every twelfth sample and thus the increased residence time does not seem to have a significant effect on dynamic breakthrough capacity. Furthermore, the method is reproducible as shown by the relatively low standard deviations expressing a mean relative standard deviation σ_R of 4.9%.

Initial breakthrough curves with a BSA concentration of 9 mg mL⁻¹ on Poros 50 D showed large deviations (> 45%) to literature data recorded for 1.5 mg mL⁻¹. When repeating the procedure with a reduced concentration of 1.5 mg mL⁻¹ good agreement with published data could be obtained where three systems showed deviations of less than 10% and only one system was measured to 25%. The impact of the initial feed concentration for Poros 50 D might be due to the position of the initial concentrations on the isotherm: at 1.5 mg mL⁻¹ the isotherm is still in the linear regime, while for 9 mg mL⁻¹ the saturation regime of the isotherm should be reached. This theory is supported by the media supplier [21] who claims saturation capacities for BSA of > 100 mg mL⁻¹.

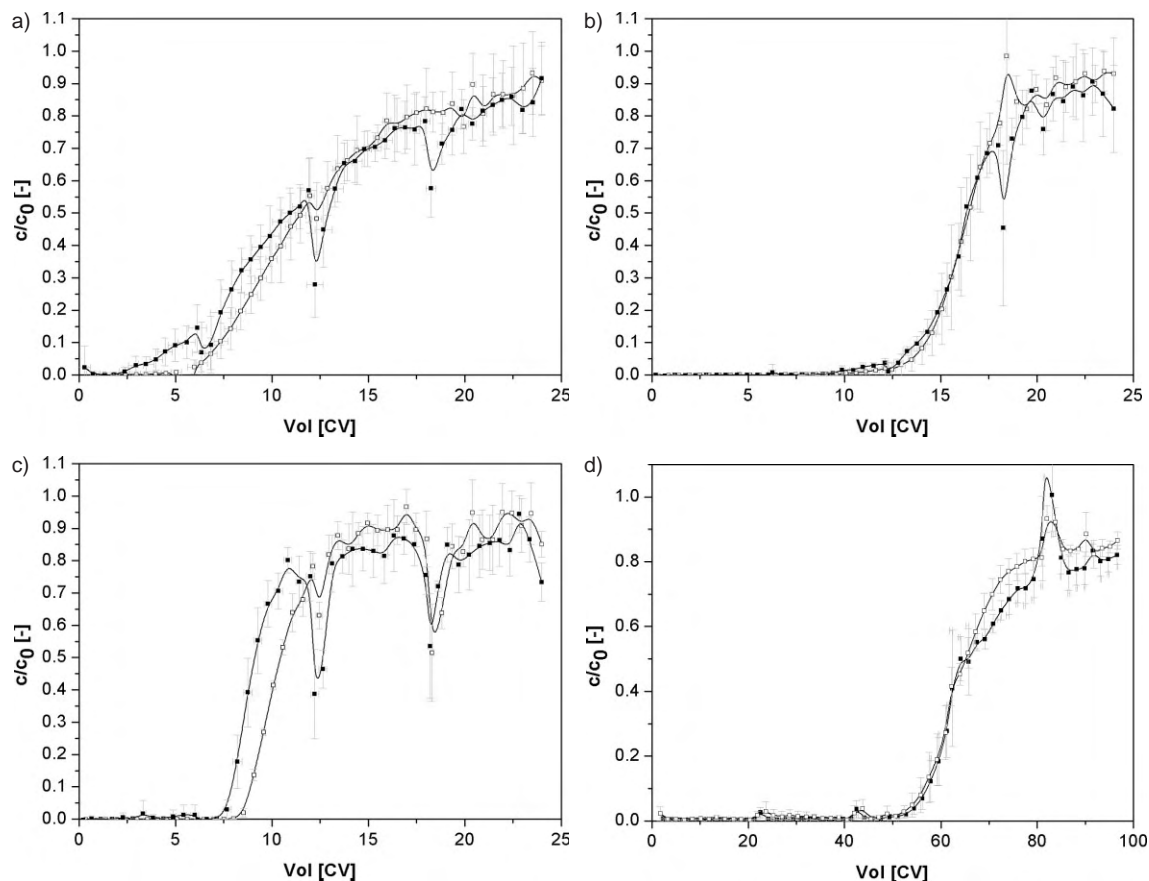


Figure 4. Breakthrough curves for BSA (9 mg mL^{-1} in 25 mM BIS/Tris propane buffer, pH 8) on different micocolumns at \square low and \blacksquare high flow rate. (A) DEAE Sepharose FF, (B) Poros 50 D, (C) Q Ceramic HyperD, (D) Poros 50 D at 1.5 mg mL^{-1} BSA initial protein concentration. Error bars correspond to standard deviations.

3.2 Elution Studies

Gradient elution is one of the most widespread chromatographic operations when high resolution is required. Typical applications lie in the use of step and linear gradients. The realization of step gradients is generally simple and does not require any additional features when applying these gradients on a LHS. Linear gradients, however, are in general technically based on in-line mixing and thus two pumps per column would be needed. This is to date not feasible on a LHS. The solution might thus lie in mimicking linear gradients by a number of small gradient steps. This procedure naturally leads to alterations in residence time when compared to in-line gradient mixing of larger systems. The impact of the latter, but also the ability to screen for elution conditions with miniaturized columns is the major theme in the following experiments.

3.2.1 Separation of BSA and Lipolase

BSA and lipolase were used as a model system to evaluate model based ‘in-silico’ process development in combination

with parameter generation on a LHS [19]. The model employed was built on the SMA methodology by Brooks and Cramer [22]. Based on the results obtained, the experimental validation of the separation process was carried out using a 1 mL column on the Äkta and the respective miniaturized column on the LHS.

Fig. 5 shows the chromatogram resulting from the LHS experiment (black dotted curve) and the same experiment conducted on an ÄKTA system with a 1 mL column (black continuous curve). The graph also contains the measured conductivity in the ÄKTA experiment (grey continuous line) and the gradient concentrations set for the experiment conducted on the LHS (grey dotted line). Both, conductivity measured on the Äkta system and experimental mimic of this gradient on the LHS superimpose well.

To ease the comparison of the two chromatograms, all absorption values measured with the flow cell on the Äkta system (light path 1 mm) were recalculated to match the theoretical light path of a $100 \mu\text{L}$ system obtained on the LHS. Due to fluctuations in the fraction volume all absorption values determined on the LHS were adjusted to the absorption value which would be obtained at a fraction volume of $100 \mu\text{L}$ (see section 3.1).

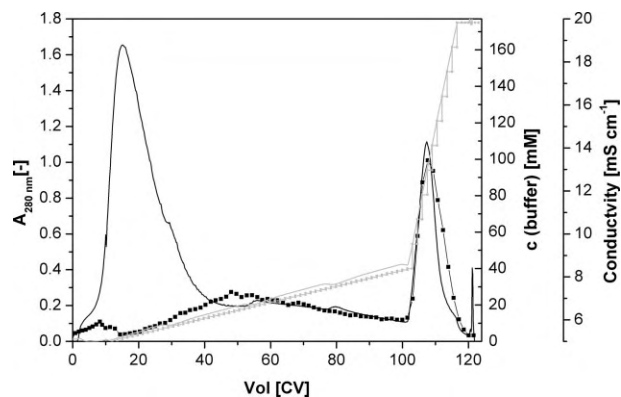


Figure 5. Separation of BSA and Lipolase on DEAE Sepharose in a complex gradient. — absorption measured during lab-scale experiment, -■- absorption measured during experiment conducted on the LHS (mean value from seven runs), — conductivity measured during lab-scale experiment, -■- step gradient applied during experiment conducted on the LHS.

The chromatogram shown in Fig. 5 can be divided into three stages. A sample application phase of 10 CV is followed by a wash phase of 4 CV and the elution gradient of 110 CV. Three peaks can be distinguished: The first peak is the peak consisting of molecules for which the resin is already saturated under the respective conditions. This peak covers the sample application and wash step and it mostly consists of Lipolase. The second peak (after approximately 50 to 60 CV) consists of Lipolase adsorbed on the resin during feed application and eluted in the gradient. The third peak, occurring after 107 CV, represents BSA eluting from the column. A rather large deviation between the two runs was obtained at the beginning of the procedure. During lab-scale experimentation a large flow-through peak occurred which could not be detected in the data of the LHS-based experiment. This might originate from a higher Lipolase concentration in the feed for the lab-scale experiment when compared to the LHS-based experiment. Solutions were prepared by dissolving wet Lipolase crystals in equilibration buffer prior to the experiment. This procedure is prone to add errors in the adjustment of defined concentrations. During the elution phase, however, the two curves match almost perfectly: The ratio of the Lipolase elution peak to the BSA elution peak and the elution times of the peak maxima are almost identical. The step gradient applied to the column seems to be sufficiently fine to resemble the linear gradient applied to the column via the ÄKTA system.

3.2.2 Separation of hGH and MEAE-hGH

Human growth hormone (hGH) is a hormone that was originally applied to the treatment of abnormally small children. Nowadays, it is further used for eleven indications including the Turner syndrome, chronic renal failure or burns and it has got an annual market value of US\$ 2.6 billion in 2004 [23]. MEAE-hGH is an hGH precursor which is extended by four

amino acids (methionine, glutamine, alanine, glutamine) at the N-terminal end compared with hGH [24].

Separation of hGH and a spiked sample of the process-related precursor MEAE-hGH is challenging because apart from the structural similarity, the separation protocol is based on the application of a low sample amount onto the column. Thus, by using this system the developed LHS method could be challenged at the lower limit of detection of the photometer. A separation screen on the LHS for human growth hormone and the related contaminant MEAE-hGH was carried out for Q Ceramic HyperD and Poros 50 D as chromatographic adsorbents with sodium sulfate, phosphate and chloride salt as elution agents. During this screen a separation between hGH and MEAE-hGH could only be achieved using sodium chloride and phosphate as elution salts. The system combining the adsorbents Poros 50 D (see Fig. 6A) and Q Ceramic HyperD (see Fig. 6B) with sodium chloride as elution salt was further verified by monitoring the chromatograms with three different system setups. Fig. 6 shows a comparison of the resulting chromatograms using microcolumns and a HR 5/100 column connected to an ÄKTA system (A1, B1) and microcolumns operated on a LHS (A2, B2). In all cases two peaks could be determined in the UV 280 signal during elution. Chromatograms where phosphate was used as elution salt were also determined, resulting, however, in a poor resolution (data not shown). Baseline or near-baseline separation was achieved with the HR 5/100 column packed with Q Ceramic HyperD. On the miniaturized columns, two peaks could be separated using either the LHS or ÄKTA system but complete baseline could not be achieved. Nevertheless, the UV curve shapes match very well for the three different experiments conducted with the same resin. The same holds true for the retention volumes: for the minor and the major peak the retention volumes superimpose well for all experiments performed.

The UV signal recorded with miniaturized columns shows significant tailing when connected to the LHS: a behavior not so prominent when connected to the ÄKTA system. Comparing Figs. 5 and 6 reveals that column tailing seems to be prominent only at low column efflux concentrations. A potential reason for this behavior might be a slight change of the liquid meniscus due to an increase in salt concentration which causes a higher interfacial tension. A higher liquid meniscus results in an increased light path during UV measurements and thus a general increase of the UV signal. This might be the reason why the UV signal does not approach a baseline value at the end of the chromatographic run. However, despite a shift in retention volume, the shapes of the chromatogram and the separation results measured for each resin using the three system setups match very well.

In Fig. 7 the influence of salt type on the elution sequence is shown using Poros 50 D (A) and Q Ceramic HyperD (B) as adsorbent matrix. Sodium sulfate, phosphate and chloride were used for elution. For phosphate a significant shift in retention time between the two resins could be observed. While on Poros 50 D the peaks elute after 9 and 11 CV, the retention volume is 10 and 18 CV on the Q Ceramic HyperD resin. For sodium chloride, the retention volume of the two peaks is the longest with 14 and 23 CV for both resins. This is easily ex-

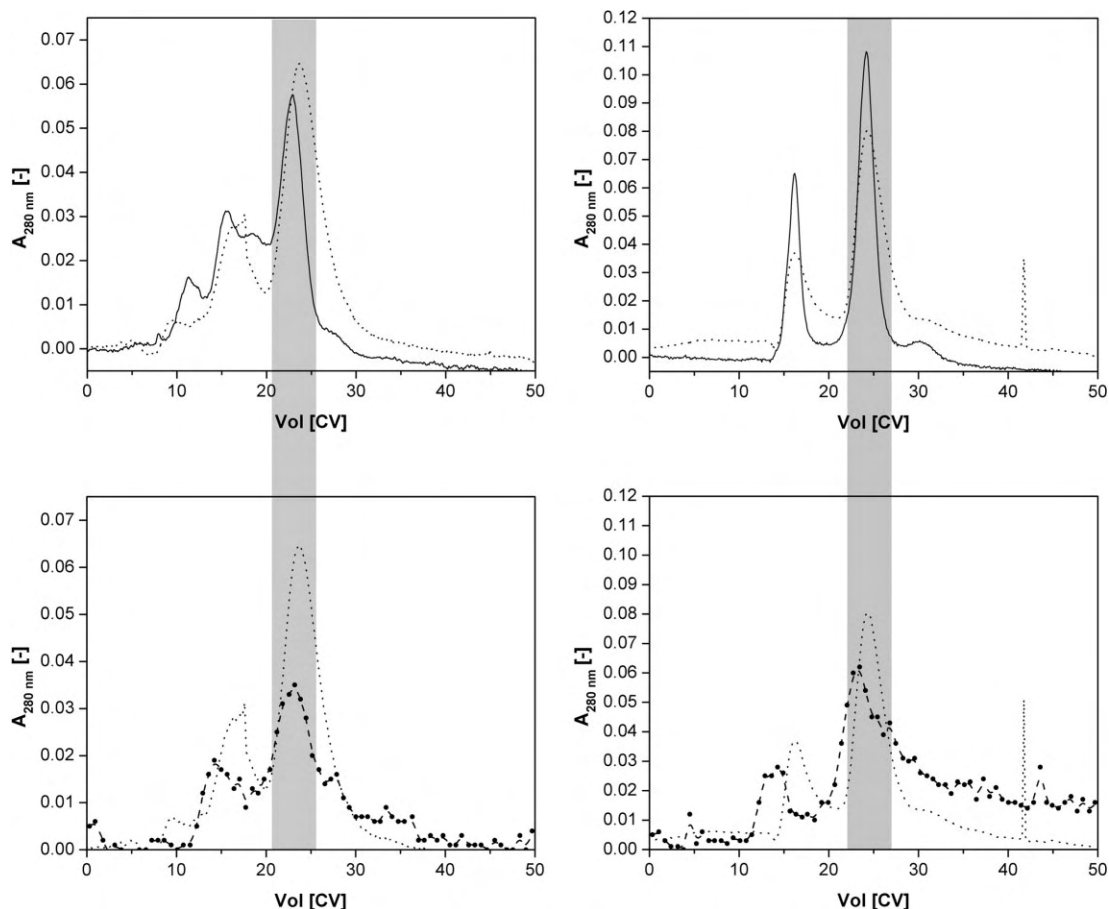


Figure 6. Separation of hGH and MEAE-hGH on microcolumns filled with Poros 50 D (A) or Q Ceramic HyperD (B) using sodium chloride as elution salt. A1, B1: ... UV 280 signal measured with miniaturized column connected to ÄKTA system, — UV 280 signal measured with HR 5/100 column connected to ÄKTA system; A2, B2: ... UV 280 signal measured with miniaturized column connected to ÄKTA system, -●- UV 280 signal measured with miniaturized column connected to the LHS; ■ position of second, major peak.

plained by the lower valency of the salt compared to the two other salts. When sodium sulfate is used for elution, no peak separation is observed. The only elution peak detected occurs after 10 CV.

3.2.3 Separation of Insulin Analogue Precursor and a Related Contaminant

The separation between the insulin analogue precursor and a process relevant modified form of the precursor was examined due to its industrial importance – human insulin was estimated to have an annual market of US\$ 4 billion [23] – and the rather complex separation task of two structurally very similar proteins. A representative example of gradient elution analysis of this system is given below using data on Poros 50 D at an elution flow rate of 15 CV h^{-1} (see Fig. 8).

Two major peaks can be distinguished: The first peak, eluting after 13 CV, represents the insulin peak whereas the second smaller peak, eluting after 21 CV, represents the contaminant peak. For Poros 50 D the insulin peak shows a significant

shoulder. A clear difference can be seen between the resins examined. The peaks eluting from Poros 50 D were much broader than the peaks eluting from finer, more regular adsorbents (data not shown).

The most important issue when miniaturizing chromatography is the effect of different scales and experimental systems on resolution. Fig. 9 shows the chromatograms resulting from the separation of the insulin analogue precursor and the contaminant on Poros 50 D at a flow rate of 15 CV h^{-1} using microcolumns operated on a LHS, microcolumns connected to the ÄKTA system as well as a reference chromatogram of a 1 mL HR 5/100 column connected to an ÄKTA system.

In all three chromatograms, again a first smaller peak can be distinguished from the main insulin peak. The retention volumes of the main peaks match reasonably well; only the first peak is significantly bigger for the run performed with the HR 5/100 column. On the other hand, peak shape and resolution potential fit well between the runs performed with the miniaturized column on the LHS (see Fig. 8A) and on the ÄKTA, demonstrating the successful transfer from the standard laboratory results to LHS-based results.

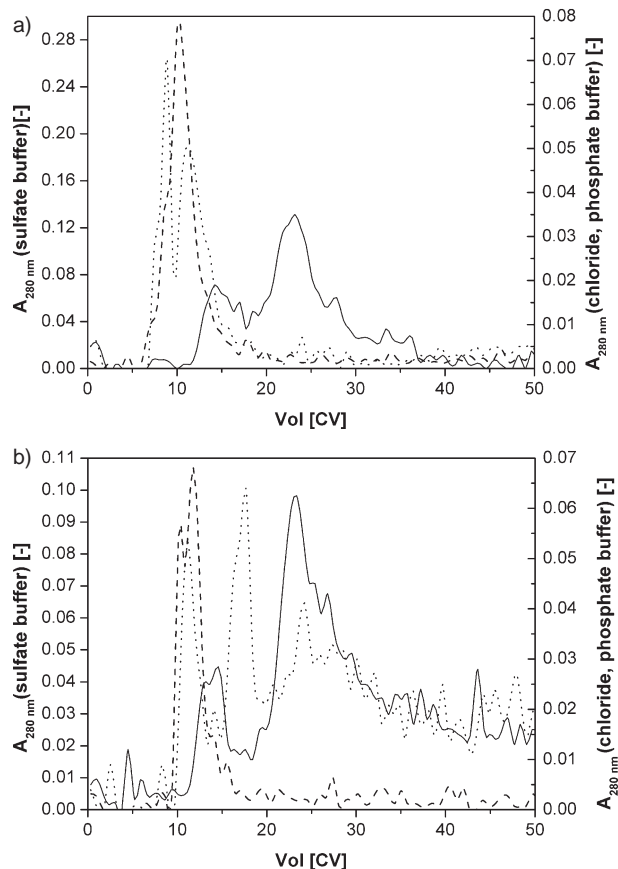


Figure 7. Separation of hGH and MEAE-hGH on microcolumns filled with Poros 50 D (A) or Q Ceramic HyperD (B) with different elution salts. — sodium sulfate as elution salt, ... sodium phosphate as elution salt, - - - sodium chloride as elution salt.

4 Summary

A chromatographic system comprising LHS compatible microcolumns, a column array carrier (CAC) for the usage of these columns and a methodology to collect fractions of the processing fluid leaving the columns has been developed, established and validated. Dynamic breakthrough curves obtained for several ion-exchange resins showed good reproducibility and good congruence with lab-scale data. Elution capabilities have been evaluated using different resins, buffer systems and industrially relevant systems exhibiting structurally similar molecules and the challenge of low protein concentrations. While both, simple and complex gradients have been used throughout the experiments, a close resemblance of lab-scale separations could be achieved. All in all, process relevant screening for elution conditions has been shown feasible. While baseline separation might not be achieved with miniaturized columns, the results obtained allow a conclusion on which system

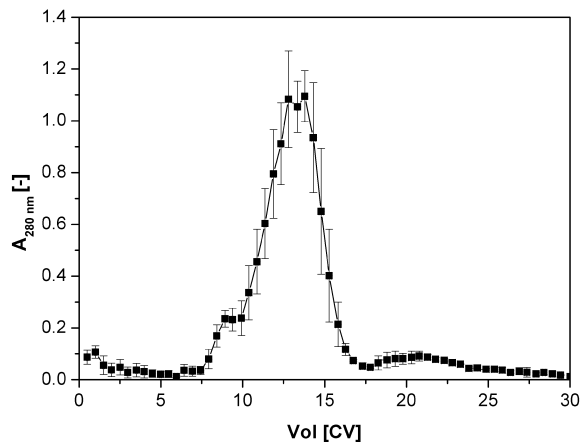


Figure 8. Separation of an insulin analogue precursor and a related contaminant using the robotic system at a flow rate of 15 CV h^{-1} (equivalent to 9.9 m h^{-1}). Microcolumns were filled with Poros 50 D (A). Error bars correspond to standard deviations.

would be a primary candidate for lab or pilot scale processes. Taking into account that the LHS offers the possibility to determine eight breakthrough curves or elution profiles in parallel, the increased sample throughput becomes more than obvious.

When considering future developments modern LHS platforms in combination with adequately miniaturized systems promise a significant progress towards a rapid, directed and optimized process development in the biopharmaceutical industry. A major issue in the coming years will be the use of statistical and mathematical methods for 'intelligent' parameter generation, either to determine an optimum system point or to aid model-based development [17–19]. The integration of more sophisticated analytical methods such as enzymatic as-

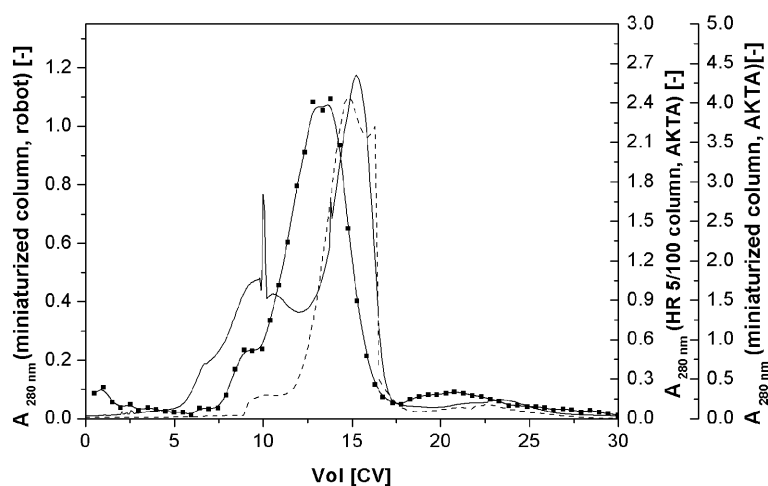


Figure 9. Comparison of insulin analogue precursor/contaminant-separations on Poros 50 D at flow rates of 15 CV h^{-1} . -■- microcolumn operated on the LHS, — miniaturized column connected to ÄKTA system, - - - HR 5/100 column connected to ÄKTA system.

says, ELISA or LC/MS techniques is generally possible and can be expected to be integrated into LHS based screening processes [16].

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