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Application Note

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This Application Note describes the use of the Ambr® Crossflow in combination with DoE software to design and run multiple parallel experiments to optimize EnPCs® quality and quantity in the downstream process.

Optimization of EnPCs® Purification With Ambr® Crossflow and MODDE®

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Abstract

NEUWAY Pharma has developed Engineered Protein Capsules (EnPCs®) as a promising system for delivering drugs into the brain. This Application Note describes the use of the Ambr® Crossflow in combination with MODDE® Design of Experiments (DoE) software to design and run multiple parallel experiments to optimize NEUWAY Pharma's EnPCs® quality and quantity in the downstream process. All UF | DF experiments were successfully performed using the Hydrosart® 300 kDa Ambr® CF Filter cassettes. The experiments showed the benefit of processing the EnPCs® in a UF | DF step as fresh product directly after harvest and clarification.

In addition, the results confirmed that adding Benzonase® prior to the UF | DF step was beneficial to both processing time and the quality and quantity of the product yield. Finally, using MODDE® software optimal buffer conditions (pH, NaCl, and L-arginine concentration) were defined.

This Application Note demonstrates that the combination of the Ambr® Crossflow with MODDE® provides a fast and cost-effective method to determine optimal ultrafiltration buffer compositions and pre-treatment conditions for NEUWAY Pharma's EnPCs®. The Hydrosart® 300 kDa Ambr® CF Filter cassettes used are well suited for the UF | DF of EnPCs®, yielding a high quality and quantity of EnPCs® in the final product.

For more information, visit
www.sartorius.com/ambr-crossflow

Introduction

Ambr® Crossflow

Benchtop tangential flow filtration (TFF) systems are commonly used as a scale-down model for UF | DF purification processes. While TFF systems can efficiently buffer the exchange of large volumes at one time, most systems are single-channel and require preparation, e.g., setup, cleaning, manual intervention between uses, etc. This limits TFF system efficiency when assessing a variety of parameters or producing many discrete formulations as is necessary when preparing formulation robustness studies.

The Ambr® Crossflow has 4, 8, 12, or 16, small-scale channels. Each is fully equipped to act similar to any traditional bench-scale TFF filtration set-up. The system is fully automated, with each channel independently controlled in terms of product input, buffer streams, and process conditions such as recirculation rate, pressure, load volume, diafiltration set point, and final product volume. The Ambr® Crossflow has a minimum recirculation volume of 5 mL and works with Ambr® CF single-use filter cassettes with a membrane area of 10 cm².

Being able to study the impact of process parameters, buffer types, and protein concentration on an automated, small-scale, high-throughput process allows scientists to determine if their biologic molecules can be formulated more cost-effectively.

Ambr® Crossflow Software

Ambr® Crossflow software has been designed to provide scientists with a user-friendly way to set up multi-parallel experiments. Recipe design is intuitive, with drag-and-drop, pre-programmed, flexible phases that allow researchers to design their own phases and recipes from scratch. Each channel operates independently, allowing individual control of process conditions, set points, and control strategy. The software provides some pre-programmed methods to enable the system to carry out routine tasks independently such as flux scouting or identification of the optimal diafiltration point to ensure optimal time savings and best recovery. Additionally, Ambr® Crossflow software can be installed on researchers' PCs so they can write process sequences at their desks before transferring to the control system.

Data is automatically collected and stored within the experiment folder, and can be viewed on the control system and from the user's PC with Ambr® Crossflow software installed. Alternatively, data can be exported as a .csv file and viewed using third-party data analysis packages (OPC control and data collection incorporated).

Visualization of multiple experiments in 'result viewer' allows scientists to look at data from different trials simultaneously to enable a better analysis of the impact of buffer type and protein concentration. MODDE® software can also be used to create and analyse DoE experiments for the Ambr® Crossflow system.

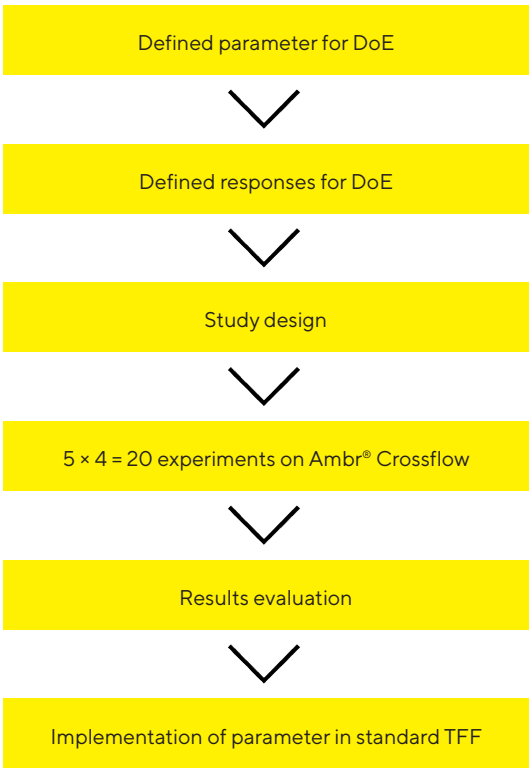
Ambr® CF Filter

The Ambr® CF single-use filter cassette has been designed to study the impact of buffer type and protein concentration, allowing researchers to explore a large experimental design space even at small-scale operation. This supports scientists in determining at a very early stage of development if their formulations will affect product quality.

In larger flat-sheet TFF devices, woven net spacers are applied. Due to material limitations, it is challenging to transfer those structures to the small-scale screening cassette. Thus, an alternative structure is used as an embedded spacer in the small-scale, single-use filter to mimic conventional spacers.

The embedded spacer structure was designed to achieve a high-mass transfer and low-pressure drop over the flow field, which is beneficial for processing high-viscous solutions up to 50 cP and above. The properties of the flow field prevent protein damage and maintain a constant shear rate at the whole membrane surface. To reduce void volume and avoid edge effects a single-layer membrane is integrated. The total membrane area is 10 cm².

Figure 1: Ambr® CF Filter



Umetrics MODDE® for DoE

MODDE® is a software tool for Design of Experiments (DOE). DOE is a rational and cost-effective approach to practical experimentation to quantify the effect of variables. Based on the structured approach, factors can be assessed using only the minimum of resources. In addition, for some questions it is not possible to describe the system's behavior in a closed mathematical form. Therefore, a step-wise statistical approach such as DoE is preferred for efficient quality by design (QbD) implementation strategies for upstream and downstream. The final specifications for a region where all specifications are fulfilled to a defined risk level is called Design Space .

Case Study

NEUWAY Pharma has developed Engineered Protein Capsules (EnPCs®) as a promising system for delivering drugs into the brain. This Application note describes the use of the Ambr® Crossflow in combination with MODDE® Design of Experiments (DoE) software to design and run multiple parallel experiments to optimize NEUWAY Pharma's EnPCs® quality and quantity in the downstream process. The experiments were performed using the Ambr® CF Filter Hydrosart® cassettes with a Molecular Weight Cutoff (MWCO) of 300 kDa.

Process conditions tested included the pH, NaCl concentration, and the addition of L-arginine and | or Benzonase®. Additionally, the influence of a freeze | thaw cycle on the ultrafiltration | diafiltration (UF | DF) process was investigated. The quality of the product after TFF filtration was defined by the protein contaminant removal rate and EnPCs® stability (polydispersion index and Inflection temperature).

This application note demonstrates that the combination of the Ambr® Crossflow system with MODDE® provides a fast and cost-effective method to determine optimal ultrafiltration buffer compositions and pre-treatment conditions for NEUWAY Pharma's EnPCs®.

Materials

EnPCs®

EnPCs® were manufactured by protein expression using a Sf9 insect cell line derived from the fall army worm (Spodoptera frugiperda) (Thermo Fischer scientific). EnPCs® were produced by infecting the cells with recombinant Baculovirus containing a John Cunningham virus VP1-protein expression cassette. The recombinant Baculovirus was prepared by using the Bac-to-Bac® Baculovirus expression system (Thermo Fischer Scientific). EnPCs® were produced at pH 6.3 in a 2L bioreactor (Biostat B, Sartorius). Air flow and temperature (26°C) were controlled over the time. To remove cells and cell debris, the suspension was centrifuged at 4°C, 5.000g, and the supernatant containing EnPCs® was harvested.

Methods

EnPCs® Characterization

For verifying EnPCs® stability, dynamic light scattering (DLS) and polydispersity index (PDI) were measured using a Zetasizer Nano ZS (Malvern). Additionally, inflection temperatures for each sample were assessed by nDSF using a Tycho NT.6 (Nanotemper). EnPCs® titre was measured with help of hemagglutination assay. To analyze sample composition, SDS PAGE was prepared.

Test Conditions

Ambr® Crossflow
A four-channel Ambr® Crossflow system was used in combination with 300 kDa Hydrosart® Ambr® CF single-use filter cassettes with 10 cm^2 filter area. The TFF experiments were set up using the pre-programmed Con-Di-Con phase provided by the Ambr® Crossflow software. TMP was set to 200 mbar with a feed flow of 10 mL/min. At the start of the experiment, the system pumped 100 g EnPCs® solution into the tank. After five minutes of recirculation, the permeate valve automatically opened and the first concentration step was initiated until a retentate weight of 25 grams was reached. Next, the diafiltration was started and the concentrate was diafiltrated against 16 Diafiltration (DF) volumes of buffer. A final concentration step started once the DF was finished, reducing the EnPCs® solution to a final volume of 5 mL.

Statistical Analysis

In the given case, DoE was applied to find optimal operating conditions for purifying EnPCs®. One of the driving forces to use DoE was that the procedure reduces the number of needed experiments to a minimum while getting maximal information from the gathered data. Therefore, we selected a screening design. Screening designs often will result in linear models with few experiments. They are a good way to get a first impression of the relevance of different factors while keeping the number of experiments low. It is possible to complement such a model in MODDE® with suggested interaction terms and higher-order terms in case they are needed. The need will be flagged automatically by MODDE®. Therefore, we decided to take a two-step approach with the factors in the table 1 of the following chapter and the corresponding linear and interaction terms of the regression function in the first step.

MODDE® Investigation Screening Design

The factors in the experimental setup could not be set deliberately, so it does not feature center points for all factors, and quantitative factors were tested on several settings (quantitative multilevel). The factors in Table 1 were investigated. A reduced combinatorial linear model with 16 experiments was proposed by the software (Table 2).

PH [pH]	NaCl concentration [mMol NaC]	L-arginine concentration [mMol Arg]	Freezing [fre]	Benzonase® [Ben]
Four steps quantitative multilevel	Four steps quantitative multilevel	Three steps quantitative multilevel	Qualitative (yes | no)	Qualitative (yes | no)

Table 1: Overview of the tested experimental conditions and plan

Exp No	pH	NaCl	Freeze	Benzonase®	L-arginine
1	5	30	no	yes	0
2	6	30	yes	no	500
3	7	30	yes	yes	250
4	8	30	no	no	250
5	5	120	yes	no	0
6	6	120	no	yes	0
7	7	120	yes	yes	500
8	8	210	yes	yes	0
9	5	210	yes	no	500
10	6	210	yes	no	250
11	7	300	yes	no	0
12	8	300	no	yes	500
13	5	300	yes	yes	250
14	8	30	yes	no	250
15	6	30	yes	no	500
16	5	300	yes	yes	250

Table 2: Initial experimental design (Screening)

The responses defined were permeate flux, titer, inflection temperature, and a first rough estimate of contaminants content after filtration (low | middle | high), divided into contaminants < 50 kDa and contaminants > 50 kDa. To be able to map this to a quantitative measure, the three steps (low | middle | high) have been translated into levels 0 | 1 | 2.

The TFF was performed with an Ambr® Crossflow system equipped with a Sartorius 300 kDa Hydrosart® crossflow membrane. Required diafiltration buffers were prepared and pre-filtered through a 0.2 µm filter. The non-frozen, EnPCs® containing supernatant was ready to use. The frozen EnPCs® supernatants were thawed and pre-filtered through 0.45 µm filter prior to TFF. The starting material for all TFF with both frozen and non-frozen supernatants consisted of 50 mL supernatant diluted with 50 mL of diafiltration buffer. The volume was reduced from 100 mL to 25 mL during the initial concentration phase. The product solution was then diafiltrated against 400 mL (16 diafiltration volumes (DV)), and final concentration step reduced the sample to 5 mL. Concentrated EnPCs® containing samples from the diafiltration were dialysed overnight against standard sample buffer (10 mM Tris-HCl, 150 mM NaCl pH 7.5) to ensure EnPCs® stability for storage and purification.

Results & Discussion

Figure 2 shows that the process time for the different experiments strongly varied, ranging from 12 hours to over 25 hours, depending on test conditions.

This shows the influence on processing time for the different conditions tested.

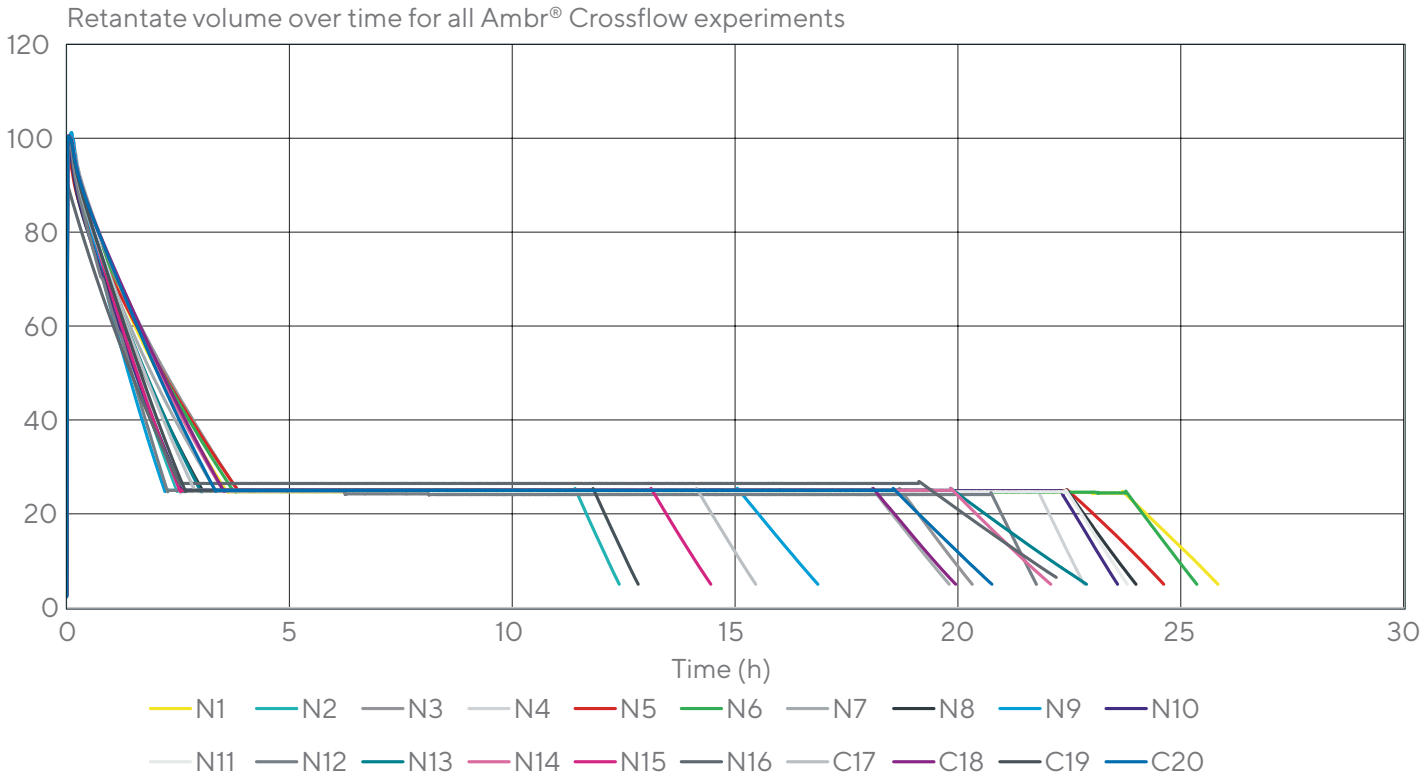


Figure 2: Retentate volume over time for all Ambr® Crossflow experiments.

An important result of the screening experiment was that obviously quadratic and interaction terms should be included in the model to improve it and to find better | more accurate settings for optimization. The following terms were suggested by MODDE® based on the screening results (excluding some of the terms not relevant for the respective responses).

This evaluation clearly showed that, for three responses, higher-order terms are relevant. It also shows that pH does not have a high-order effect. Therefore, the complement experiments only need to focus on the sodium chloride and the L-arginine. The plots below always come as set of two: freeze = yes | no. Working with unfrozen material doesn't change the nature of the model; it does scale the responses (Table 3).

Response	Model Terms (abbreviation, see Table XY)	Potential Higher-Order Term (to be confirmed with additional experiments)
Titer	NaC, Arg, NaC*Arg	NaC*NaC, Arg*Arg
Inflection Temperature	NaC, fre, Arg	Arg*Arg
Contaminants > 50 kDa	NaC, Arg, , NaC*Arg	NaC*NaC, Arg*Arg
Contaminants < 50 kDa	pH, NaC, fre, Ben, Arg	

Table 3: Significant model terms after screening

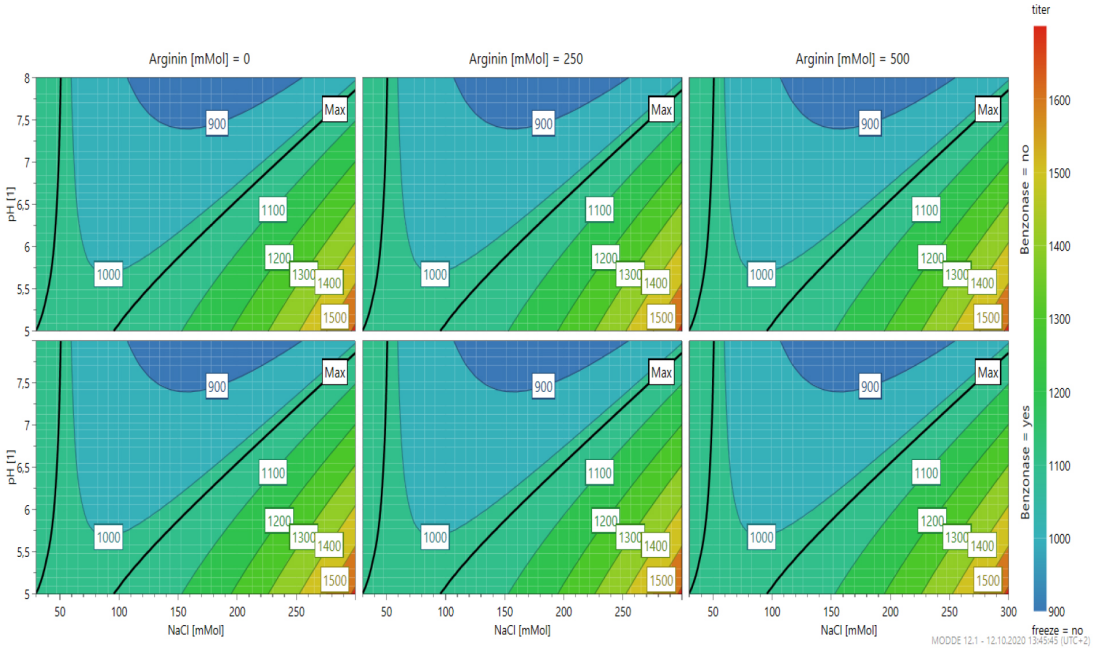


Figure 3: 4D response contour plot of titer, with unfrozen material (screening model)

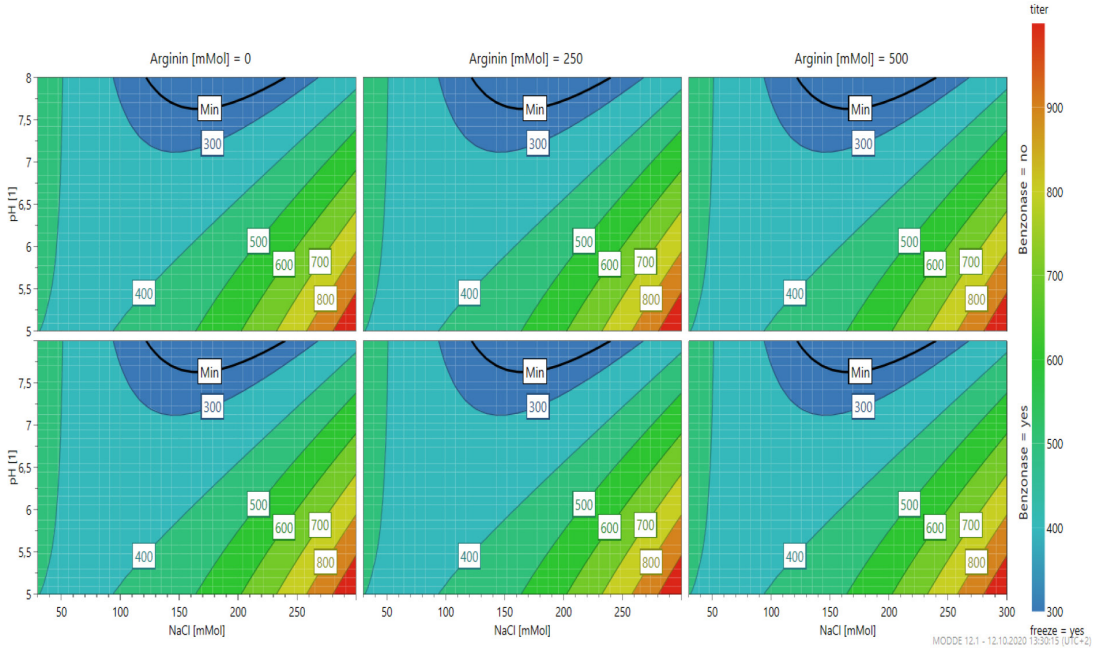


Figure 4: 4D response contour plot of titer, with frozen material (screening model)

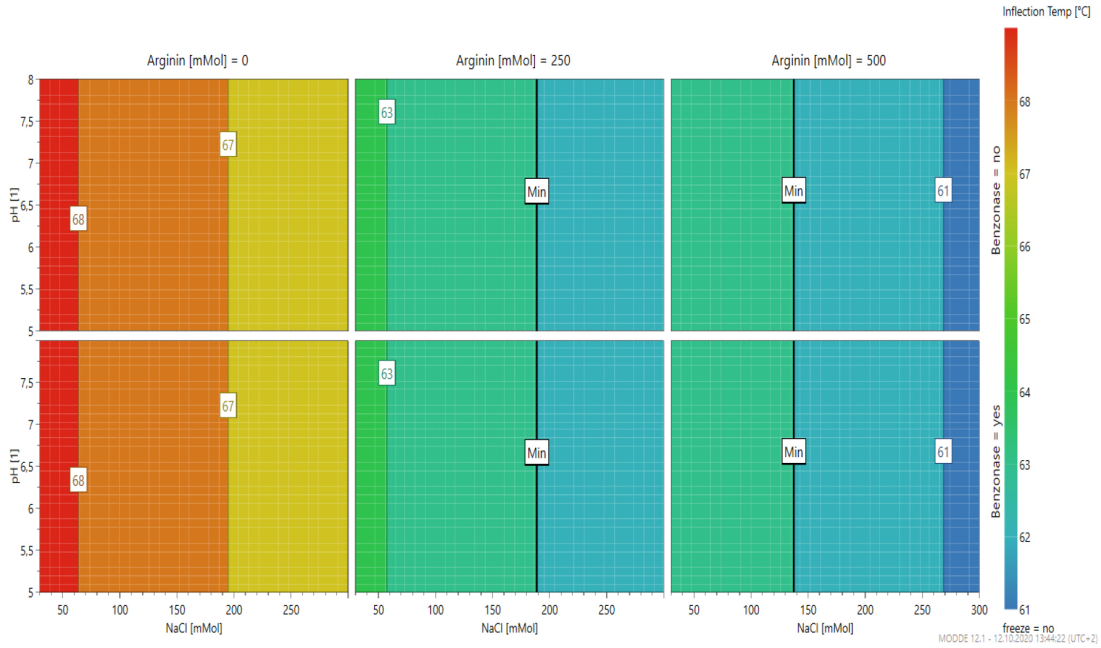


Figure 5: 4D response contour plot of unfrozen material - inflection temperature (screening model)

The evaluation of the data showed that the pH factor in this model was of minor influence due to the high numerical error. The pH was taken out as factor in this first step. As a result, the graph shows straight vertical contour lines without any slope.

Note that the complemented experiments (Table 4) and the enhanced evaluation of the experimental results as described below reintroduced linear and quadratic terms for pH that were not visible at this stage.

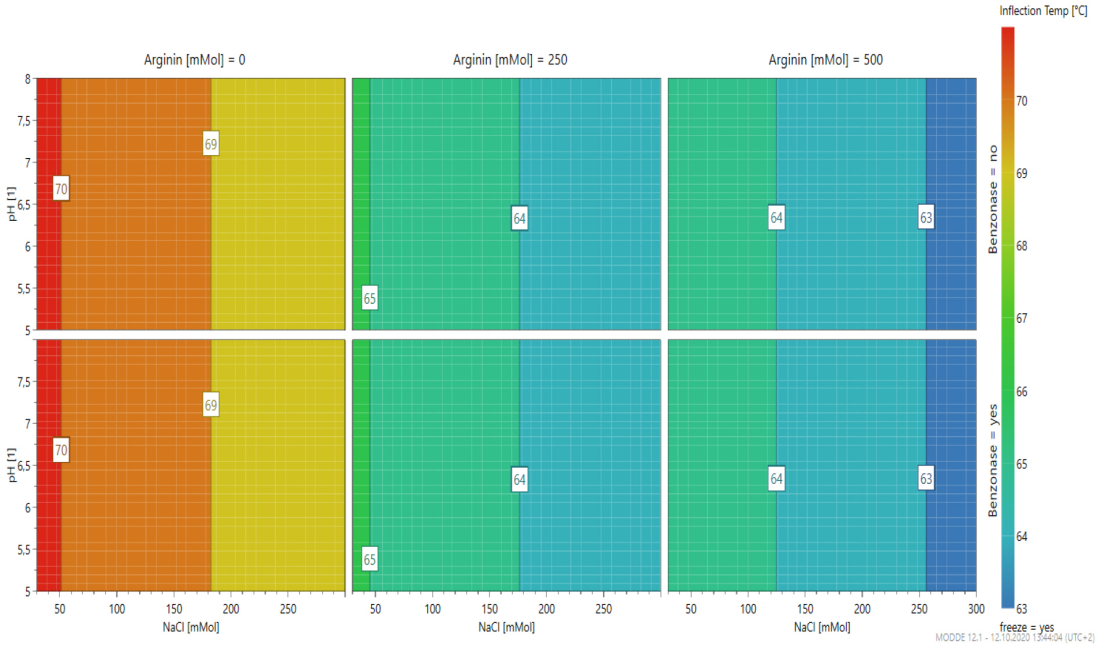


Figure 6: 4D response contour plot of frozen material - inflection temperature (screening model)

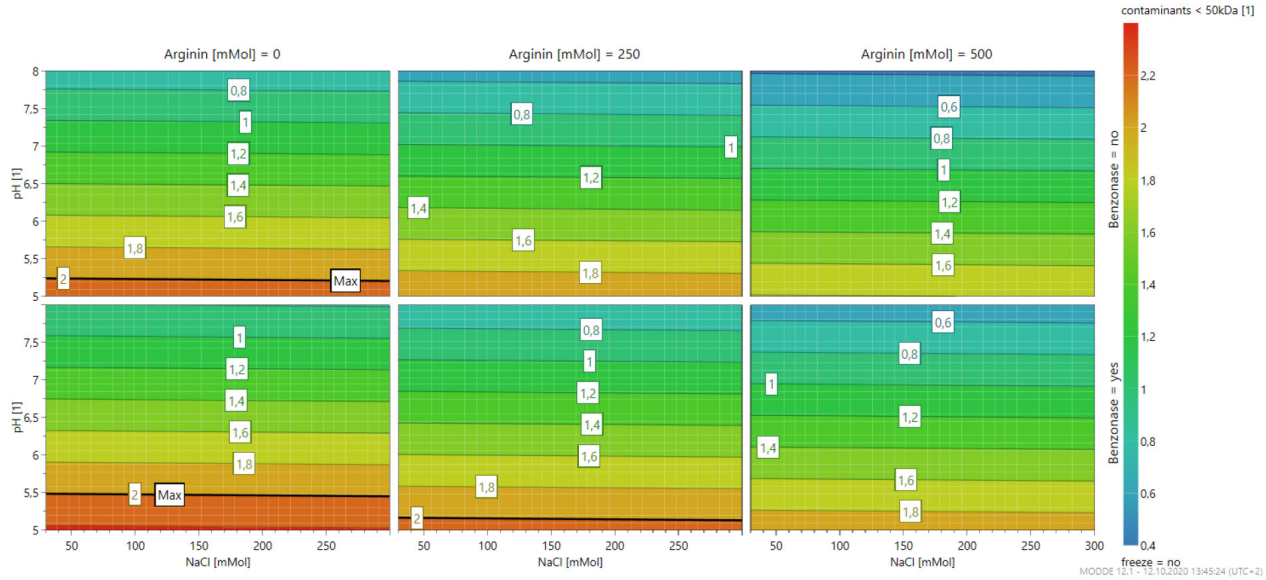


Figure 7: 4D response contour plot of unfrozen material - contamination < 50 kDa (screening model)

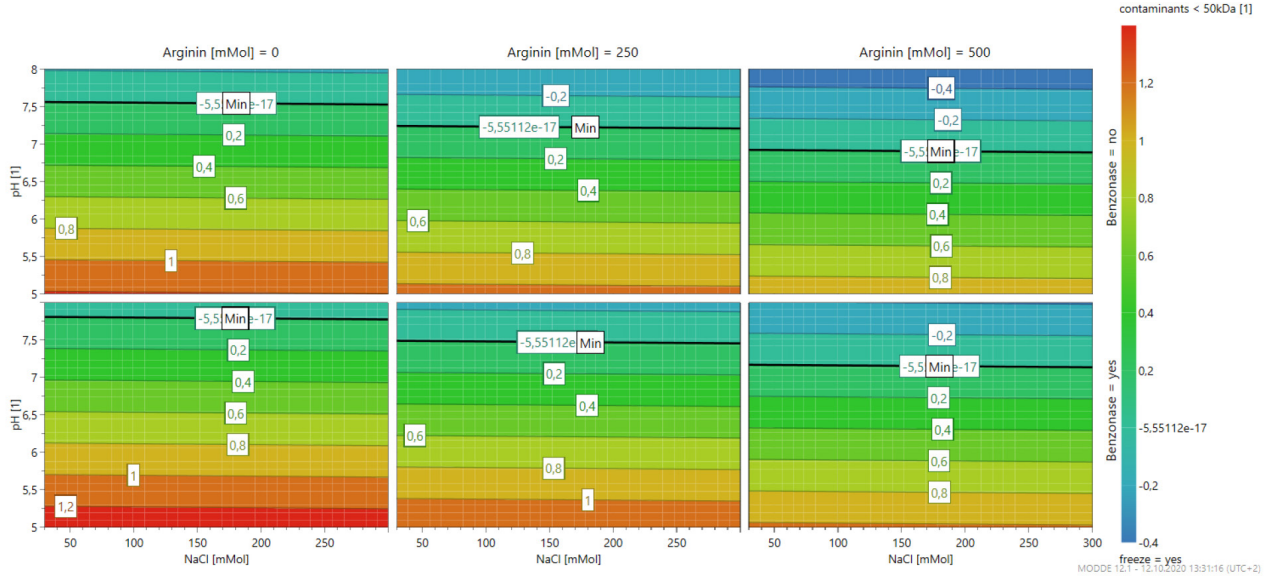


Figure 8: 4D response contour plot of frozen material - contamination < 50 kDa (screening model)

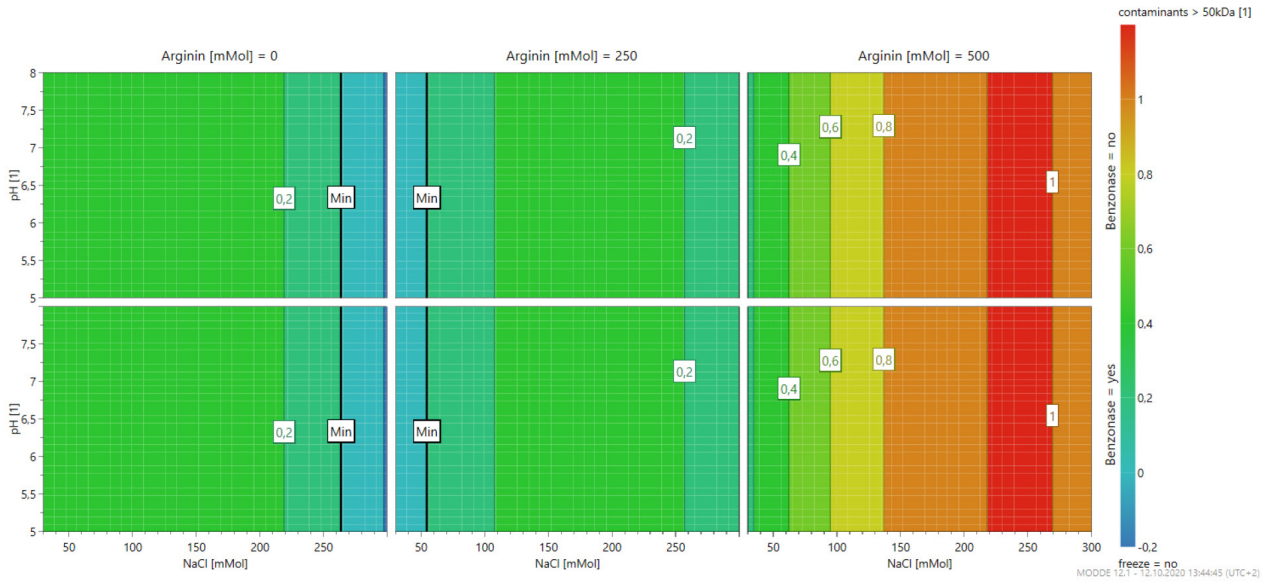


Figure 9: 4D response contour plot of unfrozen material - contamination > 50 kDa (screening model)

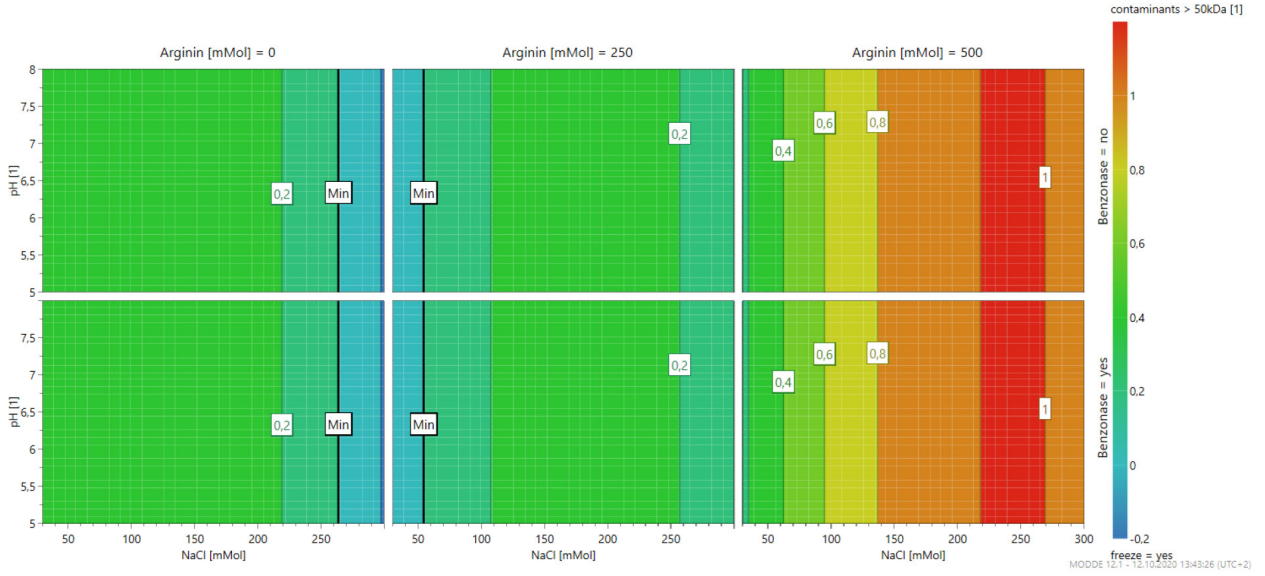


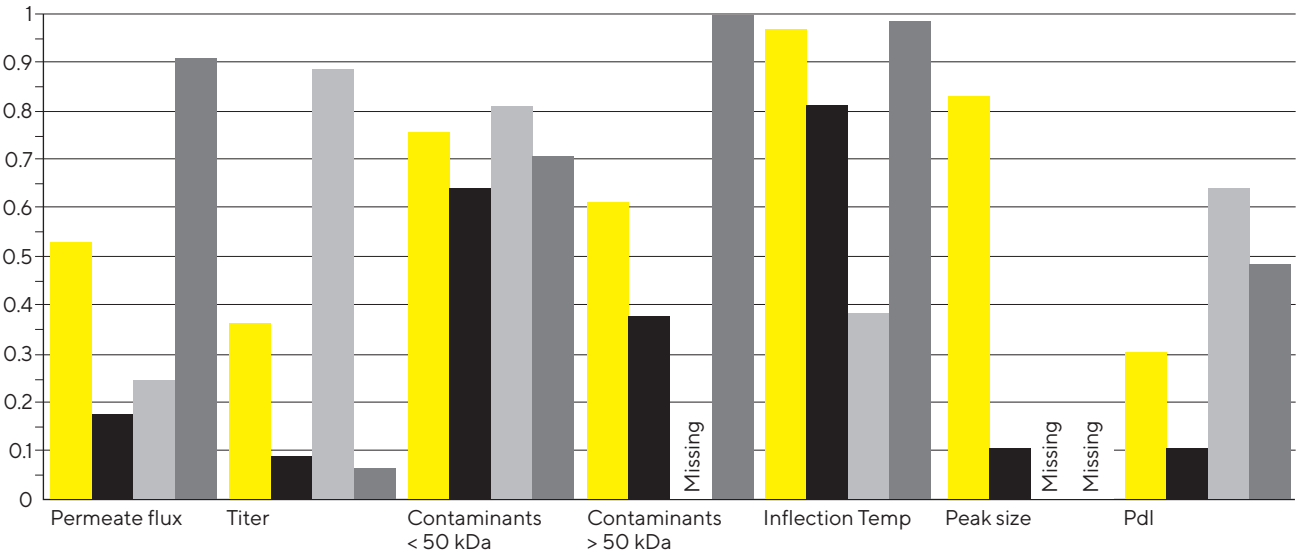
Figure 10: 4D response contour plot of frozen material - contamination > 50 kDa (screening model)

Complemented Model and Optimization

In order to get a better resolution for the higher-order terms, the experimental plan was complemented by experiment C17-C21.Exp No	pH	NaCl	Freeze	Benzonase®	L-arginine
17	7	300	yes	no	0
18	7	210	yes	no	0
19	7	210	yes	no	250
20	7	30	yes	no	250

Table 4: Complement experiments for initial screening design.

Additional responses for peak size and polydispersion index were added to the evaluation to monitor the difference between sample composition and particle size after TFF.



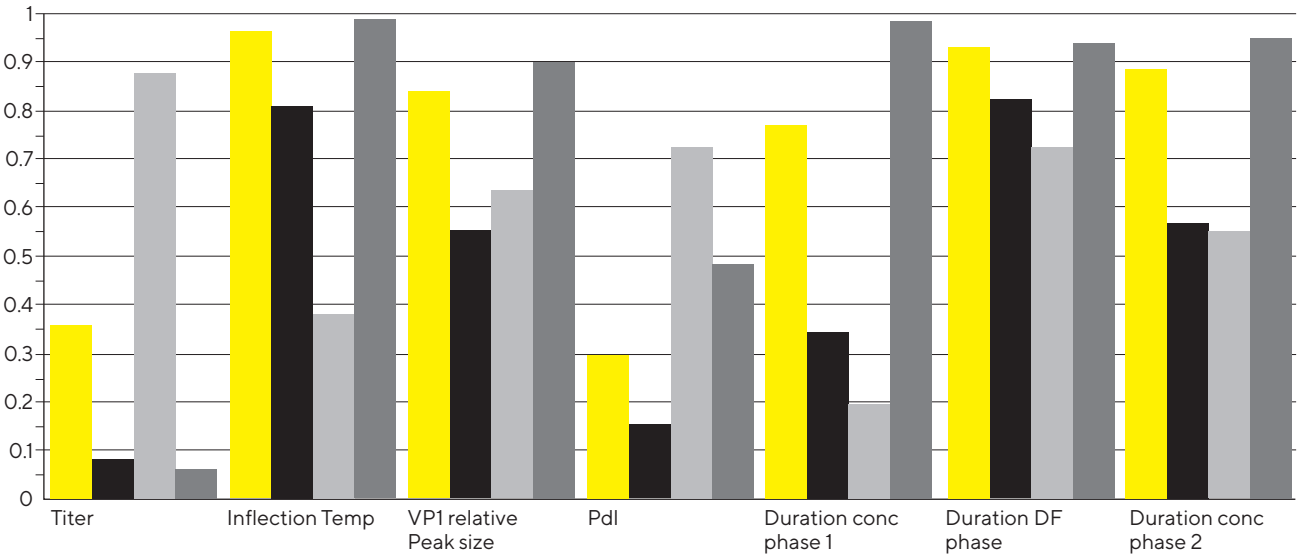
permanente flux (N=20, DF=13, R2=0), Titer (N=20, DF=14, R2=0, 36), contaminants < 50 kDa (N=20, DF=13, R2=0, 75), contaminants > 50 kDa (N=20, DF=14, R2=0, 61), InflectionTemp (N=20, DF=12, R2=0, 96), Peak size (N=9, DF=0, R2=0, 84), PdI (N=9, DF=0, R2=0, 84)

R2 Q2 Model validity Reproducibility

Figure 11: Summary of Fit (PLS) - complemented model

From the summary of fit plot of the resulting models, it was obvious that the experimental evaluation should be enhanced. The permeate flux response measurement at the beginning of the filtration needed time to level off and was error-prone due to one single value taken.

Instead of measuring the flow at one point in time or taking an integral norm of the trajectory over time it, we decided to describe the objective in a different way: find the set of factors that gave the lowest times for concentration and diafiltration. This was the description that led to a more stable model for the flux optimization.



Titer (N=20, DF=14, R2=0, 36), Inflection Temp (N=20, DF=12, R2=0, 96), VP1 relative Peak size (N=20, DF=13, R2=0, 85), PdI (N=20, DF=15, R2=0, 30), Duration conc phase 1 (N=16, DF=11, R2=0, 77), Duration DF phase (N=16, DF=8, R2=0, 94), Duration conc phase 2 (N=16, DF=8, R2=0, 88)

Figure 12: Summary of Fit (PLS) - complemented model including updated responses

The EnPCs® titer measurement used in the first model was stepping up by the power of two (result sorted into bins). To get more accurate measurements, the grey-scale values of the images generated for EnPCs® titer determination were fed into a script to calculate the inflection point of a sigmodal curve fitted to the values. This resulted in an enhanced statistical model for that response.

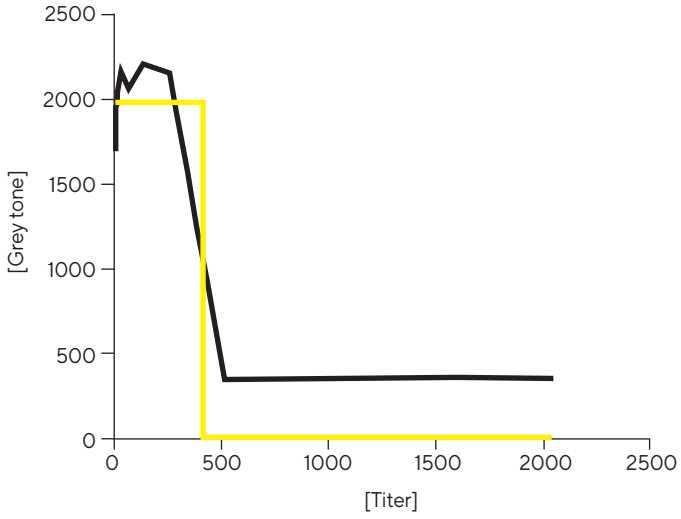


Figure 13: Derived titer values from image data (grey tone color change)

The important qualitative factor "contamination" was statistically weak in the existing model. Using numeric levels for the contaminants (0 for low to two for high) gave a first idea of influencing factors. However, since this was only a rough estimate, it was clear that the resulting statistical model would be weak. In order to enhance this situation, in the second approach the image data of the SDS PAGE gels were evaluated with the help of ImageJ and were used to calculate the relative measure of VP1-protein concentration and contaminants (in the plot below labeled "VP1 relative Peak Size"), which resulted in a stronger model that we used for the final optimization.

Optimization

For optimization, we decided to complement the screening design to address the following three objectives:

- Enhance the promising results with higher-order terms.
- Redefine the problematic responses and add additional ones that were of interest for final product quality. The new optional response Polydispersity (PdI) gave an acceptable model, although the span of measured values and accuracy were low. Since this was not in the focus of the investigation but rather an interesting topic for future experiments, it was a valuable outcome.
- Enhance the experimental evaluation.

For process optimization, we made two basic decisions. The choice between using a freeze step or fresh material is fundamental and not easily changed. Therefore, we chose to split the optimization for the two cases--"freeze" and " no freeze"--where the factor is set constant for each case. We also decided to exclude the addition of Benzonase®, which was also a qualitative factor (yes | no) for the optimization since the contour plot showed that using Benzonase® is beneficial for the process in every aspect.

Optimization Results

We see in the plot the setpoint and the suggested operating ranges narrow down to a sharp band of values when running the process with a freeze-and-thaw step in between. Due to the number of factors (three), the optimization result cannot be shown in one 2D-plot but has been split into two where the third factor was set to the constant determined by the optimizer functionality in MODDE®.

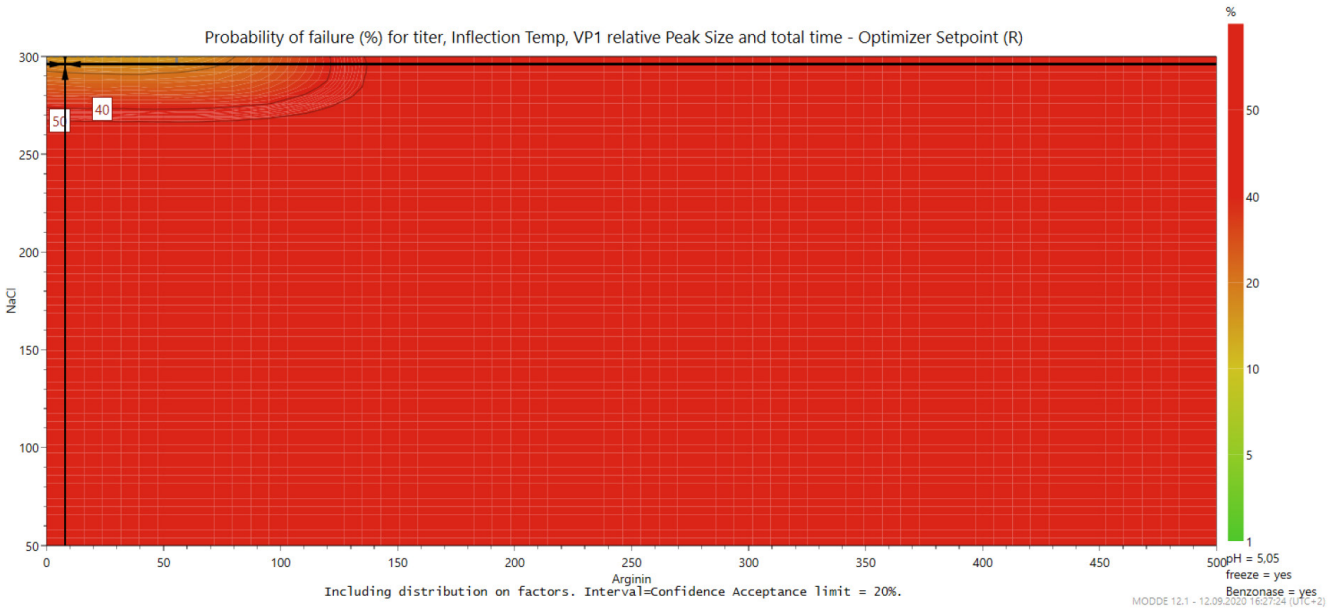


Figure 14: Design space and setpoint calculation - frozen material (pH constant: 5,05)

As a result, we see that L-arginine should be added, but with care, in order to sustain particle stability. A high salt concentration especially had a beneficial effect on the titer, and the saturation point at which it might have negative effects on the process was not reached in our experiments. A low pH value at 5.05 was found optimal.

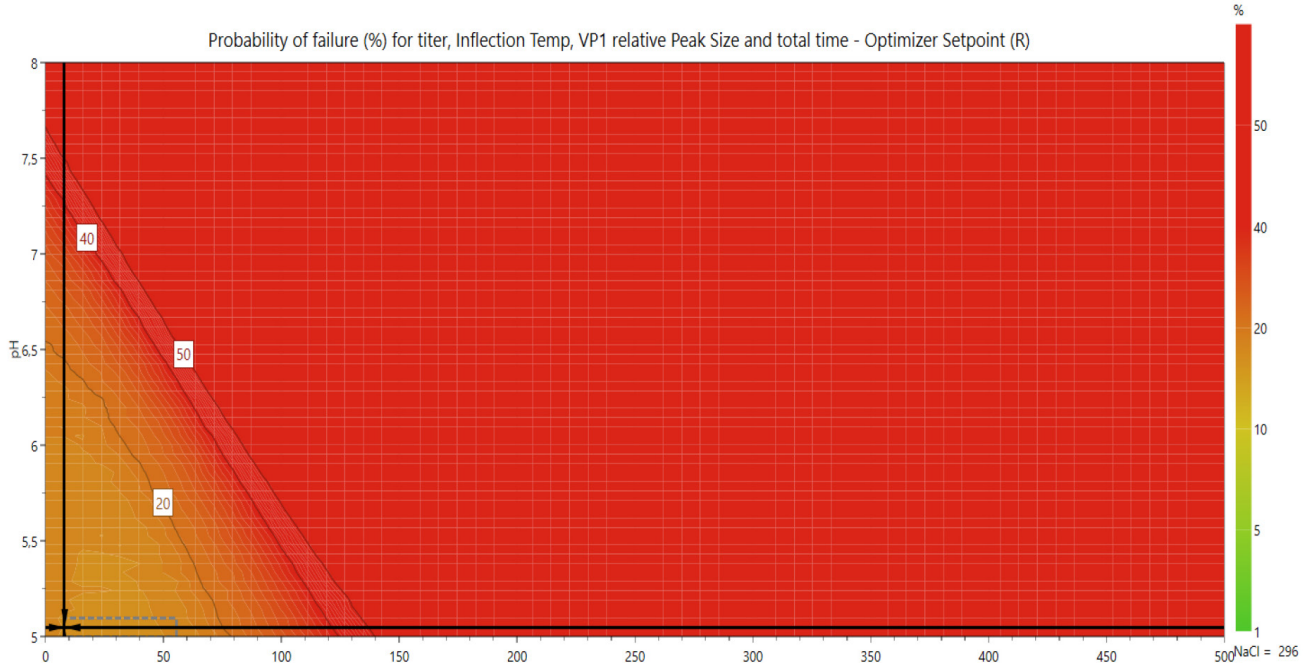


Figure 15: Design space and setpoint calculation - frozen material (constant values for NaCl: 296 mMol)

Working with unfrozen material makes the overall process more complex since the time between the upstream and downstream phases must be characterized and defined. Planning both phases must be aligned with respect to the amount of harvest material to be processed.

However, the product quality is far better, and working with unfrozen material extends the design space and gives more operative freedom. Again, L-arginine was shown to help, but with a dosage at the lower end of our experimental plan, and the pH value was at the lower end, with optimum at 5.52.

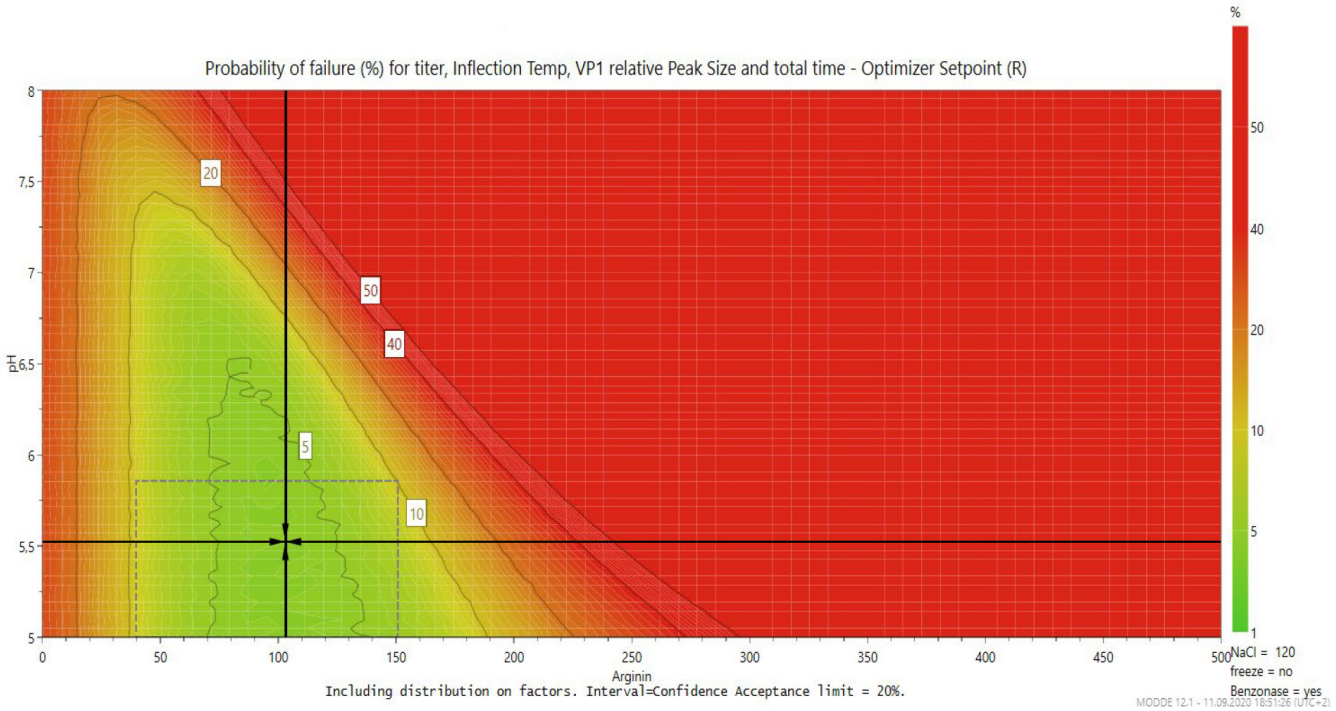


Figure 16: Design space and setpoint calculation - unfrozen material (constant values for NaCl: 120 mMol)

From what we see in the lower plot, we have two optimal values. This is more a side effect of the fact that we were optimizing several responses at the same time – some of them being less important e.g., fast processing time (business risk) versus the quality of the end product (manufacturing risk). We can interpret the whole area from the top left corner to the left middle as one corridor of operating options.

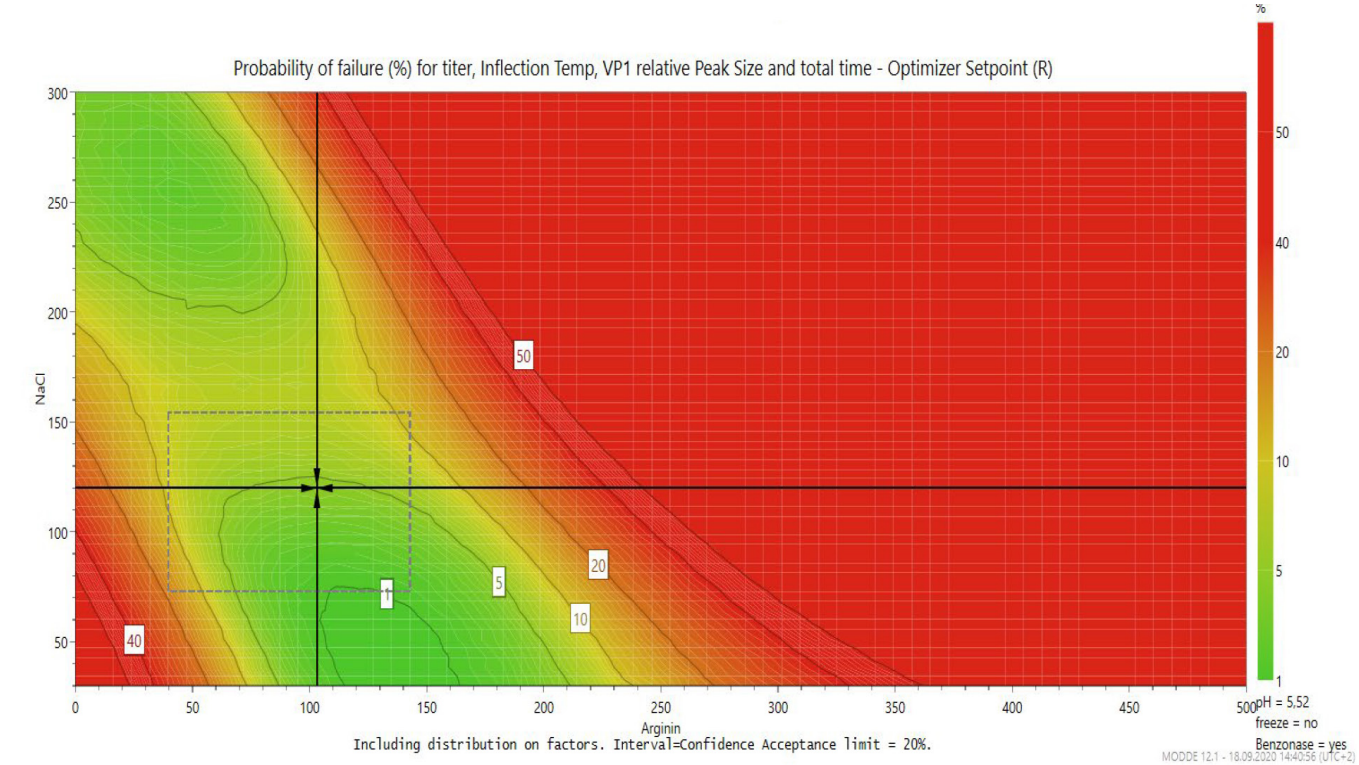


Figure 17: Design space and setpoint calculation - unfrozen material (pH constant: 5,52)

Conclusion

The goal of the described study was to optimize NEUWAY Pharma's EnPCs® quality and quantity in the downstream process using the Ambr® Crossflow multi-parallel TFF system in combination with Umetrics MODDE® DoE software. The study showed a clear benefit from processing non-frozen EnPCs® supernatant as the efficiency, quality, and quantity for the product was superior under most conditions tested when compared to material that had been frozen prior to the UF | DF step. As expected, the Benzonase® treatment also strongly improved the EnPCs® quality and quantity.

Further analysis using MODDE® software defined the optimal buffer conditions with respect to pH and NaCl. Also, the addition of low amounts of L-arginine to the solution further improved the final product.

The study demonstrated the power of the Ambr® Crossflow in combination with Umetrics MODDE® software for buffer optimization studies leading to increased EnPCs® product quality and quantity. The Hydrosart® 300 kDa Ambr® CF Filter cassettes are well suited for the UF | DF of EnPCs®.

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