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An *in-silico* approach towards multivariate acceptable ranges in biopharmaceutical manufacturing

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Abstract

Multivariate interactions between process parameters can heavily impact product quality and process performance in biopharmaceutical manufacturing processes. Thus, multivariate interactions should be identified and appropriately controlled. This article describes an *in-silico* approach to establish multivariate acceptable ranges; these ranges help to illustrate the combined impact of multiple input variables on product quality and process performance. Additionally, this article includes a case study for a monoclonal antibody polishing application.

Proven acceptable ranges are set by changing only one input parameter at a time while keeping all others constant to understand the impact of process variability on product quality or process performance, but the impact of synergistic variables are not evaluated. Within multivariate acceptable ranges, any combination of input parameters of a unit operation yields the desired product quality and process performance. The layered approach applied in this article is based on risk assessment and statistical models to leverage prior knowledge and existing data. The risk assessment is specific for a manufacturing facility but is applicable to multiple products manufactured in the same facility. No additional wet-lab experiments are required for building the statistical models when development and process characterization are executed using a design of experiments approach, compared to a univariate evaluation of data. The established multivariate acceptable range justifies revised normal operating ranges to ensure process control. Further, the determination of multivariate acceptable ranges adds to overall process knowledge, ultimately supporting the implementation of a more effective control strategy.

Keywords Biopharmaceuticals, Control strategy, Multivariate interactions, *In-silico* calculation, Process characterization, Risk assessment, Design space

Introduction

Various institutions, such as the International Council for Harmonization (ICH) and the United States Food and Drug Administration (FDA), have published guidance for development and validation of pharmaceutical processes. Further, regulatory agencies, pharmaceutical industry and academia collaborate to improve clarity and common understanding. Such working groups are another source for conceptual guidance (Glodek et al. n.d). Published papers describe key concepts and authority expectations for regulatory submissions. For instance, ICH Q8 (R2) introduces the Quality by Design (QbD) concept (Guideline and Q8 (R2) Pharmaceutical Development. n.d). QbD is a systematic approach to development and manufacture of biopharmaceuticals (Yu et al. 2014). Process design and understanding are integral parts of the QbD concept. To this end, process characterization studies are

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conducted in late-stage process development. Process characterization studies aim to ensure process robustness and process control by investigating the impact of previously identified relevant process inputs on process outputs. Relevant process inputs include potentially critical or key process parameters, whereas process outputs are critical quality attributes and process performance indicators (Mitra and Murthy 2022). In this context, proven acceptable ranges (PARs) are defined. According to the ICH definition of the PARs (Guideline and Q8 (R2) Pharmaceutical Development. n.d; Patil and Pethe 2013), this is a characterized range of a process parameter meeting acceptable product quality and process performance, while all other process parameters are held constant. Deliberate changes of one input parameter are possible within PARs. In contrast, authorities expect normal operating ranges (NORs) to represent only the uncontrollable manufacturing variability, rather than introducing manufacturing flexibility (Questions and Answers: Improving the Understanding of NORs, PARs, DSp and Normal Variability of Process Parameters. n.d). Additional flexibility in the manufacturing conditions requires justification by means of a design space. A design space, as defined in (ICH guideline Q8(R2), assures quality for a multidimensional combination of process inputs (Guideline and Q8 (R2) Pharmaceutical Development. n.d). The design space can be filed in regulatory submissions. Within an approved design space, manufacturers can adapt the operating space of the commercial process (Cooney et al. n.d). Such alterations of the commercial operating space do not require authority review or approval. Design spaces may apply to one unit operation or a complete process. If no such mitigation is in place, process input settings should be controlled at target operating conditions/within tight NORs.

ICH and regulatory bodies have shared the concept of a design space for more than a decade. The FDA's guidance for industry paper "Process Validation: General Principles and Practices" recommends design of experiment (DoE) studies to investigate multivariate interactions (Services, U.S.D, n.d). According to the FDA's guidance document, understanding multivariate interactions is relevant process knowledge to ensure effective process control. The PAR does not sufficiently support multivariate scenarios; potential multivariate interactions of parameter excursions are generally not considered for the definition of PARs. This is because PARs are established based on univariate analysis. Despite the limitations associated to PARs, manufacturers seem to adopt multivariate concepts rather hesitantly. Horst et al. investigated the implementation of QbD in EU marketing applications between 2014 and 2019 (Horst, J.P. 2021). Only around one third of all dossier submissions uses full QbD application and only around 22 % of these submissions are biotechnology-derived products.

The hesitant use of multivariate data evaluation is not specifically related to the biopharmaceutical industry. Looking at other industries, such as the service industry, chemical industry and the automotive industry in the 2000's, most statistical evaluation methods in quality engineering practice were univariate (Yang and Trewn 2004; Yang n.d). In the meantime, these industries have adopted statistical tools for multivariate data analysis. Applications focus on data-driven fault detection and diagnosis (Qin 2012). Recent advancement in artificial intelligence may further support this trend. Biegel et al. use deep learning to enhance multivariate process control approaches for reconstruction of errors in a sheet metal forming process (Biegel et al. 2022).

In biopharmaceutical processes, the multivariate analysis aims to establish an allowable parameter range, reproducibly yielding product with acceptable quality and process performance. Many articles assert that an established design space can improve process quality and flexibility, but the number of publications specifically describing an application methodology in the biopharmaceutical industry is limited (Lee et al. 2022). This could be due to the required effort and complexity. If the number of relevant process inputs is high, extensive wet-lab experiments need to be conducted to characterize multivariate interactions and to establish a design space. Statistical models aim to reduce the number of wet-lab experiments that are required to investigate a given multi-parameter space (Politis et al. 2017; Kontoravdi et al. 2013; Madenius and Brunding 2008). Nevertheless, establishing a design space yielding acceptable product quality and yield may require iterative DoE studies (Horvath et al. 2010). Abu-Absi et al. establish a design space for a cell culture process (Abu-Absi et al. 2010). In an extensive study comprising several DoEs, the authors investigate how the identified worst-case cell culture conditions extend to product quality in purified mAb pools. Jiang et al. establish a design space for a preparative hydrophobic interaction chromatography step (Jiang et al. 2010). Contour plots describe correlations of two process inputs, while other process inputs are set at their worst levels. Finally, the design space is defined from a combination of the plots, each correlating 2 process inputs. As the number of process inputs increases, this exercise becomes more complex. Nagashima et al. enhance statistically derived contour plots with Monte Carlo simulations (Nagashima et al. 2013). The presented case evaluates four input parameters and four responses in a cell culture process. Amadeo et al. evaluate multivariate interactions in the purification process of biopharmaceuticals. Similar to previously referenced publications,

correlations of two process inputs are investigated. However, the described strategy systematically identifies the design space (Amadeo et al. 2014). In several evaluation rounds occurring in parallel, interactions are evaluated between every combination of two process inputs. This procedure is repeated for every process output. All process outputs are assigned a relative importance. Finally, a desirability function selects the most suitable parameter setting. This optimization strategy involves mutual interactions between two process inputs. Further, desirability functions return optimal process conditions rather than an allowable parameter space. For this reason, confirmation of a suggested multivariate parameter space can be useful. In general, the number of process inputs affects computational complexity. At some point, alternatives to commonly applied response-surface models become attractive. Huang et al. suggest the complimentary utilization of principal component analysis and partial least squares (Huang et al. 2009) to reduce dimensions. This methodology enables evaluation of multivariate interactions between many process inputs.

Application examples for multivariate analysis extend to the definition of multivariate acceptable ranges (MAR) (Horvath et al. 2010; Wurth et al. 2016).

In this study, the MAR is defined as a parameter range that yields acceptable product quality and process performance, while other relevant process parameters can be held at any setting within their corresponding MARs. According to this definition, the MAR is a control element comprised of process inputs. This includes process parameters and material attributes. MARs are established unit operation-wise. Ideally, the MAR equals the PAR. In this case, all combinations of input parameter settings within PAR yield acceptable product quality and process performance. In any case, the MAR needs to support the NOR to ensure robust manufacturing at normal operating conditions. Further, the MAR could also justify NORs wider than the uncontrollable manufacturing variability observed in the limited data sets normally available at Investigational New Drug Application and or Biologics License Applications submissions. However, the MAR as defined in this study is not intended for regulatory filing.

The herein described approach is based on DoE and statistical models (e.g. linear mixed models). This approach may be used with other model types. Infact, it can be used for every model type in which the critical quality attributes can be described by the process parameters. The approach leverages prior knowledge to reduce computational complexity. All process inputs previously identified as potentially critical or key in a general failure mode and effects analysis are again assessed with regard to potential multivariate interactions.

Materials and methods

The MAR assessment comprised three major parts. As a first step, relevant process inputs were identified. The identification of relevant process parameters utilized a risk assessment approach. Details of this process are described below in the "Parameter selection". The relevant process parameters were included in an *in-silico* calculation of MARs. For the applied methodology, see section "In-silico calculation". The calculated MARs were then compared to the NORs. If MARs were tighter than the NORs, mitigations were put in place to ensure appropriate process control. Fig. 1 provides a high-level overview of the approach.

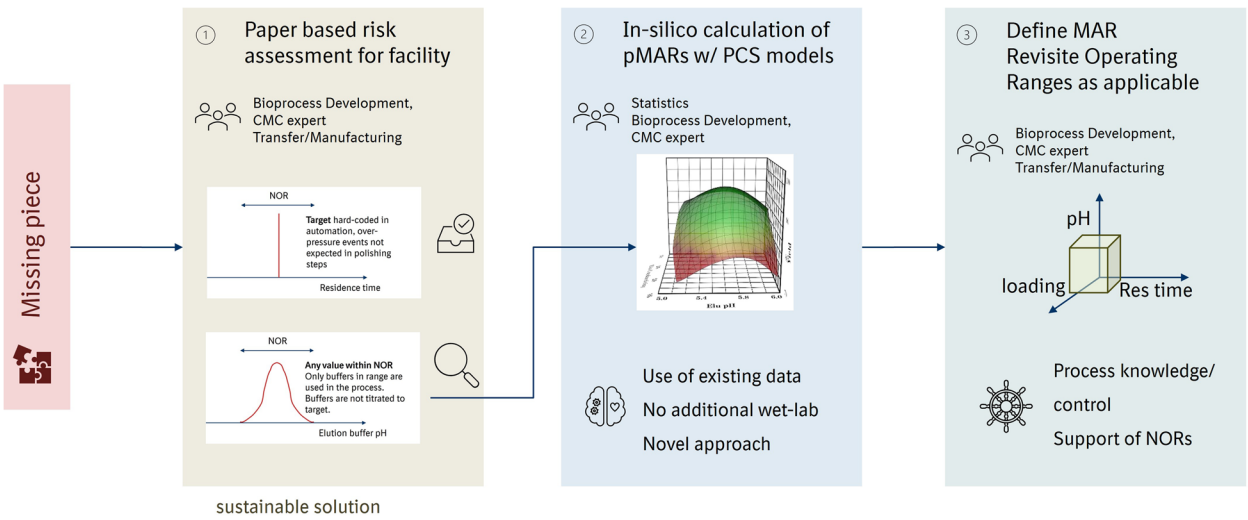

Fig. 1 MAR establishment approach. The three different steps are summarized in the grey, blue and green boxes

The approach was applied to a purification process for a monoclonal antibody (mAb). The purification process included 3-column chromatography and several filtration unit operations. The mAb was captured using Protein A affinity chromatography. Polishing steps were based on cation and anion exchange chromatography. In general, the approach could also be applied to other process steps in biopharmaceutical manufacturing.

Parameter selection

The process inputs included in the MAR evaluation were selected based on a risk assessment. Only relevant multivariate scenarios were considered in the *in-silico* calculation. This focus on relevant multivariate scenarios reduced complexity and saved computational capacity.

Appropriate justification to exclude or include process inputs in the MAR evaluation was based on three criteria: read-outs from large-scale runs; the probability of excursions off target; and the potential to interact with other process parameters. The process parameters were assigned relevant or non-relevant with regards to multivariate interactions. Relevant process parameters were included in the *in-silico* calculation. Non-relevant process parameters were set to their target setting for further evaluation. Table 1 lists the included process parameters of the elution phase from a cation exchange step for a mAb. The table provides justification and the outcome of the risk assessment.

As described in Table 1, elution buffer pH, elution buffer conductivity and loading density were assigned relevant for multivariate interactions. Peak collection criteria and the residence time were not relevant for the evaluation of MARs. Peak collection start and stop are governed by the automation software. As soon as the UV signal exceeds the pre-defined limit, the software switches the valve position. Hence, accuracy of this process parameter is ensured. Similarly, flow rates are hardcoded in the automation system. Unless unexpected overpressure events occur, flow rates are operated at target conditions. Potential overpressure events could result from resin fines or precipitates that block the column frits or detoriate the column packing. Based on platform experience with the resin and column packing, overpressure events are highly unlikely. Read-outs of the operational variability (data not included) further confirmed the provided rationales.

The applied risk assessment reduced the number of relevant input parameters for the evaluation of MARs. The further evaluation included the capturing step, acid treatment, the other polishing step and the formulation unit operation. As an outcome of the risk assessment, a maximum of 5 process parameters was evaluated per unit operation.

Table 1 Risk assessment for relevant process parameters of the elution phase from a mAb polishing step

Process Parameter	Mitigation	Relevant for multivariate interactions
Peak collection criteria	Peak collection is governed by automation. Peak collection starts/stops as soon as the criterion is reached. No variation, only neglectable variation due to rounding of read-out, e.g. peak collection start criterion is 0.2 OD and read-out is 0.21.	no
Elution buffer pH	Buffer pH is determined through the addition of buffer components. The range of adding buffer components is tight and well-controlled. Buffer pH is controlled through off-line measurements. However, buffer pH is not titrated to target. Any buffer meeting the specified pH range is used in the process. Buffer pH is well-known to interact with buffer conductivity in cation exchange chromatography.	yes
Elution buffer conductivity [mS/cm]	Buffer conductivity is determined through the addition of buffer components. The range of adding buffer components is tight and well controlled. Buffer conductivity is controlled through off-line measurements. However, buffer conductivity is not titrated to target. Any buffer meeting the specified conductivity range is used in the process. Buffer conductivity is well-known to interact with buffer pH in cation exchange chromatography.	yes
Residence time	Residence times are a result of flow rates and column dimensions. Residence times are normally operated at target. Flow rates are hardcoded in the automation system and will be adjusted for any column re-pack. Higher residence times resulting from over-pressure regulation are highly unlikely at the given particle size and with pre-purified pools. Shorter residence time than target will not be used in the process.	no
Loading density	Column loading is mainly determined by process design, scale (dimension of column, dimension of bioreactor) and previous unit operations. Higher/lower titer or higher/lower volume can lead to changes in column loading. Conductivity and pH are expected to affect resin capacity and selectivity. Potential effects may occur.	yes

Major parts of the risk assessment were generic. These data could be used again for other products that are processed with the same platform and facility, which contributed to the efficiency and sustainability of this approach. Process parameters that were assigned relevant with regards to multivariate interactions were included in the *in-silico* calculation of MARs.

Linear mixed model

While the risk assessment is applicable to the manufacturing facility and the mAb platform process, building of statistical models is dedicated to a specific molecule. The statistical models were constructed using the Ordinary Least Squares method considering all process parameter main effects, two and three factor interactions and quadratic effects. Data from multiple fermentation batches were used and possible differences were considered as fixed block effects. The statistical model for each critical quality attribute can be described as followed:

$$Y = \beta_0 + \sum_{i=1}^{p} \beta_i x_i + \sum_{i=1}^{p-1} \sum_{j=i+1}^{p} \beta_{ij} x_i x_j + \sum_{i=1}^{p-2} \sum_{j=i+1}^{p-1} \sum_{k=j+1}^{p} \beta_{ijk} x_i x_j x_k + \sum_{i=1}^{p} \beta_{ii} x_i^2 + \sum_{i=1}^{b-1} \delta_i s_i + \varepsilon$$

where

Y is the observed output parameter (e.g. critical quality attribute)

β_0 is the model intercept

β_i is the ith main effect

β_{ij} is the ijth two factor interaction effect

β_{ijk} is the ijkth three factor interaction effect

β_{ii} is the iith quadratic effect

δ_i is the ith block effect

x is the input parameter setting of the ith, jth or kth parameter

s_i is the ith block setting

p is the number of investigated input parameters

b is the number of fixed block effects

ε is the residual error term, assuming $\varepsilon \sim N\left(0, \sigma_\varepsilon^2\right)$

If at least one block was significant, all blocking effects were included in the model and transformed to random effects by using a linear mixed model. Changing the equation to a linear mixed model resulted in the following equation:

$$Y = \beta_0 + \sum_{i=1}^{p} \beta_i x_i + \sum_{i=1}^{p-1} \sum_{j=i+1}^{p} \beta_{ij} x_i x_j + \sum_{i=1}^{p-2} \sum_{j=i+1}^{p-1} \sum_{k=j+1}^{p} \beta_{ijk} x_i x_j x_k + \sum_{i=1}^{p} \beta_{ii} x_i^2 + u + \varepsilon$$

where

u was the random block term, assuming $u \sim N\left(0, \sigma_u^2\right)$

The equation showed that the fixed block effect term $\sum_{i=1}^{b-1} \delta_i s_i$ was substituted by the random term u. This term described the random batch-to-batch variability assuming a normal distribution with mean zero and variance σ_u^2.

In-silico calculation

The *in-silico* calculation of MARs used the established statistical regression models (i.e. linear mixed models) to define PARs. A linear mixed model was built for each relevant critical quality attribute and yield. Linear mixed models are able to combine global and group-level trends with fixed (i.e. investigated input parameters) and random effects (i.e. batch-to-batch variability) (Oberleitner et al. 2023). Linear mixed models converged to ordinary least square models in the absence of random effects. In this case, no batch-to-batch variability could be observed. Statistical uncertainty intervals were applied to take this variability into account so that predictions for future batches could be made. The statistical models for process characterization studies were built with DoE data from process characterization experiments and scale-down experiments. Scale-down qualification experiments were run with settings close to target; this enabled integration of scale-down qualification data into the statistical models for definition of PARs. The integration of scale-down data made it possible to investigate the impact of batch-to-batch variability.

Based on the statistical models, predictions for relevant critical quality attributes and yield were made. The linear mixed models returned, for instance, mAb aggregate predictions for any given input parameter set and space that was included. The model-based predictions were compared against target ranges. Target ranges were deduced from large scale data; an arithmetic mean ± 3 standard deviation range was calculated based on large scale runs. These target ranges were applied as acceptance criteria for the process outputs, including critical quality attributes and yield. The same target ranges applied for establishment of the PARs and for the MARs.

During *in-silico* calculations, non-relevant process inputs were set to their corresponding target value.

Process inputs relevant for MARs were evaluated at different settings. A uniform distribution of process inputs was assumed. The evaluation was performed in parallel for the different critical quality attributes and yield. The technical realization of the MARs was conducted according to the following protocol:

- Discretize the screening range per input parameter into n equidistant grid points (grid size n). The input parameter calibration tolerance can be used to find a good estimation for n. (Fig. 2a and b)
- Consider presence of quadratic effects and locate extreme values of the statistical model (i.e. vertex of the parabola). In case extrema are located between two grid points: add the extrema as additional points to the grid for the respective parameter (Fig. 2c)
- Build the Cartesian product of all discretized input parameter ranges. The Cartesian product represents all possible combinations of input parameter settings within the design space (with grid size n).
- Calculate the prediction of the statistical model with the corresponding model uncertainty interval for each grid point in the design space.
- Set the grid point to true, if the uncertainty interval of the linear model is within the target range. Otherwise set the grid point to false (Fig. 2d)
- Determine all possible orthotopes (i.e. with values true) that include the target conditions (see section Geometric Exemplification, Fig. 4)
- Normalize orthotopes to the screening range and sort according to their volume.
- Perform the calculation for every relevant critical quality attribute and process performance parameter.

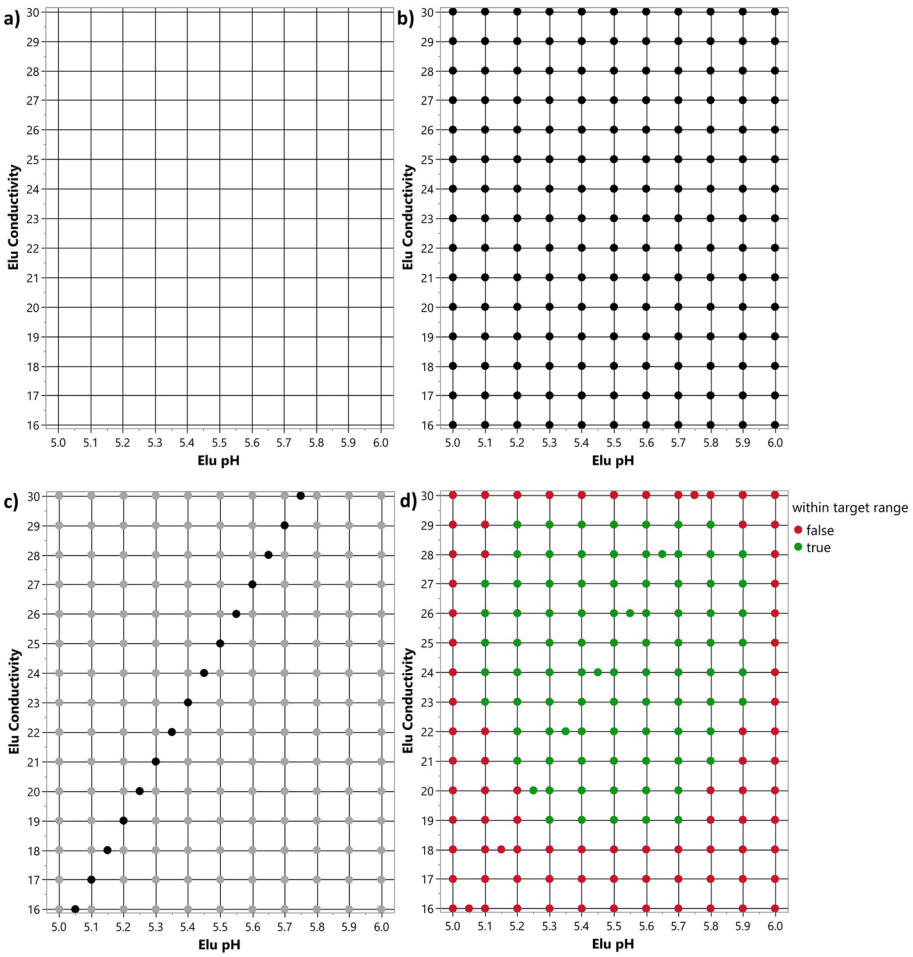

Fig. 2 Simplified example for the individual steps of the technical MAR realization for two input parameters. **a** The screening range is discretized into equidistant grids. **b** Grid points with the input parameter settings for the model predictions are added. **c** To consider the presence of quadratic effects, additional grid points were added at the vertex of the parabola. d) Classification if the model prediction with uncertainty interval falls at the corresponding grid point location within the target range

Geometric exemplification

Figure 3 shows an example for the greatest possible space for yield. For simplicity, the graphic example involves only two process inputs. Based on the contour plot of the model and the acceptance criteria for yield, the largest possible space had a circular geometry. The actual *in-silico* calculation involved all previously identified parameters relevant for MARs.

The herein described approach did not investigate or describe the geometric shape of the largest possible space within which no critical multivariate interactions occur. Instead, ranges were established for each relevant process parameter or material attribute. For consideration of 2 process parameters, the MAR resulted in a rectangle. Figure 4 illustrates the different possible rectangles for the given example from Figs. 2 and 3. For 3 process parameters, a rectangular cuboid was established. For consideration of more than 3 = N process parameters, the cuboid turned into a N-orthotope.

MARs equaled the PAR if no multivariate interactions were observed. If multivariate interactions were present within the PAR of one or several process inputs, the MAR was tighter than the PAR. MARs tighter than PARs could occur for one or more process parameter. Usually, several different cuboids or orthotopes were possible. For instance, a tighter MAR for process parameter 2 allowed more flexibility for process parameter 3; meanwhile the MAR for process parameter 1 remained unchanged. Another combination, i.e. cuboid, allowed for more flexibility for process parameter 2, while the MAR for process parameter 3 was tightened. Figure 4 illustrates different possible rectangles for a set of 2 process inputs.

Results and discussion

The statistical models for the cation exchange chromatography step described the dependency of 7 process outputs based on the settings of 10 process inputs. The process inputs were parameters from the loading, equilibration, and elution phase of the cation exchange chromatography step for an example mAb. Within this paper, the establishment of a MAR for the elution phase is described. The model was built based on a DoE approach. The DoE comprises center point experiments, and experiments that are conducted at deflected process input settings. The high number of investigated parameters led to 52 wet-lab experiments. Additional wet-lab experiments, investigating all potential multivariate interactions at their corresponding minimum and maximum level, were hardly practicable. Such additional wet-lab experiments would need to represent all possible combinations of parameter excursions; summed 2^{10} = 1024 wet-lab experiments.

The *in-silico*-based calculation resulted in a large list of possible orthotopes per process output, which were sorted by the volume. The methodology was applied to all critical quality attributes of the mAb and step yield. Process input settings, which favored high step yield, led to higher aggregate levels in the product pool of the cation exchange step.

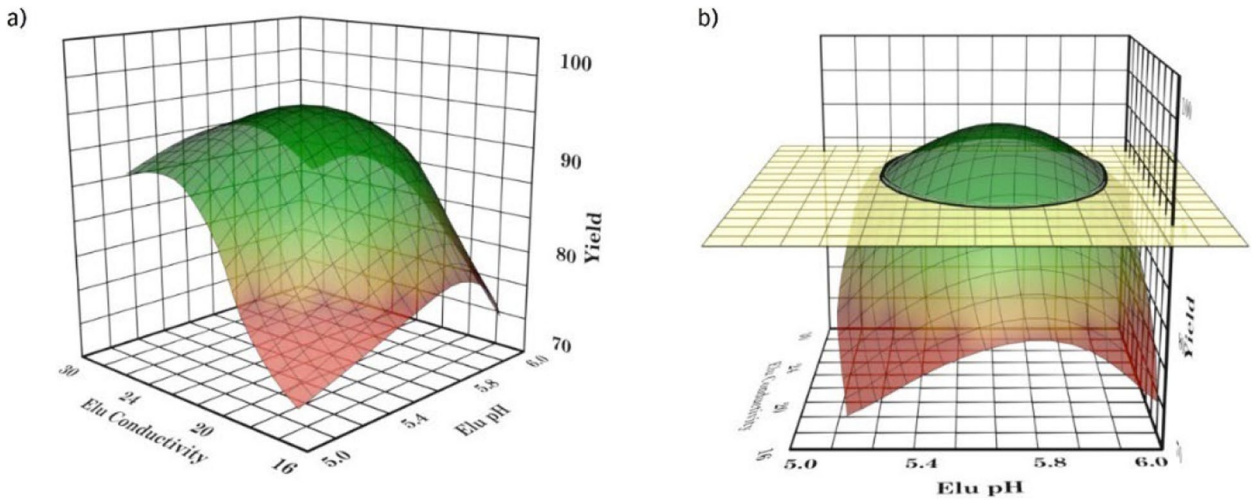

Fig. 3 Schematic drawing of the largest possible space between two process inputs. The contour plot for yield **a**) intersected the acceptance criteria for yield, represented by the yellow surface in **b**). The herein described approach did not investigate or describe the geometric shape of the largest possible space within which no critical multivariate interactions occur. Instead, ranges were established for each relevant process parameter or material attribute. For consideration of 2 process parameters, the MAR resulted in a rectangle. Figure 4 illustrates the different possible rectangles for the given example from Figs. 2 and 3. For 3 process parameters, a rectangular cuboid was established. For consideration of more than 3 = N process parameters, the cuboid turned into a N-orthotope

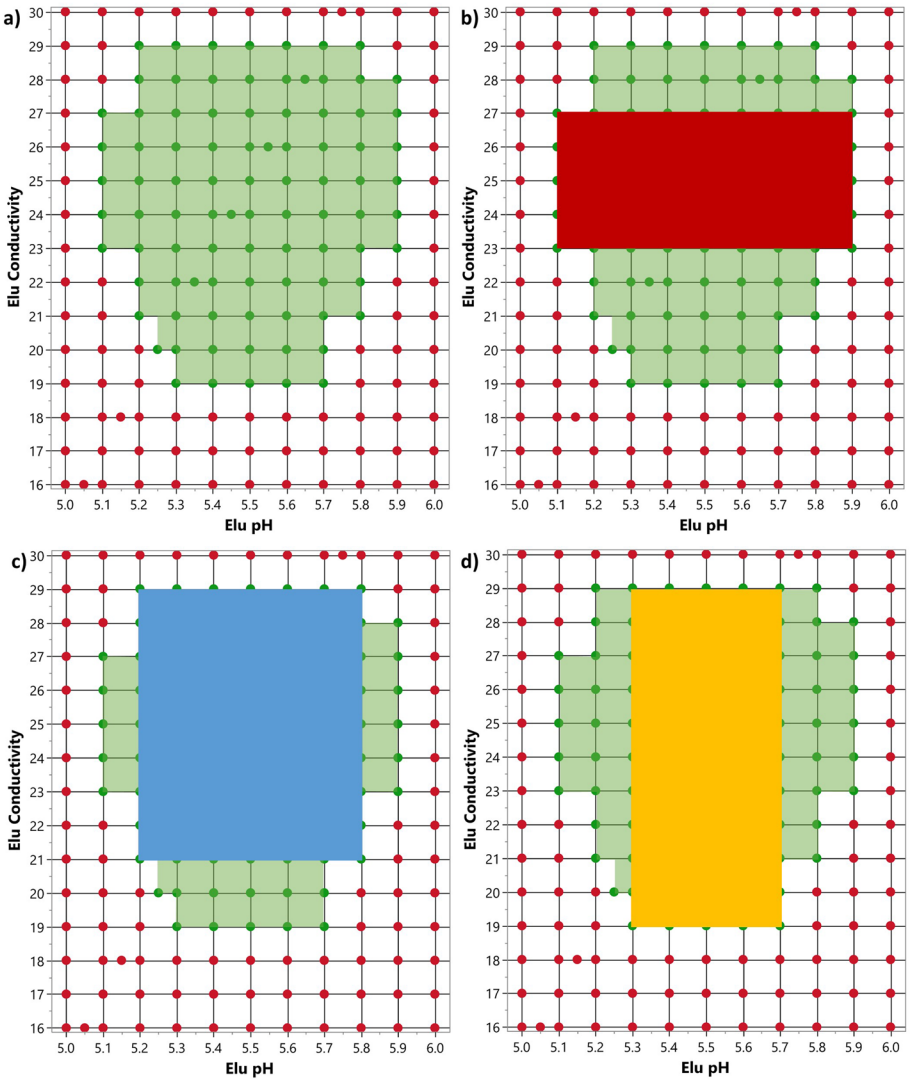

Fig. 4 Schematic representation of different rectangles within the largest possible space in green **a**). The red rectangle **b**) has the largest range for elution pH. The blue rectangle **c**) has the largest surface area and shows the best compromise in terms of ranges between elution buffer conductivity and pH. The yellow rectangle **d**) allows a comparatively wide range for the elution buffer conductivity in combination with a tighter range for the elution buffer pH. For graphical simplicity, the example involves only two process inputs

The impact of multivariate interactions was more complex with regard to the level acidic mAb variants. Tables 2, 3, 4 show results for aggregates, acidic mAb variants and step yield.

The screening range (SR) is listed in the first row. The 10 largest cuboids were sorted according to their volume. Volumes were normalized to the screening range. Process input settings highlighted in red indicate MARs that are tighter than the screening range.

For the presented unit operation, the resulting cuboids were smaller than the screening range. For mAb aggregates, volumes of the 10 largest MARs ranged from 44 – 52 % of the screened parameter space. Especially, the upper end of the tested parameters led to critical multivariate interactions. At least 2 of the 3 parameters had to be tightened at their upper end. The MAR was tighter than the PARs.

The possible MARs for acidic mAb variants were comparatively small with respect to volume. Allowable volumes comprised approximately 2 % of the screened space. Thus, multivariate interactions had a greater impact on acidic mAb variants compared to the impact on mAb aggregates. All input parameters had to be restricted on each end of the screening range. The allowable ranges for elution buffer pH and elution buffer conductivity were centered around the mid-point of the screening range.

Table 2 Cuboids for aggregates. The selected cuboid is highlighted by a double-lined, green frame

	Elution buffer cond. [mS/cm]		Elution buffer pH [pH]		Loading density [g/L]		Volume
	lower	upper	lower	upper	lower	upper	normalized
SR	11.50	13.50	5.40	5.59	22.99	66.01	1.000
1	**11.50**	**13.05**	**5.40**	**5.52**	**22.99**	**66.00**	**0.519**
2	11.50	13.27	5.40	5.50	22.99	66.00	0.494
3	11.50	13.50	5.40	5.50	22.99	61.22	0.494
4	11.50	12.83	5.40	5.54	22.99	61.22	0.461
5	11.50	13.05	5.40	5.52	27.77	66.00	0.461
6	11.50	13.05	5.40	5.52	22.99	61.22	0.461
7	11.73	13.05	5.40	5.52	22.99	66.00	0.444
8	11.50	12.83	5.40	5.52	22.99	66.00	0.444
9	11.50	12.61	5.40	5.56	22.99	61.22	0.439
10	11.50	13.27	5.40	5.50	27.77	66.00	0.439

Table 3 Cuboids for acidic mAb variants. The selected cuboid is highlighted by the double-lined, green frame

	Elution buffer cond. [mS/cm]		Elution buffer pH [pH]		Loading density [g/L]		Volume
	lower	upper	lower	upper	lower	upper	normalized
SR	11.50	13.50	5.40	5.59	22.99	66.01	1.000
1	12.39	13.05	5.45	5.50	51.67	61.22	0.025
2	12.17	12.83	5.47	5.52	51.67	61.22	0.025
3	12.17	12.83	5.49	5.52	46.89	61.22	0.025
4	12.39	12.83	5.47	5.52	46.89	61.22	0.025
5	12.39	13.05	5.47	5.50	46.89	61.22	0.025
6	12.39	12.83	5.45	5.52	51.67	61.22	0.022
7	12.39	12.83	5.49	5.52	42.11	61.22	0.022
8	11.95	12.83	5.49	5.52	51.67	61.22	0.022
9	12.17	13.05	5.47	5.50	51.67	61.22	0.022
10	12.17	13.05	5.45	5.50	56.45	61.22	0.016

In contrast, the allowable ranges for the loading density were in the upper part of the screening range, i.e. higher than 40 g/L. Compared to the PARs, the MARs for the elution buffer conductivity and the loading density had to be tightened.

The identified cuboids for yield ranged from 3 – 7 % of the screened volume. The lower end of the process parameter was found critical to multivariate interactions. This applied to all the 10 largest possible cuboids. Elution buffer pH, elution buffer conductivity and the loading

Table 4 Cuboids for step yield. The selected cuboid is highlighted by a double-lined, green frame

	Elution buffer cond. [mS/cm]		Elution buffer pH [pH]		Loading density [g/L]		Volume
	lower	upper	lower	upper	lower	upper	normalized
SR	11.50	13.50	5.40	5.59	22.99	66.01	1.000
1	12.39	13.50	5.49	5.59	56.45	66.00	0.069
2	12.39	13.50	5.49	5.56	56.45	66.00	0.055
3	12.39	13.27	5.49	5.59	56.45	66.00	0.055
4	12.39	13.27	5.49	5.56	56.45	66.00	0.044
5	12.39	13.50	5.49	5.54	56.45	66.00	0.041
6	12.39	13.05	5.49	5.59	56.45	66.00	0.041
7	12.39	13.50	5.49	5.59	56.45	61.22	0.034
8	12.39	13.27	5.49	5.54	56.45	66.00	0.033
9	12.39	13.05	5.49	5.56	56.45	66.00	0.033
10	12.39	13.50	5.49	5.56	56.45	61.22	0.027

density had to be restricted at their lower end, compared to the screening range. Cuboids 2 – 9 were also restricted at the upper end of at least one process parameter. Especially low loading densities were found to be critical with regard to multivariate interactions. The lowest allowable loading density was 56.45 g/L.

The observed multivariate interactions were attributed to the known impact of salt ions and pH in cation exchange chromatography. The results of the (MAR) evaluation agreed (generally) with the theory of cation exchange chromatography (Staby et al. 2006; Rounds and Regnier n.d; Kopaciewicz and M.A., R., Fausnaugh, J., al., n.d; Brooks and Cramer 1992).

Numerous publications investigate the adsorption and desorption of proteins to a charged stationary phase. One of the most straight-forward models is the stoichiometric displacement of the adsorbed protein by salt ions (Rounds and Regnier n.d; Kopaciewicz and M.A., R., Fausnaugh, J., et al., n.d). The pH in the liquid phase affects protein charge. Proteins have a negative net charge below their isoelectric point. This net charge decreases with increasing pH. Hence, displacement requires fewer salt ion at higher pH, provided cation exchange chromatography is operated below the isoelectric point of the protein. The steric mass action model is more detailed; the steric mass action model considers several steric effects, such as the multi-point adsorption of proteins to the stationary phase. Steric effects lead to local ion concentrations in the close environment of adsorbed proteins (Brooks and Cramer 1992). The protein charge and local ion concentrations influence protein adsorption and desorption equilibrium. Further, steric effects affect the maximum available resin capacity. Thus, load capacities depend on the salt concentration, pH, shape and size of a protein.

The MAR evaluation in this case study revealed multivariate interactions between elution buffer conductivity, elution buffer pH and loading densities. High elution buffer pH, high elution buffer conductivity and high loading densities were beneficial to achieve a high yield, where conductivity is a measure for the salt concentration. These findings agreed with the general theory of cation exchange chromatography. High pH decreases the net charge of the protein and high conductivity supports displacement. High loadings further support protein desorption and lead to a relative decrease of protein that remains adsorbed to the resin, also due to unspecific interactions.

However, high elution buffer pH, high elution buffer conductivity and high loading densities induced multivariate interactions that were critical with regard to mAb aggregate removal. The product pool contained relatively higher amounts of mAb aggregates. The amount of mAb aggregates exceeded the applied acceptance criteria. The observed multivariate interactions enhanced mAb aggregate desorption. In turn, the resolution decreased between mAb monomers and mAb aggregates.

Multivariate interactions between elution buffer conductivity and the elution buffer pH were critical with

regard to acidic mAb variants. The allowable ranges for the loading density were similar to the PAR. The comparatively small allowable cuboids for acidic mAb variants and step yields were due to strong multivariate interactions in the cation exchange unit operation. The acidic mAb variants were determined as a relative measure. Low elution buffer pH and low conductivity decreased recovery of basic species. This effect was enhanced by low load densities. The relative share of acidic mAb species increases. Similarly, high elution buffer pH and high elution buffer conductivity led to an increasing level of basic species. High load densities added to this effect. Consequently, acidic mAb species were below the applied acceptance criteria.

The observed impact of multivariate interactions on yield and the level of acidic mAb species could extend to other mAbs, as well. The principles of ion exchange apply to proteins, in general. The observed effect for mAb aggregate purity were more complex. Aggregates are not necessarily more basic, compared to monomers. Net charge of aggregates can differ from one mAb to another. Thus, observed effects can be different for other mAbs.

The herein presented approach returned orthotopes, i.e. an allowable rectangular volume. These allowable volumes were conservative, rectangular approximations of the true allowable parameter space. Yet, the applied grid enabled *in-silico* calculations to be conducted within few hours. On the other hand, the rectangular constraint might drastically reduce the returned allowable parameter space compared to the true allowable parameter space.

Review of operating ranges

The *in-silico* evaluation returned MARs for all process outputs of the mAb polishing step. The resulting MARs were smaller than the established PARs. A discussion between development, process transfer, and manufacturing was initiated. The discussion addressed the observed variance of several factors: process parameter read-outs in large-scale runs; process parameter governance; and manufacturing variability demands. As per the process layout, particular process parameters required higher variability than others. For instance, high operational variability was found for the chromatography column load densities. The column load capacities depended on the harvest titer, the yield of unit operations upstream, the column volume, and the cycling strategy. Thus, variability for the loading density was prioritized over variability for elution buffer pH and conductivity. To this end, cuboid 7 was selected for acidic mAb variants (see Table 3).

The selection procedure was repeated for each critical quality attribute and yield. For the given example, cuboid 1 was selected for yield (see Table 4), based on the volume. Cuboid 4 was selected for aggregates (see Table 2). This cuboid was a trade-off between tightening the ranges for elution buffer pH and elution buffer conductivity. The selected cuboids were combined to one MAR. In some cases, the cuboid selection was reviewed. This re-evaluation aimed to achieve the largest overlap between the cuboids. The MAR for a process parameter was established based on the tightest range among all selected cuboids. Table 5 summarizes the MAR for the herein discussed mAb polishing step.

The *in-silico* calculated MARs were compared again to operational variability. The MAR for the elution buffer pH was tighter than the desired operational range. Thus, eight wet-lab experiments were conducted. The experiments aimed to confirm the desired multivariate range: elution buffer pH 5.45 – 5.55; elution buffer conductivity 12.0 – 13.0 mS/cm; and loading density 40 – 58 g/L. Table 6 summarizes the experimental parameter settings and Fig. 5 shows the results. The wet-lab experiments were performed using a qualified scale-down model.

The wet-lab results verified the results from the *in-silico* calculation of MARs, also demonstrating the appropriateness of the approach. If loading density, elution buffer pH and conductivity were set to their upper end, mAb aggregates exceeded the applied acceptance criteria. Similarly, setting the process parameters to the lower end led to exceeding the acceptance criteria for the acidic mAb variant level. Process-wise, appropriate measures had to be applied. These measures ensured that product quality was consistently met. The NORs were revised. If the pH of the elution buffer exceeded pH 5.52, conductivity of the elution buffer had to be below 12.70 mS/cm. The established NORs were set based on the MARs and confirmed by wet-lab experiments yielding

Table 5 Combined MAR for all considered CQAs and yield. The lower limit for yield was not considered for the overall MAR

Process Parameter	Aggregates [%]	Acidic variants [%]	Step yield [%]	Combined MAR
Elution buffer pH	5.40 – 5.54	5.49 – 5.52	5.49 – 5.59	**5.49 – 5.52**
Elution buffer conductivity [mS/cm]	11.50 -12.83	12.39 – 12.83	12.39 – 13.50	**12.39 – 12.83**
Load density g protein/L resin [mg/mL]	22.99 – 61.22	42.11 – 61.22	56.45 – 66.00	**42.11 – 61.22**

Table 6 Parameter settings for the MAR verification wet-lab experiments

No. Exp	Elution buffer conductivity [mS/cm]	Elution buffer [pH]	Load density [g/L]
1	12.4	5.48	58
2	12.0	5.45	40
3	12.3	5.45	40
4	13.0	5.55	58
5	12.7	5.54	58
6	12.0	5.46	40
7	13.0	5.46	58
8	12.5	5.50	58

acceptable product quality. This guaranteed that multivariate interactions would not affect future manufacturing runs.

Establishing the MARs for the given mAb polishing, required eight additional wet-lab experiments. The *in-silico* data was used to design the wet-lab experiments. If no preliminary data was available for the experimental design, the number of wet-lab experiments will likely be higher. For the given mAb downstream process, MARs were also established for the capture step, acid treatment and the first polishing column (data not shown). These unit operations did not require confirmation of MARs in the wet lab.

Process knowledge and control

The evaluation of MARs provided in-depth understanding of multivariate interactions among process inputs. The MAR increased process knowledge and supported process control. For most of the investigated process inputs, multivariate interactions were non-critical. The established MARs were equal to the PARs or at least wider than the NORs. If MARs were tighter than the NORs, another round of *in-silico* calculations was conducted. This second round of *in-silico* calculations used a smaller grid size. The first round of *in-silico* calculations used at least 10 equidistant grid points. The resulting calculated MARs were conservative ranges. The application of a higher resolution grid size increased the MARs. Naturally, it also increased the computational effort. Finally, a good balance was established between process variability requirements and the computational complexity. If the established MARs remained tighter than the normal operation ranges, mitigations were put in place.

Mitigations included the adaptation of NORs, addition of further controls or the confirmation of multivariate parameter excursions in the wet lab. As the wet-lab data confirmed the multivariate interactions determined *in-silico*, NORs were adapted. Potential opportunities to adapt NORs included redevelopment of the affected process step, improved calibration tolerances of the pH and conductivity probes, or a change of equipment. Further, allowable loading densities could be tightened,

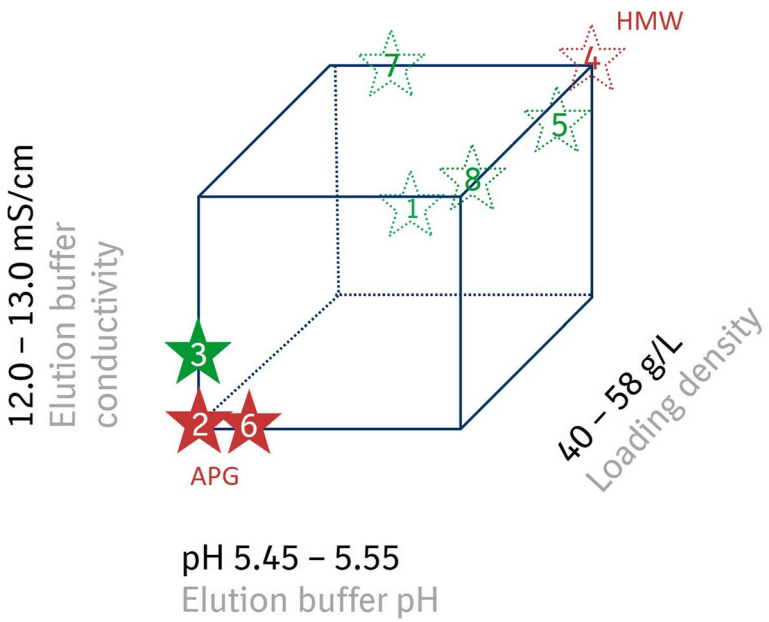

Fig. 5 Schematic drawing of the MAR confirmation experiments. The blue cuboid represents the investigated design space of the confirmation experiments. Red stars indicate experiment with conditions leading to product quality data outside the acceptance criteria. Green stars represent experiments with conditions leading to acceptable product quality. Numbers indicate experiment numbers

which was considered the least preferred option regarding manufacturing flexibility. In the presented case study, a what-if condition was introduced. This what-if- condition applied to the elution buffer pH and conductivity. If the elution buffer exceeded a defined pH limit within the NOR, the conductivity had to be below a defined limit. This additional control of process inputs ensured product quality was met despite the observed multivariate interactions. Mitigations implemented upon the assessment of MARs varied on a case-by-case basis. In any case, the MAR delivered a criterion to assign process inputs that are subject to critical multivariate interactions.

The MAR as a measure for multivariate interactions was further considered for the criticality assessment of process parameters and material attributes. This added substantial value to process robustness in manufacturing scale. For the given example, the revised NORs for elution buffer pH and elution buffer conductivity ensured process control. The adapted NORs prevent multivariate interactions that could cause inconsistent product quality. Both process parameters were classified as critical material attributes. The identification of process inputs prone to critical multivariate interactions led to significantly increased process understanding and a much stronger control strategy. On the other hand, the data driven approach justified deprioritization of criticality of several other process parameters.

Conclusions

Although the concept of a design space has been laid out in ICH Q8 (R2) for almost 15 years, the interpretation of the regulatory expectation remains challenging. Instead of regulatory relief, applicants have experienced complete application rejection and other consequences. Currently, speed to market impacts many decisions in late-stage development. Additionally, both resources and time for process characterization are limited. Therefore, an intermediate approach to defining MARs, instead of claiming a full QbD design space, is of high interest as the applicant files only PARs and NORs. Additionally, the MAR, which is established from small-scale experiments conducted for scale-down model qualification, and definition of PARs, supports manufacturing flexibility within NORs. Data is augmented with prior platform knowledge at manufacturing scale. The available DoE data considers 3-way interactions that were not reflected in NORs and PARs as established in the past. Thus, the MAR fully explores the DoE data according to the intended purpose of the DoE methodology. The evaluation of MARs was conducted *in-silico*. Any input parameter setting was treated equally possible, which allowed for prediction about whether a combination of process input settings would lead to meeting the applied acceptance criteria for product quality and process performance.

Based on the extended process knowledge, the scientific risk assessment of parameter changes could be enhanced. The MAR was tighter than the PAR for several process parameters, indicating a relevant impact of multivariate interactions. This additional process knowledge was applied for an improved process control. Further, the process knowledge obtained from the evaluation of MARs supports the identification of established conditions according to ICH Q12 (Guideline and Q12 on Technical and Regulatory Considerations for Pharmaceutical Product Lifecycle Management. n.d) and categorization of post-approval chemistry, manufacturing, and control changes.

Abbreviations

DoE	Design of experiment
FDA	Food and Drug Administration
ICH	International Council for Harmonisation
mAb	Monoclonal antibody
MAR	Multivariate acceptance range
NOR	Normal operating range
PAR	Proven acceptable range
QbD	Quality by design

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Authors' contributions

MK developed the *in-silico* methodology and prepared the original draft. JT conceptualized the methodology and manuscript and wrote the original manuscript draft. SL performed wet-lab experiments, curated and validated data. JMS supervised the project, provided capacity and reviewed the manuscript. BP supervised the project, acquired internal funding and reviewed the manuscript. JS conceptualized the methodology, administered the project, edited the original draft and reviewed the manuscript.

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Availability of data and materials

The full datasets generated and/or analyzed during the current study are not publicly available, due to confidentiality reasons. The presented case study includes a reasonable amount of data to demonstrate that the modeling approach is suitable.

Declarations

Ethics approval and consent to participate

The article does not report information or data from studies involving human participants or human tissue.

Consent for publication

All authors consent to publication.

Competing interests

All authors are paid employees of Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany.

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Questions and Answers: Improving the Understanding of NORs, PARs, DSp and Normal Variability of Process Parameters.

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