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From the conference chairs

Following the success of the first conference on high-throughput process development (HTPD) that was held in Kraków, Poland, in 2010, we decided to entertain the idea of organizing a second conference, also exclusively devoted to HTPD. The decision was actually fairly easy. Both the HTPD 2010 survey and comments from industry experts clearly showed the need for a scientific conference series with the goal of establishing a forum for discussions and exchange of ideas surrounding the challenges and benefits of employing high-throughput techniques in the development of production processes for biological products. Following suggestions from the survey, we decided to expand the conference program to cover upstream processing and protein formulations, in addition to downstream processing. We also followed a unanimous request to continue organizing HTPD conferences at UNESCO World Heritage sites. The outcome became HTPD 2012, held in Avignon, France.

The conference program for HTPD 2012 included a quality by design (QbD) workshop, a keynote lecture, from Professor Jürgen Hubbuch, on the current and future developments in HTPD, two case study sessions devoted to downstream processing, one session focused on QbD, one session devoted to upstream process development, and one session where the application of high-throughput techniques in protein formulations was discussed. In total, 29 oral presentations were delivered and 25 posters were presented. Additionally, following the great example from the first conference, a morning panel discussion offered an opportunity to discuss common challenges encountered in executing HTPD experiments.

This extended abstract book captures some of the presentations from this very exciting conference. We hope that this book will serve as a resource and summary of the first-rate talks and discussions, as well as encourage you to participate in the next meeting in the HTPD conference series, a series that already has established itself as a leading forum within its field.

Our thanks go the session chairs for their efforts in putting together excellent sessions, the presenters for their contributions, and the participants for making this a truly valuable and enjoyable event. We also would like to thank the conference sponsors.

Looking forward to seeing you at HTPD 2014.

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Launching an automated platform for enhanced bioprocess optimization

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Within bioprocess development, many biological and bioengineering parameters influence the expression and secretion of a protein of interest, for example, promoter strength and induction strategy, fused signal peptides, temperature, different substrates and feeding profiles, as well as the protein and expression host itself. This leads to a high number of possible parameter combinations to be tested for each new target protein de-novo. To manage the high cultivation effort, miniaturized, but scalable, parallel fermentation systems, called microbioreactors (MBR), with increased throughput became an important field of research during the last decade. The key aspects of MBR are parallelization, miniaturization, and online-detection of key parameters like pH, pO₂, and biomass (1), bridging high-throughput techniques and high controlled conditions in bioreactors for an easy and fast upstream bioprocess development. The bottleneck of such parallel cultivation experiments is often the high experimental effort of peripheral procedures like media preparation, sample handling, and offline protein analytics. Thus, a speed-up of experimental work can be achieved with integration of MBR into an automated liquid-handling platform.

Fig 1. An automated microbioreactor set-up, enabling a basic workflow for upstream bioprocessing. The overhanging rail extension on the right side of the JANUS™ Integrator allows the interaction of the pipetting arm and the microplate cultivation system BioLector, enabling triggered sampling and dosing events.
Automated microbioreactor set-up
In our work, we have implemented the microplate-based cultivation system BioLector™ (2), within a liquid-handling robotic set-up. The system was extended with a centrifuge, cooling devices, a plate reader, and a surrounding sterile enclosure, enabling the whole basic microbial workflow (Fig 1). Besides water, a second disinfectant robotic system fluid facilitates the fast decontamination of the eight steel needles, after pipetting cell suspensions. Although the set-up could be universally applied to microbial bioprocess development, the optimization of microbial protein production was one of the primary targets.

Automated media preparation
Optimization of media component concentrations is a typical task during the upstream part of bioprocess development. To identify optimal media composition, we used an iterative approach using a branch-and-bound optimization algorithm as a framework, together with the automated media preparation on the robotic deck. The standard minimal media CG XII was used as reference. Most strikingly, we found that by leaving out three salt components, the biomass-specific green fluorescent protein (GFP) and cutinase productivity could be increased with about a factor 1.5.

Enhanced reliability in strain selection and process parameter optimization
As a key functionality, the BioLector online measurements can be used as a trigger signal for the robot’s individual sampling or dosing events during 48 running cultivations (3). These triggered sampling events were used to select recombinant Corynebacterium glutamicum strains producing and secreting cutinase from Fusarium solani pisi. The secretion efficiency is strongly dependent on the combination of protein and the fused signal peptide, making a de-novo screening of signal peptides necessary with each new target protein (4). Normally, cultivation in microplates (MP) is limited by the small cultivation volume that limits the sampling to a single harvesting event. The easiest and most common sampling procedure for MP is the synchronous harvest of all cultivations at the end of the stationary growth phase. One drawback of this method is the different lengths of stationary phases caused by non-parallel growth characteristics, which can lead to unfair comparison between fast and slow growing cultivations. One possibility is to harmonize the growth patterns using glucose-release media (5). Another alternative for harmonization is to sample at defined time points based on the growth phases of each single cultivation, which is discussed here.

To establish a screening method, three signal peptides, with high, mid, and low secretion efficiency, were used as reference constructs for cutinase secretion. Three approaches were compared regarding the statistical robustness with 12 biological replicates (Fig 2): (I), the classic approach of synchronous sampling in the stationary phase, (II), individual sampling in the early stationary phase based on a biomass trigger, and (III), individual, biomass triggered induction in the early exponential phase together with triggered sampling as described in (II). Samples were stored at 4°C on the robotic deck until all cultivations were sampled. The stored samples were centrifuged and lipolytic activity in the supernatant was carried out with a pNPP-based assay according to Winkler and Stuckmann (6).

The resulting mean cutinase activities of the three approaches showed a similar range, but the variance of the values was significantly different depending on the sampling approach (Fig 2). While the classic synchronous sampling (approach I) led to a relative standard deviation of 30%, the triggered procedures led to a much lower statistical error of 10%, leading to a more reliable strain characterization and strain selection in early bioprocess development of secretory protein production.

The influence of other process parameters, like media and temperature, on the ranking of 32 signal peptides was investigated. While the results show no drastic difference, when cultivated in minimal CG XII media or complex BHI, the ranking of signal peptide
performance was drastically disturbed when the temperature was lowered from 30°C to 23°C (data not shown), strongly pointing to the close connection between biological and bioprocess parameters. Furthermore, automated methods were established, such as media preparation, induction optimization, and feed rate screening. The combination between a robotic station and the BioLector enables the induction optimization with 48 different induction time/IPTG concentrations in only one MP (Fig 3). Here, GFP and cutinase were investigated with this optimization method, showing a clear difference of the optimum.

**Conclusion**

The combination of MP-based microbioreactors and robotic systems enable the successful optimization of bioprocess development, as shown for microbial cutinase production. Such bioprocess optimization is often done in a step-by-step approach (Fig 4), but the results clearly indicate that there is a strong dependency between the results of the different steps. Consequently, when parameter settings are changed after signal peptide screening in a bioprocess optimization, this could favor another signal peptide. Thus, the identified optimum will not be the global optimum, clearly demonstrating the demand for information rich, high-throughput bioprocess development.

**References**

Whenever our immune system is not able to detect malignant mutations, we lose an important protective shield and cancer cells can proliferate uncontrolled. This is the case with non-Hodgkin-lymphoma (NHL). B lymphocytes become malignant, escape the immune system, grow uncontrolled, and accumulate in lymph nodes or other tissue. NHL is ranked fifth among the causes of death resulting from tumors. The tumor B lymphocytes are not recognized by the patient’s immune system. A logical approach is to try and activate the immune system through individualized therapeutic vaccines. The antigen in this vaccine is the surface immunoglobulin of the neoplastic B lymphocytes carrying a unique idiotype, that is, the drug is patient specific.

The plant-based magnICON™ technology is a rapid and versatile protein expression platform, which allows fast production of numerous pharmaceutical proteins with reliable high yield and purity. As such, it is particularly well-suited for the manufacture of these individualized therapeutic vaccines. This requires the correct idiotype sequence identification from tumor biopsy material and subsequent cloning in viral vectors for transfection of Nicotiana benthamiana. After an appropriate incubation time, the N. benthamiana plants are harvested, the biomass separated, and the immunoglobulin isolated and purified. After the final formulation step, the vaccine is filled and packaged for distribution.

The production of such individualized vaccines poses unique challenges during both developmental and later commercial production. Each patient requires a new product and a new production batch, fully compliant with cGMP. Consequently, requirements on process robustness, data management, segregation of processes, and release of the product are more demanding than in a regular pharmaceutical or biotechnological production environment.

Despite these challenges, the goal remains to ensure an affordable and profitable treatment. A cost structure analysis (Fig 1) has confirmed that the idiotype sequence identification and subsequent cloning in viral vectors already accounts for a significant portion of manufacturing costs. All material and labor needed to generate a patient’s viral vectors for transfection is allocated entirely to the manufacturing cost for just this patient.

A strict concept of self-sustaining automation for these processes (Fig 2) supports cost-effective sequence identification and cloning. Moreover, manually performed cloning operations, sample handling, as well as material and data management, would in later stage clinical trials generate a bottleneck and represent a significant hurdle for widespread commercialization. It was, thus, decided to start the development of self-sufficient automation concurrent with commencing phase I clinical trials.
The goal was to provide a robust system to satisfy the requirements of a routine production and to develop an appropriate control system. Main design requirements for the equipment included:

- self-sufficient and fully integrated automation for those processes that cause bottle necks during clinical phases II and III or pivotal studies, respectively
- future expansion capability to cover the entire process
- reliability, validity, and robustness suited for an industrial production environment
- parallel performance of molecular biology unit operations for up to eight patients/day

An interdisciplinary and cross-functional team from the Bayer Group was formed in order to elaborate the needs and to perform a thorough vendor evaluation and selection process. A robotic system vendor was selected in a bidding process, which was based on a detailed User Requirement Specification.

A crucial task for the team was to transform the existing laboratory process to one that supports a fully automated and integrated solution. Process steps not suited for automation were replaced. The feasibility of the novel methods was experimentally verified on a laboratory scale. The final process included unit operations, such as:

- thermal cycling for PCR
- capillary electrophoresis for nucleic acid fragment analysis
- purification with magnetic beads
- plating and cultivation of bacterial cells
- picking of recombinant bacteria
- microplate sealing and peeling
- pipetting
- carrier feed and transport

Several technical solutions had to be developed from scratch in order to meet the project goals. To name a few examples, the following was constructed, disposable free plating, integrated pipetting, capper and grabber device, and automated colony picking. Use of programmable logic controller-based industrial controls for steering of the process, monitoring, as well as data management, was also crucial to meet the project goals.

The system was successfully implemented and commissioned (Fig 3). The challenges of developing novel automated and integrated solutions for autologous treatment were met. In summary, the following factors have been proven essential for success:

- forming a team that covers all disciplines and areas of expertise needed
- providing sufficient time for defining the project scope
- pursuing the path of developing tailor-made solutions
- selecting the most suitable technology
- managing development, engineering and project execution concurrently (Concurrent Engineering)
- maintaining patience and persistence during implementation
Experimental investigation of scale effects in high-throughput process development studies

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Introduction
High-throughput process development (HTPD) has already proven to be successful, mainly because it not only allows for investigating a larger experimental space of process conditions much faster, but also HTPD does not require large amounts of sample (1).

In general, HTPD for developing chromatography steps can be performed using different miniaturized formats that support parallel experiments. However, when choosing the right format for a particular study, some intrinsic features of the formats need to be considered, as these could impact results obtained at small-scale, making them not representative for large-scale operations (2).

In this work, we have analyzed effects related to local hydrodynamic conditions around resin particles packed in columns on the performance of different chromatography resins. The analysis was based on the comparison of results obtained with mini- (200 μL) and laboratory-scale (1 mL) columns operated at different linear velocities, but under otherwise identical conditions, including the residence time.

Materials and methods
The columns used in this study were packed in-house. For the 1 mL and 200 μL columns, Tricorn™ 5/50 and Tricorn 5/20 columns were used, respectively. In total, seven cases were considered, covering different combinations of resin and protein types (Table 1). For each case, three residence times (1, 3 and 8 min), and two protein concentrations (0.5 and 2 g/L) were investigated, yielding in total six runs per column size. All experiments were performed on an ÄktA micro™ system. The columns were packed following recommended packing procedures and packing quality was determined by a standard height equivalent to a theoretical plate (HETP) method. Some experimental conditions were repeated at different occasions to avoid potential column usage history effects.

All experiments were performed following a standard procedure for frontal analysis method. The column was equilibrated using 10 column volumes (CV) of buffer. Following the equilibration step, protein sample was loaded onto the column until the sample concentration in the effluent from the column reached 85% of the

<table>
<thead>
<tr>
<th>Case 1A</th>
<th>Protein type</th>
<th>Resin type</th>
<th>Particle size</th>
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<tbody>
<tr>
<td>Case 1B</td>
<td>Large (hIgG)</td>
<td>Macroporous</td>
<td>Small (Capto™ SP ImpRes)</td>
</tr>
<tr>
<td>Case 2A</td>
<td>Small (Lysozyme)</td>
<td>Macroporous</td>
<td>Large (SP Sepharose™ FF)</td>
</tr>
<tr>
<td>Case 2B</td>
<td>Large (hIgG and Mab)</td>
<td>Macroporous</td>
<td>Small (Capto SP ImpRes)</td>
</tr>
<tr>
<td>Case 3</td>
<td>Large (hIgG)</td>
<td>Gel Composite</td>
<td>Large (SP Sepharose FF)</td>
</tr>
<tr>
<td>Case 4</td>
<td>Small (Lysozyme)</td>
<td>Large (MabSelect SuRe™)</td>
<td></td>
</tr>
<tr>
<td>Case 5</td>
<td></td>
<td></td>
<td>Large (Capto S)</td>
</tr>
</tbody>
</table>
sample concentration in the feed. The loading step was followed by wash and elution steps. Finally, after each run, the column was stripped using 0.1 M sodium hydroxide solution. The same columns were used for all conditions tested (residence times and proteins).

**Results**

Reduced plate height data for the small (200 µL) and larger (1 mL) columns packed with two resin types are shown in Figure 1. No significant differences between the two scales can be seen in either case. No change in reduced plate heights were measured after the end of the study, indicating that packing quality did not contribute to the results obtained during the frontal analysis runs.

![HETP plot](image)

**Fig 1.** HETP plot obtained for the two column sizes and two types of resins.

Two examples of frontal analysis data obtained in this study are shown in Figures 2 and 3. Both figures show breakthrough curves obtained at different residence times and at two protein concentrations. Figure 2 shows breakthrough curves of hIgG on Capto SP ImpRes (Case 1A), while breakthrough of lysozyme on Capto S (Case 5) is shown in Figure 3.

As shown in Figure 2, the difference in the shape of breakthrough curves, obtained with the two column sizes, increases with the increase in residence time. A very well pronounced shoulder on the breakthrough curves for the 200 µL column is visible. The lower the concentration, the more pronounced is the shoulder. As a result of the difference in shape of the breakthrough curves, the relationship between the dynamic binding capacities and residence time for the two scales considered is not the same.

![Breakthrough curves](image)

**Fig 2.** Breakthrough curves of hIgG on Capto SP ImpRes determined using either 200 µL or 1 mL columns at two protein concentrations and three residence times. (A) 1 min; (B) 3 min; (C) 8 min.
As shown in Figure 3, the differences between the two scales are already visible at short residence times and seem to become larger with the increase in residence time. However, despite significantly different shapes of breakthrough curves at the two scales for the same residence time and protein concentration, still similar dynamic binding capacities are measured at 5% or 10% breakthrough.

**Conclusions**

The data obtained in this study have shown that, in some cases, results obtained at the two scales were not the same. Despite this fact, it can be concluded that mini-columns can be used for HTPD studies of dynamic binding capacities and/or elution conditions. However, investigations of longer residence times, where the linear velocities drop below 20 cm/h, should be avoided. At these low velocities (equivalent to longer residence times), the deviation between results obtained with the small and larger columns increases with: (i) increase in protein MW, (ii) decrease in protein concentration, and (iii) decrease in resin particle size. The observed deviations are independent of resin type and ligand chemistries.

**References**


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**Fig 3.** Breakthrough curves of lysozyme on Capto S determined using either 200 μL or 1 mL columns at two protein concentrations and two residence times. (A) 1 min; (B) 8 min.
Fast track process development using high-throughput system—a case study

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Development and process economics continuously need to be optimized, in particular by reduction of development times and process costs, respectively. Accordingly, accelerated development scenarios have been set up by the pharmaceutical industry, including enhanced speed in downstream process development. However, at the same time, due to requirements of health authorities, more knowledge of process development needs to be accumulated. To overcome this apparent conflict, the use of high-throughput experiments (HTE) in combination with a model-based approach, using statistical design of experiment, is indispensable. Hereby, we present a case study of a fast track process development approach for a cation exchange chromatography step of a monoclonal antibody, starting from scratch. Firstly, a broad batch screen using 96-well microplates was used to get initial information on the required chromatography conditions on the robotic system. Next, the promising ranges were transferred to mini-columns, run on the robotic system as well, and a narrower range was investigated. Also, the binding capacity was determined using the mini-columns. Finally, the HTE results were verified and proven very comparable to the standard laboratory scale.

Figure 1 shows the different downscale models used to develop a robust downstream process. Starting with the mini-columns run on the high-throughput robotic system, certain experiments were verified using the standard laboratory-scale system. An upscaling to the pilot plant was conducted before transferring the process to the manufacturing facility.

Figure 2 and Table 1 shows a comparability study of a cation exchange chromatography step using SP Sepharose™. “Pseudo-chromatograms” could be collected by automatically measuring the absorbance of the fractions of mini-columns, with 1 and 3 cm bed height, on the robotic system. In comparison to the chromatogram obtained from the AKTA™ system, the chromatogram of the 3 cm mini-column matched very well, and even though there were only five data points available for the 1 cm mini-column, the chromatogram showed a similar shape. The yield moved in similar ranges for all scales. The fractions of the mini-columns were analyzed by size exclusion high-pressure liquid chromatography (SEC) to detect the impurity level. The results show the same values for all different scales.

Fig 1. The model systems: screen, verify, and transfer.
Fig 2. Comparability of the high-throughput robotic system vs the laboratory-scale system.

Table 1. Yield and purity at different scales

<table>
<thead>
<tr>
<th></th>
<th>20 cm (ÅKTA)</th>
<th>3 cm mini-column (HTS)</th>
<th>1 cm mini-column (HTS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield (%)</td>
<td>98.0</td>
<td>91.4</td>
<td>89.4</td>
</tr>
<tr>
<td>Purity (%)</td>
<td>99.6</td>
<td>99.5</td>
<td>99.3</td>
</tr>
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</table>
Protein formulation studies: Using high-throughput technology to assess aggregation behavior, solubility and viscosity

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Introduction
Pharmaceuticals that are produced biotechnologically increased their market share to approximately 20% in 2010, with monoclonal antibodies (MAbs) representing the major part. In the production process of MAbs, formulation is the most critical step, as it is difficult to preserve the delicate steric structure of the proteins. Moreover, aggregation phenomena must be monitored. To achieve this, column-based separation methods (e.g., size exclusion chromatography, SEC) coupled to multi-angle light scattering (MALS) detection were frequently used in the past. Unfortunately, destructive shear forces and interaction of the proteins with the column material often resulted in aggregate formation and time-consuming investigations, which made screening of numerous samples difficult. This is why alternative characterization methods were recently developed and are increasingly applied. Some of these new technological approaches are asymmetric flow field flow fractionation (AF4) (1), hollow fiber field flow fractionation (HF5) (2) and dynamic light scattering (DLS), the latter of which offers the opportunity to measure a high number of samples in microplates with a tremendous increase in productivity.

In our contribution we introduce a rapid determination of various protein formulation parameters using low sample volume microplates in the Wyatt DynaPro™ Plate Reader DLS instrument. This high-throughput method enables the user to screen protein preparations and comparable solutions for aggregates, viscosity, and other crucial parameters. It will be demonstrated that this method represents a major step forward in terms of facilitating developmental and quality control procedures.

Theory
DLS detectors determine the time-dependent fluctuation of the intensity of scattered light. In general, all molecules are moving, driven by Brownian motion. Bigger molecules move slower, whereas smaller ones move faster. By measurement of the changes in this fluctuation of the scattered light, the thermal diffusion coefficient can be determined by an autocorrelation of the light scattering signal. With the Stokes-Einstein equation, the size of the particles (i.e., the hydrodynamic radius, $R_h$) can be calculated:

$$R_h = \frac{k_b T}{6\pi \eta D_T}$$

To achieve this, an avalanche photodiode is used, which provides a time resolution of 100 ns. This allows a detection range of DLS in water between 0.3 nm and 1 µm. Because the intensity of scattered light is proportional to the molar mass of the molecules, this method is ideally suited for the detection of even very low numbers of big aggregates. According to a publication of Yadav et al. (3), the dissociation constant, $k_D$, can be determined from the change in diffusion coefficient with concentration. The $k_D$ is, at least for the tested condition from Lehermayr et al. (4), proportional to the second virial coefficient, $A_2$, which describes the solution properties and interactions between the molecules of a protein solution. Moreover, the viscosity can be measured if so-called tracer particles of a known size are added to the solution.

Fig 1. Wyatt’s DynaPro Plate Reader DLS instrument.
Sample measurements

The data shown here were generated using a Wyatt Technology DynaPro Plate Reader with an industrial standard 384-well microplate. The sample was a monoclonal antibody. The wells were filled with 20 µL and the concentration was between 3 and 10 mg/mL. As viscosity tracers, polystyrene particles with a radius of 50 nm were added.

Because the measurements are performed directly in the wells, no sample transport or plate rinsing is necessary. Consequently, sample cross-contamination is completely excluded.

The measurement of the particles in different protein solutions yielded particle sizes between 50 and 63 nm (Fig 2). This variation is due to the influence of the viscosity of the different protein concentrations. The true viscosity, \( \eta \), can be calculated using the Stokes-Einstein relation:

\[
\eta = \frac{k_B T}{6\pi R_h D_T}
\]

The hydrodynamic radii can be calculated if a series of diluted antibodies is measured and the specific viscosity of each dilution step is used in the equation. The sample was measured in triplicates to minimize statistical value fluctuation. The plot in Figure 3 is generated from 24 wells with a total time of 12 min.

From the analysis of the change of the diffusion coefficient with the concentration (Fig 4) it is possible to measure several parameters:

- \( k_D \): From the slope the dissociation constant (according to Yadav et al. [3]: \( D_m = D_s + D_s k_D C \))
- \( A_2 \): From \( k_D \) and a known molar mass (according to Lehermayr et al. [4]: \( k_D = 1.06 A_2 M_w^{-8.9} \))

- Melting point (\( T_m \)): With increasing temperature, the melting point marks the transition where the stability of the protein formulation decreases and aggregation can be detected by DLS. This shows the stability of a protein in solution (Fig 5).
Summary

DLS measurement in a microplate using the DynaPro Plate Reader instrument represents a fast method to characterize protein formulations. It is especially suitable for antibody preparations which are prone to self-association and aggregate formation. This method requires a small amount of sample and yields several valuable parameters such as hydrodynamic radius ($R_h$), diffusion coefficient ($D_T$), second virial coefficient ($A_2$), melting point ($T_m$), and viscosity ($\eta$). Because the DynaPro Plate Reader DLS is an automated process, it offers the opportunity to determine a high number of different samples in a reasonable time. Extensive sample preparation is unnecessary and the danger of sample cross-contamination is avoided. Therefore, automated DLS is the first method that allows for high-throughput analysis of the shown parameters in a variety of samples with low sample consumption.

References

The effects of carboxylate buffer structure on chemical degradation of proteins: Application of QSPR to high-throughput formulation development

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High-throughput formulation development presents a unique opportunity for the rationalization of results and the formation of predictive models using quantitative modeling. By screening libraries of structurally similar compounds, quantitative structure-property relationship (QSPR) methodologies can be applied to large stability data sets to inform potential mechanisms of biochemical degradation and to create predictive models. This can be used to select for compounds with optimal stability properties.

This study explored the effects of carboxylic acid buffers on the chemical degradation rates of an IgG, monoclonal antibody (MAb1), using high-throughput methods. Sample preparation was automated using a Biomek™ FX P liquid handling system. The chemical degradation of MAb1, in the presence of each excipient at 40°C, was measured using high-throughput ion exchange chromatography. Peak analysis of degraded samples demonstrated that the primary mechanism of chemical degradation for MAb1 was asparaginyl deamidation. The rate constants for MAb1 chemical degradation were calculated using first order kinetics. The results from the kinetics study demonstrate that MAb chemical stability was affected by the carboxylic acid buffer structure. In general, dicarboxylic acids resulted in higher degradation rates than monocarboxylic acids. Also, the hydroxylated dicarboxylic acids resulted in higher chemical degradation rates.

In order to further study the structural and physicochemical properties that affect MAb chemical degradation, a model hexapeptide, with sequence YGKNGG, was used to model asparaginyl deamidation rates in the presence of various excipients. The deamidation rates of the model hexapeptide, in the presence of each excipient, were measured at 30°C, 40°C, 50°C, and 60°C using reverse phase ultra-performance liquid chromatography (RP-UPLC). The rate constants for peptide deamidation were calculated using first order kinetics. The results from the kinetics study are consistent with the results from the MAb1 study. In general, dicarboxylic acids resulted in higher degradation rates than monocarboxylic acids, ketols, diones, and diols. Results from the peptide degradation study demonstrate that the topological distance between the carboxylic acids in dicarboxylic acid is important in determining degradation rates. Also, the hydroxylated mono- and dicarboxylic acids result in higher degradation rates than the unhydroxylated analogs.

Thermodynamic analysis of the temperature dependence of peptide deamidation was performed using the Arrhenius and the Eyring equations. The calculations demonstrate that the differences in deamidation rates could not be explained by differences in the energy or enthalpy of activation, respectively. These results suggest that proton acid catalysis is not contributing to the observed differences in asparaginyl deamidation. Interestingly, Eyring calculations demonstrated that the most negative entropies of activation are measured in the presence of excipients that result in the highest deamidation rates. The correlation with entropy indicates that structural stabilization of deamidation reaction intermediates by carboxylic acid buffers could be an explanation for the differences in deamidation rates.
To understand the specific structural and physicochemical properties of carboxylic acids that are responsible for the observed differences in chemical degradation rates, we implemented quantitative structural analysis. In this study it was implemented to relate the structural properties of various buffer compounds to observed rates of chemical protein degradation using linear regressions, multiple linear regressions (MLR), principal components analysis (PCA), and partial least squares (PLS). By screening the correlation between MAb chemical degradation and > 1000 molecular descriptor types, we were able to establish that the 3-D descriptors outperform 1-D and 2-D descriptors. The 3-D descriptors encode information about the molecular geometry in 3-D space, whereas the 1-D and 2-D descriptors encode information about the atomic properties and topology of each molecule, respectively.

The results demonstrate that geometric (3-D) descriptors correlate strongly with chemical protein degradation and suggest that the spatial distribution of specific functional groups (e.g., carboxyl and hydroxyl groups) on buffer molecules can determine the rate of chemical degradation reactions in proteins. This finding is consistent with the qualitative analysis of the carboxylic acid structures, where we established the importance of the topological distribution of carboxylate and hydroxyl functional groups in the determination of degradation rates. The results demonstrate that the spatial distribution of hydrogen bond donor and acceptor groups play a strong role in determining the MAb and peptide deamidation rate. This suggests that direct interactions (i.e., hydrogen bonding) between the carboxylic acid and the deamidation reactive site could stabilize the reaction site by providing alignment between the reactive atoms. This interpretation would also be consistent from the thermodynamic analysis, which emphasized the role of structural stabilization in the deamidation reaction by the reactive carboxylic acids.

These studies demonstrate the potential of high-throughput formulation development. Using high-throughput methods, we were able to easily prepare and analyze thousands of formulation samples. The results from the kinetics and thermodynamics studies provided insight into the relationship between carboxylic acid buffer structure and deamidation rates of MAbs and model peptides. Additionally, this study demonstrates the value of using QSPR analysis to supplement high-throughput, formulation screening studies. Our study shows that, by including sophisticated quantitative analysis of chemical structures, more detailed information can be extracted from screening libraries of unique chemicals, which can then be used to provide additional mechanistic insights.
Assessing in-process pool stability of monoclonal antibodies using a high-throughput approach

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The purification processing of monoclonal antibodies (MAbs) can expose proteins to a number of different stress conditions, including high and low pH, elevated temperatures, high ionic strength solutions, or buffers that might not be ideal for protein stability. Practical manufacturing processing times might necessitate holding pools at these stressed conditions for an extended time. As a result, understanding both the physical and chemical stability of proteins during in-process pool holds is important in purification process development.

In-process pool stability studies can be performed using 316L stainless steel (in-process pool tank construction material) mini-tanks, wherein the mini-tanks are incubated at specified temperatures and sampled for analysis of physical and chemical stability at desired time points. Such studies are time- and labor-intensive. They also require large volumes (approximately 50 mL of each pool per mini-tank condition). Instead, we propose using 96-well 316L stainless steel plates coupled with high-throughput high pressure liquid chromatography (HPLC) analytics as a comparable model to the mini-tanks. The 96-well plates are of the same geometry as a standard 96-well UV plate and thus can fit into most temperature-controlled HPLC autosamplers, but they are made of the same construction material as the mini-tanks and can provide a worst-case liquid contact surface-area-to-volume ratio. The ability to test multiple conditions on one plate means that a wider parameter space can be tested at the same time, for instance, multiple pH values, buffer conductivities, or chromatography pools.

In order to show comparability between the mini-tanks and the 96-well plates, we performed in-process pool hold studies at 25°C in both configurations for two chromatography pools, the low-pH affinity pool and the high-pH ion exchange pool (data not shown). In the study, each pool was titrated to eight pH values spanning the pH range 3.1 to 5.0, for the low-pH affinity pool, and pH 5.5 to 8.0, for the high-pH ion exchange pool. These samples were then aliquoted into cleaned and passivated 96-well stainless steel plates, sealed with a pierceable seal, and incubated in a temperature-controlled HPLC autosampler. Three samples were also transferred to mini-tanks, which were incubated at 25°C. Samples were taken at desired time-points for HPLC analysis. Samples in the 96-well plate were directly injected onto an interlaced ion exchange and size exclusion (SE) chromatography method.

Aggregation and fragmentation trends from the low-pH screen for MAb-1 [Fig 1] are similar between the mini-tank and the 96-well plate configurations. Specifically, for pH values 3.2 and above, there is a slight decrease in relative percent aggregates with incubation time. However, at pH 3.1, there is a significant increase in aggregation over time. There is also a pH and fragmentation trend, as the pH of the pool is lowered, there is an increase in relative percent fragments forming with time.

In viewing the trends for MAb-1’s low-pH pools, the 96-well plate format provides several advantages. Because sampling was automated, we easily acquired more data points and thus were better able to visualize degradation over time. In addition,
Fig 1. Plots of relative percent aggregate and fragments for MAb-1’s affinity pool at 25°C show degradation trends to be comparable between the mini-tank (A,B) and 96-well plate (C,D) configurations. More data points with the 96-well plates allow better visualization of pH trends with fragmentation.

because we could sample a wider range of pH, with a smaller sample volume requirement, the pH trend of fragmentation was better observed in the 96-well plate data, compared to the mini-tank data, and we were able to identify a sharp transition in aggregation rates between pH 3.1 and 3.2. Moreover, having more data points also allowed for elimination of potential outliers in the data. This rich data set also provided insight into the qualitative differences in degradation between different pH values. For example, the SE-HPLC chromatogram overlays (Fig 2) show a broadening of the monomer peak at pH 3.1, the formation of a single aggregate peak at approximately 13.8 min, and the presence of a late-eluting shoulder to the monomer peak and very small fragments.

Taking the data across five MAbs and assuming a linear rate of degradation, we can generate cross-plots of the physical and chemical degradation rates between mini-tanks and 96-well plates for the affinity pool (Fig 3) to show that most data points fall along the line of unity and thus, the two configurations are comparable for the aggregation, fragmentation, and main peak loss rates.

Performing these high-throughput in-process stability screens quickly allows us to generate a database of MAb degradation. This subsequently allows us to compare relative rates of degradation across molecules and determine their impact. As a result, this can guide the development of individual chromatography steps in both early and late-stage development projects and can avoid setting short in-process pool expiry times in manufacturing as a result of finding stability issues late in the purification development.
Fig 2. Size exclusion HPLC chromatogram overlays for MAb-1's affinity pool for different pH values in the 96-well plates allows visualization of small differences in product quality profiles. (A) pH 3.1; (B) pH 3.6; (C) pH 5.0.

Fig 3. Cross-plots comparing physical [A] and chemical degradation [B] in the 96-well plate (x-axes) and mini-tanks (y-axes) across five MAbs show comparability between the two configurations, suggesting that the 96-well plate format is a comparable model to the mini-tanks.
Development of a Pareto optimization algorithm for the downstream process development of a monoclonal antibody

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Introduction
The utilization of high-throughput screening (HTS) protocols has gained popularity in biologics process development, as it has become an invaluable tool that can significantly shorten the time to the clinic and, ultimately, time to market. HTS allows rapid exploration of larger design spaces for process characterizations that have been applied to molecules in preclinical, clinical, and commercial manufacturing processes. One unit operation in the manufacturing process of biologics that uses HTS protocols is chromatography. HTS can ensure thorough process characterization and, consequently, process robustness. Rapid progress in automation and robotic platforms has enabled this powerful tool in process development, but careful planning is required to ensure that the bottleneck is not simply shifted to data analysis and analytical throughput.

One possible solution to relieve the bottleneck of data analysis is the use of optimization algorithms that are widely used in diverse industries, as they are powerful tools for process characterization. These computational algorithms can efficiently analyze complex data sets, while reaching true optima with fewer experiments. This will effectively reduce the data and sample analysis, while ultimately saving overall development time and resources.

In this study, a Pareto optimization algorithm was used to optimize a chromatography unit operation in a monoclonal antibody purification process. The algorithm follows the Pareto principle, which evaluates results against two objectives to determine dominated and non-dominated results. Results with lower values for both objectives are inefficient and thus dominated by results with higher values. These non-dominated results are efficient and constitute the best experimental conditions measured against the chosen objective to form the “Pareto front” (Fig 1). This approach offers an advantage as it allows the simultaneous optimization of two objectives.

The Pareto optimization algorithm lays the foundation for a multi-objective genetic algorithm (MGA). The Pareto optimization algorithm performs a fitness assessment of results, in which only the non-dominated solutions are used in the MGA. The MGA then simulates the process of natural evolution, in which selection, variation, and combinations of experimental conditions are explored. This determines the next generation of experimental conditions that are necessary for optimizing the objectives to produce the next generation of non-dominated results. The MGA is an iterative process that is completed once the objectives can no longer be optimized or a sufficient optimization is achieved, as determined by the user.
The two objectives optimized in this study were protein recovery (yield) and monomer content (purity). In this study, we successfully demonstrated that a Pareto optimization algorithm and an MGA can accurately detect the optimal chromatography process parameters in less process development time, compared to traditional fractional factorial HTS.

Methods
This study was conducted in three phases. The objective of phases 1 and 2 was to generate data sets to assess and confirm the robustness of the Pareto optimization algorithm to optimize two objectives, yield and monomer content, for various process parameters. Phase 1 was a screening of three continuous and one categorical variable: pH, sodium chloride, load amount, and chromatography resin, respectively. Phase 2 investigated an additional two variables, a categorical variable (phase modifiers) and a continuous variable (concentration of the loading material). The results from phases 1 and 2 were used for the MGA in phase 3. A minimum of two generations of the MGA are required to demonstrate that the true optima for both objectives are reached. For this study, two rounds of the Pareto optimization algorithm, in combination with one generation of the MGA, was sufficient to meet the desired yield and impurity removal criteria so that no further experiments had to be conducted. Results were confirmed at a larger scale.

A$_{280nm}$-based chromatograms were created in Excel® 2007. The Pareto optimization algorithm and the MGA were created in MATLAB™ 2010b and Java™, respectively, and allowed data transfer through Excel 2007. Additional statistical analysis was performed using Excel and JMP™ 8.0.

Results
Phase 1 and phase 2 evaluated process parameters to optimize yield and monomer content. Representative chromatograms from both phases are presented in Figure 2. Representative chromatograms for phase 1 are presented for the two hydrophobic interaction chromatography resins (Capto™ Phenyl and Toyopearl™ Hexyl 650C) and two mix-mode resins (Capto MMC and Capto adhere), screened at the same buffer system and sodium chloride concentration. These chromatograms illustrate how various process conditions can affect the protein yield. Representative chromatograms for phase 2 are presented for Capto Phenyl at 50 g/L with different phase modifiers. These chromatograms show how the phase modifiers can affect the protein yield.

The protein and monomer yields for phase 1 and 2 were entered into the Pareto optimization algorithm. The outputs are presented in Figures 3 and 4, respectively. The algorithm determined the best experimental conditions, which maximized protein yield and monomer content, as highlighted by the blue boxes (Fig 3).

The results from phase 1 (Fig 3) indicate that Capto Phenyl and Capto adhere resins were superior for monomer enrichment, while Capto Phenyl distinguished itself further through highest protein recovery. The algorithm also determined that the buffer system and sodium chloride concentration did not have a significant impact on yield and monomer content, as indicated by the lack of discrete conditions in the blue box. However, the optimal pH range of pH 5 to pH 6.5 indicates a very robust operating window for this step.
Fig 3. Pareto optimization algorithm results for phase 1 analyzed the results for monomer content and yield for four process conditions. The Pareto front for each set is highlighted by a blue box. The color of each point represents the corresponding variable shown in each graph.

Fig 4. Pareto optimization algorithm results for phase 2 show the results for monomer and yield against four variables. The Pareto front for each set is highlighted by a blue box. The color of each point represents the corresponding variable shown in each graph.
The results from phase 2 (Fig 4) suggest that the use of additives had a negative effect on the yield, as indicated by the red points in the blue box highlighted in the additive plot. Results show that a load amount of 25 g/L was best for optimizing protein yield and monomer content.

Overall, the data from phases 1 and 2 indicate that the Capto Phenyl and Capto adhere were the best resins for monomer content and Capto Phenyl was the best resin for protein yield. Capto MMC and Toyopearl Hexyl 650C performed poorly for both objectives. The buffer system and sodium chloride did not have a significant impact, while the optimal pH ranged from pH 5 to 6.5. Overall, the use of additives had a negative effect on the yield. The data generated in phases 1 and 2 were analyzed in JMP 8.0 for traditional statistical interpretation of results against the Pareto analysis.

After successful demonstration of the Pareto optimization, the data generated from phases 1 and 2 were used as input for the MGA. The MGA included random combinations of additives, buffers and pH ranges, as well as the buffer system alone as controls. Although the additives from phase 2 demonstrated a negative effect on protein yield, the results were still used as input for the MGA, with the goal that the MGA's combinations of experimental conditions could improve yield and monomer content. From a possible combination of 16 416 experiments, the MGA selected 44 conditions to explore in the first generation.

Representative chromatograms for some experimental conditions with Capto Phenyl and Capto adhere are presented in Figure 5.

Compared to their controls, the MGA was able to determine experimental conditions to improve the yield based on the data generated in phases 1 and 2. The monomer content for each of the 44 conditions was comparable.

**Conclusion**

The Pareto optimization algorithm provides a strong foundation for the process development of protein therapeutics. The algorithm successfully demonstrated its utility as a powerful search and optimization tool for large, complex design spaces and it allowed for the rapid identification of processing conditions to optimize yield and monomer content. We demonstrated that the Pareto optimization algorithm, in combination with an MGA, accurately and efficiently detected the optimal chromatography process parameters. By using this algorithm-based approach, complex statistical analysis was reduced and the number of experiments required to reach the optimal process parameters was decreased by a factor of 33.

**References**

Error analysis and characterization of microliter-scale column chromatography performed on liquid handling stations

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Introduction

Within the last decade, chromatography process development in biopharmaceutical industries has been improved strongly in terms of efficiency and material consumption. The improvement was initiated by the successful miniaturization of chromatographic experiments down to the µL-scale, coupled with parallel and automated performance on liquid handling stations. Although the application of high-throughput experimentation (HTE) has been successfully demonstrated, the limitations of this technology at its present stage have not yet been characterized in detail. If µL-scale chromatography performed on robotic workstations is intended to be used not only as a screening tool, but also for process robustness studies, a detailed evaluation of precision, accuracy, and error sources is required. We have identified sources of experimental noise related to robot-specific parameters and have investigated data quality concerning retention times, mass balances, and peak resolution.

Evaluation of a miniaturized chromatography process

Protein concentration is typically determined by the absorption at wavelength 280 nm, and liquid volume can be linearly correlated to the absorption difference at wavelengths 995 nm and 900 nm. Predominantly, precision of absorption measurements in microplates is affected by the formation of a meniscus. Depending on protein characteristics and concentration, the meniscus shape changes and thus, liquid level and light path length vary. The choice of buffer or protein solution for calibration of the volume determination therefore strongly affects accuracy of microplate absorption measurement. Because composition of proteins and their concentrations vary throughout chromatographic elution, mass balances and yields cannot be determined with a precision comparable to values obtained in traditional laboratory scale. For example, in a series of 24 isocratic robotic experiments, mass balances varied by absolute +/- 5%.

One important parameter to choose, when performing HTE chromatography, is the number of eluate fractions to be collected. On the one hand, elution profiles show higher noise level with decreasing fraction volume. The dropwise collection of elution volume leads to deviations of approximately 25 µL between fractions. Thus, the path length is not constant for all fractions, which needs to be considered in the calculation of the concentration. If the absorbance value is not corrected by actual path length and eluate is collected in small fractions (e.g., 75 µL, half area microplates), the deviation has a rather large effect on the determination of correct mass balances. In large fractions (e.g., 300 µL), however, the relative deviation in fraction volume is small and might be negligible. On the other hand, fractionation using low volumes results in more information on peak shape. The influence of fraction size on the determination of peak area and retention time was investigated using Monte-Carlo simulations.

An experimentally derived chromatography peak was fitted with a common exponentially modified Gaussian (EMG) function and fractionated in-silico with varying fraction volumes (Fig 1). Experimental errors were added to the calculated concentrations of the new, superficial fractions and peak fitting was performed for each new peak.

As an example, the calculated variance in peak area is displayed in Figure 2. An expected convergence of upper and lower boundaries of the 95% confidence interval of the peak area, with increasing number of fractions, can be clearly seen. Thus, low precision in concentration determination can be partly compensated by a high number of fractions per peak width.
Fig 1. Illustration of the Monte-Carlo simulation procedure. Simulations aimed to investigate the influence of fraction volume and precision of concentration and volume determination on retention time and peak area. Randomly distributed errors of different levels on fraction volume and concentration were added in at least 100 × 100 different combinations.

Fig 2. Influence of error levels of concentration determination depending on fraction size. Lower and upper boundaries of 95% confidence intervals calculated for the peak areas of simulated peaks, related to the peak area of the starting peak, are plotted in same color. Two percent absolute error on volume determination of each fraction was used as general noise level in all simulations, except for simulations of the fit error. Fraction volumes ranged from 50 to 400 µL.
Gradient chromatography on a robotic workstation is performed by mimicking a gradient via multistep elution. This, however, comes with various flow interruptions for buffer salt uptake steps. In isocratic experiments with and without flow interruptions, it was demonstrated that the mean (n=4) retention times shifted to a lower elution volume with decreasing residence time. The results of different operational modes were statistically not significant (95% significance level). The lack of significance, however, might be explained by a rather large deviation in the determined retention volume of 0.7 column volume (CV) between the four different columns used. To fully characterize gradient or multistep elution, respectively, the robotic process was simulated including all flow interruptions and every step elution, varying step height and fraction size.

In Figure 3, simulated elution profiles of a two-protein chromatographic separation are displayed using two different fractionation schemes. Retention times calculated after peak fitting revealed a difference of 0.5 CV in retention times and a deviation of 5% in the determined peak resolution. In addition to differences in retention time, peak shape and peak width were also influenced by the choice of salt step height and fraction volume. In a next step, analytical errors will be included in these simulations to obtain an estimate of the overall precision of robotic column chromatography experiments.

**Conclusion**

It was demonstrated that data quality of “pseudo-gradient” column chromatography performed on robotic workstations is influenced by the choice of experimental process parameters, such as the number of fractions per peak width, flow interruptions, salt step heights, and analytical precision. There is no doubt that for screening purposes the use of µL-scale column chromatography definitely fulfills common requirements on accuracy. However, the results of this study also demonstrate that chosen process parameters should be critically evaluated on a case by case basis, if data are used to calibrate mechanistic chromatography models or if the technology is considered to be used for process robustness studies.
Using batch-binding chromatography and separation factor analysis to develop chromatography processes

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We developed screens using batch-binding on 96-well filter plates to map the behavior of both antibodies and impurities, as a function of pH and counterion concentration, on our chromatography resins. These screens can be used to accelerate the development of our purification processes by rapidly identifying conditions that separate the antibody from process impurities, such as host cell proteins (HCP), and product related impurities, such as high molecular weight species (HMWS). However, a hurdle to the widespread adoption of these techniques is the need for expert analysis and interpretation of very large data sets. Contour plots can be used to quickly visualize the data sets generated with batch-binding experiments. Figure 1 shows the contour plot for antibody and impurity behavior, mapped in terms of partition coefficient ($K_p$), on a cation exchange resin. These plots can be interpreted to identify conditions that resolve the antibody from impurities for an individual resin. However, it becomes difficult to identify the conditions that give optimal resolution when comparing conditions across operating pH values and different salt concentrations. This analysis can be even more challenging as additional impurities, alternative buffer systems, and chromatography resins are also considered, in particular because the binding behavior for both antibody and impurities differs between resins.

To overcome this challenge, we developed analytical tools to aid in the interpretation of our data sets. We utilized a separation factor to quantify resolution between antibodies and the impurities of interest. For our batch-binding application, the separation factor is defined as a ratio of the impurity $K_p$ to the antibody $K_p$ and can be generated from cross-plots of these parameters. Figure 2A shows a cross-plot of the log $K_p$ and log $K_p$ versus $K_p$ for the data in Figure 1. The relationship between the log $K_p$ and log $K_p$ is pH-independent, with the data points for all pH values collapsing onto a single line. This indicates that the separation between the antibody and HMWS is not influenced by pH, but only by counterion concentration. By contrast, the separation between antibody and HCP is pH-dependent, as indicated by a linear relationship between log $K_p$ and log $K_p$ for each pH value. In both cases, the linear relationship between impurity and antibody behavior can be described by the simple linear equation:

\[ y = mx + b \]

\[ \log K_p = m \log K_p + b \]

With some algebraic rearrangement, this can be transformed into:

\[ \text{Separation factor} = 10^b \log K_p \]

Using this equation, the separation factor for impurities can be plotted against the log $K_p$ (Fig 2B). Where the log $K_p$ equals the log $K_p$, the separation factor is one and there is no separation under those conditions. A separation factor of less than one indicates that the impurity is more weakly bound than the antibody, and that it may be removed during the load phase or an
Fig 1. Contour plots of Log $K_p$ illustrate the binding behavior of an antibody and its impurities under a range of pH and salt conditions. Direct interpretation of these plots can be challenging.

Intermediate wash phase. By contrast, a separation factor of greater than one indicates that the impurity is more tightly bound than the antibody. In this scenario, the impurity may remain bound following a step elution of the antibody or alternatively, the impurity may elute after the antibody in a pH or salt gradient.

Using this data processing technique, we can identify optimal conditions for separation. Coupled with existing correlations between partition coefficients, purification yield, and elution pool volume, we can rapidly identify operating conditions that can be verified in column mode (Fig 3). For the example data set presented here, the separation factor plots can be used to inform the selection of conditions to be verified on packed-bed columns. For HMWS, the separation factor decreased with decreasing Log $K_p$ as the elution buffer strength increased. As the clearance of HMWS is pH independent, elution conditions with a Log $K_p$ of 0.5 were selected at pH 5.5 to maintain a high separation factor, while maximizing yield and maintaining a reasonable pool volume of less than seven column volumes (Table 1). By contrast, the separation of HCP decreased with increasing pH as indicated by the fitted line moving closer to the line of no separation (Fig 2B). This is consistent with column runs where, at the sample Log $K_p$ of 0.5, HCP were more effectively reduced at pH 5.5 than pH 6.0 (Table 1). Interestingly, each pH separation factor increased with decreasing Log $K_p$ of the antibody. This trend can be explained with the cross-plot of the log $K_p$ of antibody and the log $K_p$ of HCP (Fig 2A). The rate at which Log $K_p$ decreased as a function of counterion concentration was faster for the antibody than for HCP, as indicated by the fitted line for each pH moving.
Results from small-scale packed-bed chromatography runs are consistent with predictions for the high-throughput model.

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Fig 3. Impurity level and pool volume predictions based on modeling with high-throughput batch-binding data.

We have demonstrated that the combination of high-throughput screening techniques and analytical tools allows us to more readily identify optimal chromatography conditions to achieve the target separation. Analyzing the data using cross-plots of antibody and impurity K<sub>p</sub>, as well as separation factor, removes the difficult and often subjective interpretation of contour plots. The identified conditions can subsequently be verified on packed-bed columns, minimizing the number of column runs necessary to optimize a purification process.
The hybrid experimental simplex algorithm for early bioprocess development

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Understanding bioprocesses early on in their development train is integral in facilitating and accelerating further development work, a feature which is of high importance for achieving fast product release. Generating this early information on bioprocess behavior can be argued to be one of the goals of high-throughput process development (HTPD). In HTPD, bench-top experimental studies, of different unit operations, aim to identify subspaces of favorable operating conditions, so-called sweet spots. Once obtained, these sweet spots can be further assessed in follow-up studies. Usually sweet spot identification during scouting studies is based on design of experiments (DoE) methodologies, in particular on response surface modelling (RSM) techniques.

RSM techniques, such as central composite designs, are resource-efficient, but they often require the employment of specialists to design and evaluate a study. This is because they are based on the derivation of mathematical models and they require rigorous data analysis steps, the duration of which is often not negligible. At the same time, due to their dependence on linear additive models, they may fail to reliably and correctly assess process behavior when process changes do not follow a linear trend, as a function of operating conditions. Such complications can be overcome if high level factorial designs are employed instead. These designs enable sweet spots to be identified by observing direct experimental measurements. In this case, however, the accrued experimental burden can be radically increased because the application of high level factorial designs requires that all test conditions are evaluated within an experimental space of interest.

The hybrid experimental simplex algorithm (HESA) can be considered to be an alternative to these DoE-based approaches, which can help overcome the aforementioned challenges. HESA is based on standard simplex methods, with added modifications to make it compatible with coarsely gridded data, like those generated with the application of high level factorial designs. It works by generating data on the fly. As a result, the deployment of HESA forms a path of test conditions within an experimental space that points towards optimal operating conditions, which are eventually located and encircled to outline a sweet spot. Due to the fact that HESA is based solely on direct experimental measurements, it does not require mathematical models, nor does it rely on linearity assumptions, or necessitate any lengthy data analysis steps. The latter makes HESA readily accessible to experimenters and lays the foundation of a walk-away operation regime on robotic stations, which can reduce turnaround times between experimental studies and, as a result, increase the throughput of development activities.

These features of HESA, together with the fact that it can be considered as an alternative approach to sweet spot identification during scouting studies, are demonstrated in this paper in two case studies. The first investigates the effect of pH and salt concentration on the binding of green fluorescent protein, isolated from E. coli homogenate, to a weak anion exchange resin. The second examines the impact of salt concentration, pH, buffer species and resin type upon the binding of a monoclonal antibody to cation exchange resins. In both case studies, HESA encircled and converged to optimal conditions and
consequently correctly identified sweet spots for further investigation. This was achieved despite the existence of nonlinear trends in the behavior of the chromatographic separations as a function of the considered operating conditions. This exemplifies the similarity between HESA application and the deployment of high level factorial designs, because with both approaches, optimal conditions can be identified based on experimental measurements without the need of mathematical models. HESA was also found to display similarities with RSM techniques regarding its associated experimental burden. In both case studies, sweet spot identification occurred while the number of test conditions, which were selected by HESA, was low and at the same time comparable to the one from the deployment of conventional RSM techniques.

In conclusion, this paper describes the hybrid experimental simplex algorithm and demonstrates its application to two case studies, depicting common bioprocess development scenarios. HESA is based on standard simplex methods, but also includes enhancements to make it applicable to coarsely gridded data. As evidenced by the two case studies, HESA is shown to combine beneficial features from high level factorial designs and other RSM techniques. Namely, it can, to a low experimental burden, correctly identify sweet spots in presence of nonlinear trends and results. This makes HESA a viable alternative approach to sweet spot identification during scouting studies with significant implications for HTPD. HESA can facilitate the generation of early information on bioprocesses, which in turn can lead to efficient bioprocess development.
Characterization of complex mixtures for bioseparation process development using modern, high-throughput compatible analytics

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The accelerating growth of the market for biopharmaceuticals, the growing interest in new, more complex protein therapeutics, as well as the increasing competition due to the market entry of biosimilars, are bringing new challenges to the downstream process development of these molecules. This results in a demand for faster, more cost-efficient, highly understood downstream processes.

Additionally, the FDA initiative for quality by design (QbD) is encouraging a smarter development of bioseparation processes. This leads to the usage of high-throughput set-ups for the screening of critical process parameters, as well as model-based approaches to develop purification processes (1). However, the determination of critical process parameters, as well as the acquisition of modeling parameters, is still a challenge when it comes to complex biological feedstocks.

This work shows the usage of pH-gradient (2) and salt-gradient ion exchange separations, linked to modern protein analytics, to determine the process and modeling parameters of the critical contaminants and the target protein in a cell lysate. The resulting parameters can be used subsequently for the design and optimization of an ion exchange unit operation to purify the target.

At first, an in silico method to generate buffer compositions, consisting of multiple buffer substances with a linear titration curve over a broad pH-range, had to be developed. Therefore, a MATLAB™ based method was set up to optimize the buffer compositions’ buffer capacity, resulting in compositions with a linear titration curve. To generate buffer compositions, commercially available ion exchange chromatography (IEC)-compatible substances, with known pKa values, was used. Using this method, a buffer system for pH-gradient anion exchange chromatography (AEC), spanning a pH-range of 10.5 to 3.5, was generated. Its experimental applicability was validated.

The buffer system was used to fractionate an insect cell lysate, containing a recombinantly expressed target protein, via pH-gradient AEC. The target protein was a 45 kDa sized fragment of nucleolin, a major autoantigen used for the diagnosis of SLE (Systemic Lupus Erythematosus). By specifically analyzing the fractions for the target protein’s presence, its elution pH could be determined (Fig 1).

**Fig 1.** pH-gradient AEC SF9-Nucleolin lysate. Chromatographic run pH 10.5 to 3.5 of 1 mL cell lysate on a Mono Q® 4.6/100 column on an ÄKTApurifier™ with online pH and UV 280 nm absorption monitoring. 5 column volume (CV) equilibration, 10 CV flow through, 15 CV gradient length, 5 CV post-gradient with a flow rate of 1.5 mL/min, gradient fractionated in 1 mL fractions. Fractions were analyzed for protein presence with a Nucleolin-specific dot blot. The elution pH of the target protein, pH 4.9 to 4.3, is marked in light-blue.
After the determination of the target’s elution pH (pH 4.9 to 4.3), a short range pH gradient (pH 5.5 to 3.5) AEC was used to focus on the target’s elution range. The gradient was again fractionated and all fractions were submitted to an SDS-PAGE after a concentration step via lyophilization. From the SDS-PAGE of the focused pH-gradient fractions, the most abundant proteins, the target protein, and the major contaminants could be identified with reverse phase liquid chromatography-mass spectrometry (RP-LC-MS) on an Orbitrap™ Velos mass spectrometer. These proteins are the most problematic contaminants for purification of the target protein via AEC due to their high degree of similarity. With the mass spectrometric identification of the major contaminants, these could be named and tabularized together with the corresponding elution pH value.

To acquire modeling parameters (SMA-parameters) for the target protein as well as the major contaminants, a new, multidimensional fractionation method had to be developed. Classically, multiple linear salt gradients with different gradient lengths are used in ion exchange chromatography to relate the single protein’s retention volume with the gradient length. This enables the determination of the protein’s characteristic charge, \( \mu \), as well as the equilibrium constant, \( k_{eq} \). This is not possible for the previously described feedstock using the classical approach, due to the feedstock’s high complexity (i.e., chromatographic peak overlapping) the contaminants cannot be clearly assigned to the single peaks. To address this problem, a multidimensional fractionation method was successfully developed (Fig 2).

The method is also based on multiple linear salt gradients with different gradient lengths, but with fractionation. The collected fractions were at first submitted to a fast RP-C18 ultra high pressure liquid chromatography (UHPLC) assay (Ultimate™ 3000 x2) to remove the salt. Afterwards, they were concentrated to dryness in a centrifuge evaporator (Martin Christ). The dried, salt-free samples were resolved in the sample buffer for a high-throughput capillary gel electrophoresis (HTS-CGE) device (LabChip™ GXII) and analyzed in the same system. The latter has the advantage, over other analytical alternatives, in that it provides extremely high speed (about 45 s/sample) with low sample consumption. The single protein chromatograms could be reconstructed from the second analytical dimension, HTS-CGE.

The method was successfully used to characterize the previously described cell lysate. At first, the elution-pH of the target protein was used to fix the pH for the salt gradient AEC slightly above its elution pH 5.2. After
the multidimensional fractionation, the single protein chromatograms of the five most abundant proteins were reconstructed. From their retention volumes in the different gradients, as well as the column parameters, the characteristic charge and \( k_{eq} \) could be determined. This is shown in Figure 3.

To summarize, at first a long range pH gradient was used to determine the target's elution pH on an anion exchange column. Afterwards, the cell lysate was separated and fractionated with a focused short range pH gradient AEC. Modern mass spectrometric protein identification was used to name the major protein contaminants in the fractions. Finally, a multidimensional fractionation set-up was developed to track the single proteins in multiple linear salt gradients in order to determine their physicochemical properties needed for further process modeling.

The usage of such a multidimensional fractionation approach generates a lot of process information in an analytical scale, while being acceptably fast. The method is fast enough to also be used as an analytical tool for the monitoring of lot-to-lot variances of different productions, while generating process relevant parameters, which can be used for process simulation.

**References**

Introduction

Traditionally, as a project matures, the clone number drops significantly just as the amount of information and cost per cultivation increases. This also corresponds to the transfer of work from screening to fermentation groups. If early stage methodologies that allow a higher throughput are predictive of those further downstream (traditionally a bottleneck within the discovery and development pipeline) then the following benefits become achievable:

- Clone evaluation may take into account process factors reducing later stage risk and attrition.
- Process information collated at an early stage is much cheaper and quicker to generate, thereby reducing costs.
- The traditional split between molecular biologists and fermentation engineers is eliminated, reducing silo thinking.

Following technological advancements, a number of miniaturized systems are now available for addressing this “predictiveness” and high-throughput (HTP) bottleneck.

Micro-24

The Micro-24 MicroReactor system (Pall Corporation) represents an enabling technology that attempts to bridge the gap between microplates (MPs) and bioreactors. Using PreSens’ non-invasive technology, the system enables the control and monitoring of the key process parameters, temperature, pH and dissolved oxygen (DO). Each individual well within a 24-well microplate can be controlled independently. The system may be used for microbial and cell culture cultivations.

A Micro-24 system was used in the following studies to assess the equipment’s suitability for incorporation into our in-house microbial scale-up processes.

Increased process knowledge

An experiment was undertaken in the Micro-24 system, which mirrored a typical in-house MP experiment, that is the Micro-24 control options were switched off, but the monitoring options were used to record online data. Figure 1 shows the process profile from one well. This profile represents the first glimpse into the dynamic culture environment of MP processes and initial attempts at MP process characterization. It also

Table 1. The traditional gap between microplates and bioreactors in their ability to investigate the design space within a commercial environment

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<th>Microplates</th>
<th>Bioreactors</th>
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<td>Critical process parameters (CPP)</td>
<td>Poor. No control or monitoring of pH, dissolved oxygen, etc.</td>
<td>Good. Many control and monitoring options for numerous CCPs.</td>
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<tr>
<td></td>
<td>Batch fermentation.</td>
<td>Fed batch fermentation.</td>
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<tr>
<td></td>
<td>Harvest time pre-determined.</td>
<td>Harvest time results led.</td>
</tr>
<tr>
<td>Critical quality attributes (CQA)</td>
<td>Poor. Limited to offline samples.</td>
<td>Good. Online and offline possibilities.</td>
</tr>
<tr>
<td>High-throughput (HTP)</td>
<td>Good</td>
<td>Poor</td>
</tr>
<tr>
<td>Cost of goods (COGs)</td>
<td>Good</td>
<td>Poor</td>
</tr>
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</table>
demonstrates how critical process parameters (CPPs) vary over time. In this situation we can see oxygen becoming limited after 10 h, acidification of the culture medium and a media limitation issue developing after 14 h. The dip in DO at after 5 h corresponds to a sample and induction time.

Another experiment demonstrates the first Micro-24 runs simulating the bioreactor culture environment within a small-scale system (4 mL batch fermentation) using the control and monitoring capabilities of the Micro-24 system. Figure 2 shows a profile with similarities to those routinely obtained using a bioreactor SCADA system and also demonstrates the effective control of temperature, DO and two-way control of pH over time.

![A microplate process profile.](image1)

**Fig 1.** A microplate process profile.

![A 4 mL bioreactor process profile.](image2)

**Fig 2.** A 4 mL bioreactor process profile.

**HTP scoping of the design space**

The combination of increased process knowledge and HTP at the mL-scale effectively opens the door to an enlarged explorable design space surrounding heterologous expression. Two-level fractional factorial designs can now be replaced with central composite and other designs, improving our ability to generate predictive statistical models.

A central composite design of experiment (DoE) was designed and executed with the aim to investigate three critical process parameters and build a small-scale expression model.

**DoE factors:**
- Temperature
- pH
- Induction strength
- DoE responses
- Titer
- Specific productivity
- Biomass

![Titer](image3)

**Fig 3.** Design space characterisation predicting optimal process ranges.
Conclusions

Developing high-throughput predictive scale-up strategies is key to the speedy progression of microbial assets. Until now, the inherent limitations of the microplates have prevented their use in developing scale-up models, but here we have shown how one miniaturized system can be used to develop expression models. By increasing the repertoire of critical process parameters and critical quality attributes, used in such experiments, and incorporating engineering aspects, such as volumetric mass transfer coefficient (KLa) and mixing times, we aim in future to characterize the microbial design space at the small scale and to validate within traditional stirred-tank bioreactors.

Tangible benefits of pursuing such an approach include increasing our design space knowledge earlier within project timelines, reducing costly fermentation development time, and accelerating process development into the quality by design arena.

Fig 4. Overlay plots showing, from A to C, how the optimal window of operation reduces with a critical process parameter.
Selectivity screening in batch format for development of an intermediate insulin purification step

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Designing a purification step for a complex target molecule is challenging, as the possible combinations of resins and conditions are numerous. Focusing on an intermediate purification step, one of the responses of major interest is resin selectivity, which relates to purity of target molecule. By using the high-throughput process development (HTPD) PreDictor™ format, it is possible to perform screening of resins and conditions in a parallel format, while keeping the sample amounts low. As demonstrated in this study, the elution experiments performed in batch format have proven highly valuable to predict mobile phase conditions for highest resolution. The knowledge gained from an elution study, utilizing 20 µL chromatography resin per well, followed by optimization in small columns, resulted in a successful 400 mL column scale intermediate purification of insulin to more than 90% purity.

Most often an elution study is preceded by a binding experiment to find good conditions for binding of the target molecule. The results from the binding study performed with crude material at overloaded conditions are shown in Figure 1, where binding of insulin is shown as a function of ethanol and salt concentration. The overloading of the resin is performed in order to reach highest possible binding (at 1 hour incubation time), that is to reach levels that are close to the capacity plateau of an adsorption isotherm. The crude material consisted of enzymatically cleaved pro-insulin, resulting in insulin, C-peptide, fragments, and truncated forms of insulin. Although the C-peptide was by far the most abundant impurity, the other impurities were also present in significant amounts. The pH during the study was 4, and was not altered, as the solubility of insulin is low at higher pH-levels. pH 4 was also used to stop the enzymatic cleavage, enabling sample application with minimum prior sample conditioning. Ethanol and salt concentrations were used as variable factors during the binding study.

From this study, one may draw the conclusion that the binding capacity for insulin is very low at high ethanol content and low salt, and that salt is needed for binding. However, when performing repeated loading of the crude sample (starting with concentrations that will not overload the chromatographic resin), it was observed that the binding at low salt and low ethanol concentration decreased with increasing number of loadings (Fig 2A). When also plotting the binding
capacities found for the C-peptide, one could observe that the C-peptide binds over the entire range of conditions investigated (Fig 2B). Thus, the decreased binding of insulin at higher number of loadings may be explained by competition from C-peptide, which appears to bind stronger than insulin. This may also be shown by comparing adsorption isotherms for the two main components, insulin and C-peptide (data not shown).

The stronger binding of the C-peptide as compared to insulin, was also confirmed by results obtained from the elution study, where it was observed that in order to elute the C-peptide from the Capto SP ImpRes resin, elevated salt concentrations were needed.

The elution study was performed using non-overloaded conditions and 20 µL resin volume. The enzymatically cleaved sample was loaded after addition of 10% ethanol. The factors studied were salt and ethanol concentration, keeping pH at 4 during elution. The yield of insulin obtained at all conditions investigated is shown in Figure 3A and the purity achieved is shown in Figure 3B. From the purity plot it is clear that the highest purity is achieved at intermediate salt conditions. A closer examination of the results shows that the contribution to low purity at high salt conditions is mainly caused by the C-peptide, which starts to elute at higher salt concentrations. In the region where the highest insulin purity was observed, the impurity profile predominantly showed presence of the truncated fragments.

By combining the yield and purity plots and setting the acceptance criteria above 80% and 77%, respectively, a window of operation for further optimization in column studies was identified (Fig 4). It should be mentioned here that while yield of 80% could be considered low when performing purification in a column format, yields at this level are often observed in plate studies. However, the effect of conditions on the relative yield, as identified in a plate format, are very informative as long as they are above 50% to 60% in the first elution step, because the higher the yield from that step the more narrow the elution peak is expected to be in a chromatographic run performed in a column format.

The conditions found in the batch study were used to design an experiment utilizing the design of experiment tools available in ÄKTA™ avant 25 chromatography system. Nine different combinations of ethanol and salt concentrations were investigated in a central composite circumscribed (CCC) design with three center points. The results were conclusive, resulting in a step elution at 47.5% ethanol and 130 mM salt for elution of insulin, at a purity of 91%. The C-peptide was eluted after elevating salt concentration to 1M and was thus not present in the insulin elution pool.

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**Fig 2.** Binding study on Capto SP ImpRes performed as repeated loading of crude sample, consisting of enzymatically cleaved pro-insulin (analysis of flow through fractions). The binding capacities found at fourth loading (overloaded conditions) indicate that the C-peptide binds as strongly as insulin.
Fig 3. Elution study on Capto SP ImpRes performed at a loading of 20 mg cleaved sample/mL resin at 10% ethanol and pH 4 as loading condition. (A) Yield of insulin after two repeated elution steps; (B) Purity after first elution step.

Fig 4. Overlay of yield above 80% (blue area) and purity above 77% (shadowed area)

Conclusions
Results presented in this work represent a very successful example of application of HTPD workflow based on combination of high-throughput screening, using PreDictor plates, followed by a final optimization of the purification step, studied in larger columns. The purification process developed following this workflow resulted in a successful scale-up purification (400 mL scale) of insulin on Capto SP ImpRes.
List of posters presented at HTPD 2012†

* Denotes author for correspondence.
† As listed in the HTPD 2012 conference program. Some differences between the list and the final posters presented might exist.

1. **Process development balancing solubility and partitioning aqueous two-phase extraction of proteins**
   Stefan Oelmeier
   Karlsruhe Institute of Technology, Engler-Bunte-Ring 1, Karlsruhe, Germany

2. **Characterization of lysozyme PEGylation reactions with a high-throughput approach**
   Benjamin Maiser*, Florian Dismer, and Jürgen Hubbuch
   Karlsruhe Institute of Technology, Biomolecular Separation Engineering, 76131 Karlsruhe, Germany

3. **Fluorescent protein as model systems for high-throughput bioprocess optimization: Chances and pitfalls**
   P. Rohe*, U. Krauss, K. Klein, W. Wiechert, and M. Oldiges
   1) Institute for Bio- and Geosciences, IBG-1: Biotechnology Forschungszentrum Jülich, Germany
   2) Institute for Molecular Enzyme Technology, Heinrich-Heine Universität, Düsseldorf, Germany

4. **High-throughput screening of HIC media in predictor plates for capturing recombinant green fluorescent protein from E. Coli**
   Charlotte Brink, Carina Engstrand, Eva Heldin, and Susanne Nyholm Westin
   Presented by Sara Ullsten
   GE Healthcare Life Sciences, Björkgatan 30, 751 84 Uppsala, Sweden

5. **Mechanism of interaction between proteins and multimodal chromatographic media**
   Nils Wallménius, Kristina Nilsson-Välimaa*, Enrique Carredano, Karol Łacki, Hans Rogl, Susanne Konrad, and Eggert Brekkan
   1) GE Healthcare Life Sciences, Björkgatan 30, 751 84 Uppsala, Sweden
   2) Roche Diagnostics GmbH, Penzberg, Germany
   3) Presently at Boehringer Ingelheim Pharma GmbH & Co, Biberach an der Riss, Germany

6. **Application of high-throughput technology in purification process development**
   Cindy Xin Li* and Yan-Ping Yang
   Bioprocess Research and Development, Sanofi Pasteur, Toronto, Canada

7. **High-throughput cell separation in aqueous two phase systems (ATPS)**
   Sarah Nagel*, Stefan Oelmeier, and Jürgen Hubbuch
   Karlsruhe Institute of Technology, Biomolecular Separation Engineering, 76131 Karlsruhe, Germany

8. **Issues encountered and key understanding for implementation of automated high-throughput process development**
   Nicola Roberts
   Downstream Process Development, Biopharma Process Sciences, UCB Pharma, U.K.

9. **How to establish a full scale design space**
   Conny Vikström
   Umetrics, Umeå, Sweden

10. **Quality by design and design space in formulation**
    Conny Vikström
    Umetrics, Umeå, Sweden
11 Use of multivariate technology to utilize the information content in several and connected data sources
Conny Vikström
Umetrics, Umeå, Sweden

12 HTPD for weak partitioning chromatography
Susanne Nilsson* and Bernd Kalbfuss-Zimmermann
Protein Processing, Novartis Pharma AG, Basel, Switzerland

13 Biosensor and chromatography based strategies for high-throughput measurement of protein self-association during formulation development
Krisztina Kovacs-Schreiner*, Olatomirin Kolade1, Brendan Fish2, and Daniel G. Bracewell1
1) Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, U.K.
2) GlaxoSmithKline, Barnard Castle, U.K.

14 Faster development of flocculation recipes in a high-throughput set-up
DSM Biotechnology Center, Dep. Downstream Processing, A. Fleminglaan 1, 2613 AK, Delft, The Netherlands

15 Automated platform for cell culture process development high-throughput analysis
Caroline Sellin*, Carole Borosvek, Eric Calvosa, and Jean-Marc Guillaume
Sanofi Pasteur Bioprocessing Research & Development-Upstream, 1541 Avenue Marcel Merieux, FR- 69280 Marcy l'Etoile, France

16 Keeping up with the screeners: High-throughput evaluation of newly discovered enzymes for product development
Angela Cifelli, Sang-Kyu Lee, Sandra Ramer, Doug Dale, and Sergey Paramonov*
Genencor / DuPont, 925 Page Mill Rd, Palo Alto, CA 94304, U.S.A.

17 Prediction of the dynamic binding capacity for a column chromatography from a filter plate based experiment
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Synthion Biopharmaceuticals BV, Microweg 22, 6503 GN, Nijmegen, The Netherlands

18 Effect of column size on determination of chromatographic selectivity using high-throughput experimentations
Charlotte Brink* and Eggert Brekkan
GE Healthcare Life Sciences, Björkgatan 30, 751 84 Uppsala, Sweden

19 Overcoming the analytical bottleneck in high-throughput process development MARS1 and ELECSYS™
Markus Haindl*, Michael Wiedmann, and Harald Wegele
Roche Diagnostics GmbH, Pharma Technical Development Europe Analytics (PTDEA), Penzberg, Germany

20 Strategy for implementing automated high-throughput process development for purification of a lysosomal enzyme
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**21** High-throughput screenexpertsm platform using robotics and analytics for the development of sorbent and membrane chromatography purification strategies  
Virginie Brochier*, Vincent Ravault, Anthony Schapman, and Clément Despres  
PALL BioSepra, France

**22** High-throughput viscosity measurement for assessing biologic formulations  
Presented by David Yamane  
Freeslate, Inc., Sunnyvale, CA, U.S.A.

**23** A parallel, microscale, automated approach to biologic formulation development and stress-test studies  
E. Carlson, S. Lambert, and S. Cypes  
Presented by Grant Gavaranovic  
Freeslate, Inc. Sunnyvale, CA, U.S.A.

**24** The hybrid experimental simplex algorithm (HESA) for ‘sweet spot’ identification in early bioprocess development: applications, challenges and recommendations  
Spyridon Konstantinidis*, Sunil Chhatre, Ajoy Velayudhan, Eva Heldin, and Nigel Titchener-Hooker  
1) The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, Torrington Place, London, United Kingdom, WC1E 7JE  
2) GE Healthcare Life Sciences, Björkgatan 30, 751 84 Uppsala, Sweden

**25** Membrane adsorber based HTPD for antibody purification  
Martin Leuthold, Louis Villain, and Claire Roulin*  
Sartorius Stedim Biotech GmbH, Göttingen, Germany
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