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Optimisation and robustness analysis of a hydrophobic interaction chromatography step

Niklas Jakobsson, Marcus Degerman, Bernt Nilsson *

Department of Chemical Engineering, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden

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Abstract

Process development, optimisation and robustness analysis for chromatography separations are often entirely based on experimental work and generic knowledge. The present study proposes a method of gaining process knowledge and assisting in the robustness analysis and optimisation of a hydrophobic interaction chromatography step using a model-based approach. Factorial experimental design is common practice in industry today for robustness analysis. The method presented in this study can be used to find the critical parameter variations and serve as a basis for reducing the experimental work. In addition, the calibrated model obtained with this approach is used to find the optimal operating conditions for the chromatography column. The methodology consists of three consecutive steps. Firstly, screening experiments are performed using a factorial design. Secondly, a kinetic-dispersive model is calibrated using gradient elution and column load experiments. Finally, the model is used to find optimal operating conditions and a robustness analysis is conducted at the optimal point. The process studied in this work is the separation of polyclonal IgG from BSA using hydrophobic interaction chromatography.

Keywords: Robustness analysis; Optimisation; Modelling and simulation; Hydrophobic interaction chromatography; Antibody purification

1. Introduction

Today, when a protein purification process is to be adopted as an approved pharmaceutical production process a great deal of experimental work is performed to study the robustness of the purification process. The use of modelling and simulation makes it possible to reduce the number of labour-intensive experiments, and thereby shorten the development time and reduce the cost. This requires a methodology employing accurate models validated by carefully designed experiments. The methodology employed should be based on an understanding of the underlying physical mechanisms of the separation process. One advantage when using this approach is that the model may be applicable for larger variations in the process parameters compared with empirical modelling.

A major cost in the production of biopharmaceuticals is the cost of downstream processing, which usually consists of several steps. Therefore, the optimisation of each step in the purification process is of great importance. The optimisation procedure can be based solely on experimental work exploring one parameter at a time or can be performed using a factorial experimental design [1,2]. There are a number of examples in the literature describing how modelling and simulation of a chromatography column can be used to find the optimal running conditions [3].

The US Food and Drug Administration (FDA) [4] recently published guidelines in which the importance of process understanding is emphasised when validating a process. These guidelines promote the use of process analytical technologies such as multivariate data acquisition and analysis, modern process analysers and process monitoring. The FDA also states that the ability to predict process behaviour shows process understanding, and a greater process understanding gives more freedom in changing process conditions within the scope of the original approved validation documentation. The cost of validation often hinders process development and implementation of new process equipment in existing production processes for pharmaceuticals. The reluctance to use new process technologies in the pharmaceutical industry is undesirable from a public health perspective. Efficient pharmaceutical manufacturing is of great importance in achieving effective health care [4]. The guideline

* Corresponding author. Tel.: +46 46 222 8088; fax: +46 46 222 4526.
E-mail address: bernt.nilsson@chemeng.lth.se (B. Nilsson).

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also suggests that experimental process development databases could be used to develop process simulation tools, which will help us gain knowledge of the process reduce the overall process development time from laboratory to production scale.

The aim of the present study was to evaluate how a mathematical model of a hydrophobic interaction chromatography step can be used to assist in process optimisation and robustness analysis. The aim was also to use the experimental techniques normally employed in process development to calibrate the model, and to keep the demand for additional experiments to a minimum. Many authors have addressed the issues concerning a model-based approach to chromatography [3,5–8]. This work aims to present a modelling approach that is useful in dealing with the optimisation and robustness analysis work normally performed in the biopharmaceutical industry.

This study focuses on hydrophobic interaction chromatography as it is a common unit operation in biopharmaceutical production today. A mixture of BSA and polyclonal IgG was used as a model system to evaluate the advantages of using the proposed methodology.

2. Theory—models and simulation technique

The model of the hydrophobic interaction used in this study consists of a description of the interaction between the protein and solid phase and a description of the dispersion in the column. The solid-phase interaction is modelled based on solvophobic theory using an interaction model including kinetics [9].

2.1. Column model

The kinetic-dispersion model describing a column contains one part describing the dispersion and convection in the mobile phase, and another part describing the adsorption as a reaction including kinetics. In the model used in this study, the shape of the elution peaks and breakthrough curves are dependent on a dispersion coefficient, which is determined from an empirical correlation, and on the adsorption rate. The column model for component i is described by the following equation,

$$\frac{\partial c_i}{\partial t} = D_{\text{ax}} \frac{\partial^2 c_i}{\partial x^2} - v_{\text{int}} \frac{\partial c_i}{\partial x} - \frac{1-\varepsilon_{\text{c}}}{\varepsilon_{\text{c}}} \frac{\partial q_i}{\partial t} \tag{1}$$

where ε_{c} is the void fraction in the packed bed (m^3 mobile phase/m^3 column), x the axial coordinate along the column (m), v_{int} the interstitial velocity (m/s), D_{ax} the apparent dispersion coefficient (m^2/s), c_i the concentration of component i in the mobile phase (mol/m^3), q_i the concentration of component i in the stationary phase (mol/m^3 stationary phase) and t is the time (s).

The column equation is subject to the following boundary conditions. A Robin condition describes the column inlet,

$$\frac{\partial c_i}{\partial x} = \frac{v_{\text{int}}}{D_{\text{ax}}}(c_i - c_{\text{inlet},i}), \quad \text{at } x = 0 \tag{2}$$

where $c_{\text{inlet},i}$ is the inlet concentration (mol/m^3) and c_i is the concentration just inside the column (mol/m^3), which may be slightly lower than $c_{\text{inlet},i}$ due to dispersion at the inlet. At the outlet where x is equal to L, the length of the column (m), only convective transport is considered and can thus be described by a Neumann condition (see Eq. (3)).

$$\frac{\partial c_i}{\partial x} = 0, \quad \text{at } x = L \tag{3}$$

2.2. Adsorption—the Langmuir MPM model

The description of the adsorption is based on solvophobic theory [9]. The protein mixture studied contains proteins of various sizes. It is assumed that the binding sites are uniformly distributed. In the present study where two components are loaded onto the column it was found that the most suitable model was a variant of the original competitive Langmuir model.

A Langmuir kinetic model (see Eq. (4)) describes the adsorption and desorption of the protein and these are regarded as competitive processes in which the salt concentration affects the retention of the protein. Because salt is considered to be inert, dq_{salt}/dt is 0. During the binding step, $k_{\text{ads},i}$, the adsorption coefficient of component i (m^3/kmol s), is much larger than $k_{\text{des},i}$, the desorption coefficient of component i (s^{-1}), while at elution $k_{\text{des},i}$ dominates.

$$\frac{\partial q_i}{\partial t} = k_{\text{ads},i} \cdot c_{x,i} \cdot q_{\text{max},i} \left(1 - \sum_{j=1}^{N} \frac{q_j}{q_{\text{max},j}}\right) - k_{\text{des},i} \cdot q_i \tag{4}$$

where $q_{\text{max},i}$ and $q_{\text{max},j}$ are the maximum concentrations of components i and j in the stationary phase (kmol/m^3 solid phase); q_i and q_j are the concentrations of components i and j in the stationary phase (kmol/m^3 solid phase) and N the number of interacting components.

In the current model, the adsorption sites are divided into two different types. In the first type, IgG adsorbs without competition. In the second type IgG and BSA compete for the available sites, and BSA experiences competition from IgG adsorbed on both sites, see Eqs. (5)–(7). This model effectively "weakens" the ability for BSA to compete with IgG and "strengthens" the ability for IgG to displace BSA. In the competitive part of the stationary phase IgG and BSA have the same maximum capacity (q_{max}). The equilibrium coefficient, K_{eq}, see Eq. (10), and the hydrophobicity coefficient, γ, as well as the kinetic coefficient are the same for both types of sites.

$$\frac{\partial q_{\text{IgG},1}}{\partial t} = k_{\text{ads,IgG}} \cdot c_{x,\text{IgG}} \cdot q_{\text{max,IgG},1} \times \left(1 - \frac{q_{\text{IgG},1}}{q_{\text{max,IgG},1}}\right) - k_{\text{des,IgG}} \cdot q_{\text{IgG},1} \tag{5}$$

$$\frac{\partial q_{\text{IgG},2}}{\partial t} = k_{\text{ads,IgG}} \cdot c_{x,\text{IgG}} \cdot q_{\text{max,IgG},2} \times \left(1 - \frac{q_{\text{IgG},2}}{q_{\text{max,IgG},2}} - \frac{q_{\text{BSA}}}{q_{\text{max,BSA}}}\right) - k_{\text{des,IgG}} \cdot q_{\text{IgG},2} \tag{6}$$

$$\frac{\partial q_{\mathrm{BSA},2}}{\partial t} = k_{\mathrm{ads,BSA}} \cdot c_{x,\mathrm{BSA}} \cdot q_{\mathrm{max,BSA}} \times \left(1 - \frac{q_{\mathrm{IgG},2}}{q_{\mathrm{max,IgG},2}} - \frac{q_{\mathrm{BSA}}}{q_{\mathrm{max,BSA}}} - \frac{q_{\mathrm{IgG},1}}{q_{\mathrm{max,IgG},1}}\right) - k_{\mathrm{des,BSA}} \cdot q_{\mathrm{BSA},2} \quad (7)$$

The model can be used for the loading step as well as the elution step by using mobile-phase modulators [9], defined by Eqs. (8) and (9),

$$k_{\mathrm{ads},i} = k_{\mathrm{ads0},i} \mathrm{e}^{\gamma_i S} \quad (8)$$

$$k_{\mathrm{des},i} = k_{\mathrm{des0},i} S^{\beta_i} \quad (9)$$

$$k_{\mathrm{eq},i} = \frac{k_{\mathrm{ads0},i}}{k_{\mathrm{des0},i}} \quad (10)$$

where S is the concentration of the elution component, often salt, and $k_{\mathrm{ads0},i}$ (m^3/kmol s) and $k_{\mathrm{des0},i}$ (m^3/kmol s) are parameters accounting for the reaction rate. β_i is a parameter describing the ion-exchange characteristics and γ_i (m^3/kmol) describes the hydrophobicity. Under loading conditions, S is given by the buffer salt concentration and salt from the protein solution. In this study, it was assumed that there are no ion-exchange interactions between protein and stationary phase, which means that β_i is equal to 0.

2.2.1. Model calibration

When developing a hydrophobic interaction separation step for protein purification, the initial part of the development consists of choosing a suitable stationary phase, pH and salt. In the present study this was conducted as a factorial experiment, evaluating two columns, two different salts at two different concentrations and two pH values.

The model calibration procedure comes into use when the operating conditions have been selected. The first step is to determine the equilibrium constant K_{eq} and γ for each component. These parameters are traditionally determined by performing isocratic retention experiments at different salt concentrations, and analysing the results by linear regression [10,11]. In this study, the parameters K_{eq} and γ were determined by loading a small amount of protein onto the column and thereafter performing linear gradient elution experiments and adjusting K_{eq} and γ to fit the peak positions using a least squares method. The shapes of the elution peaks were fitted by adjusting the kinetic parameter k_{des0} in the interaction model [12].

The second step is to determine the maximum capacity in the interaction model for each protein. In this part the protein solution is loaded onto the column and the breakthrough of each protein is detected, allowing q_{max} to be determined for each protein. In the present work the concentration of proteins in the gradient elution experiments was so high that the retention time was slightly affected by the capacity parameter, q_{max}. The parameter estimation had therefore to be performed as an iterative procedure between the gradient elution experiments and the column load experiment.

To determine the dispersion in the column an empirical flow-rate-dependent correlation using the particle Peclet number was implemented to calculate the dispersion coefficient, see Eq. (8) [13,14],

$$Pe = \frac{v_{\mathrm{int}} d_{\mathrm{p}}}{D_{\mathrm{ax}}} \quad (8)$$

where d_{p} is the particle diameter of the column packing.

2.3. Optimisation and robustness analysis

Optimal conditions for chromatographic separation can be determined using computer simulation. The mathematical model used in the simulations is often based on physical phenomena and is calibrated using data from chromatography experiments. The optimal point is determined by finding the minimum value of the objective function. The objective could for instance be to find the operating conditions that maximises the yield of the desired product, or to find the maximum productivity under a constraint of a minimum required purity [15–18]. In optimisation of a chromatography step one limitation is often the number of decision variables that can be altered to find the optimal point, i.e. there are too many degrees of freedom for the full optimisation problem to be solved. In practice, the key variables are considered for optimisation and the remaining variables are excluded from the optimisation procedure.

In the pharmaceutical industry, one very important aspect of any unit operation is the robustness. The normal procedure for determination of the critical process parameters involves a number of steps [19,20]. The first step is to find the normal variation in the performance of the process equipment and to determine the normal operating range (NOR) defined as the deviation from the normal operating point for each process parameter. The normal operating range may involve variations in flow rate, pH, conductivity, column load, etc. When the NOR has been defined from knowledge about the process equipment, laboratory experiments are usually conducted to find the parameter range over which the product meets the demands on purity, activity, yield, etc. This range is evaluated for variations and co-variations between the process parameters to determine the proven acceptable range (PAR) for each process parameter, which defines the limits for each process parameter that are acceptable in the process. When a variation in a process parameter leads to process failure, i.e. the requirement on purity, activity, yield, etc. is not met, the edge of failure (EOF) is determined.

In the case of chromatography, this means that the separation of the components must meet the specifications defined for the chromatography step despite variations in process parameters such as flow rate, conductivity, pH, temperature and so on. The robustness analysis is usually performed experimentally, and can often be conducted using a factorial experimental design where the different process parameters are altered according to their expected variations in the actual process, to determine the PAR for the different parameters. The experimental work is usually conducted on laboratory scale

with an appropriate scale-down of the chromatography process [21,22].

For a chromatography column the optimal point is often situated at the edge of one of the constraints for optimisation. This point may be the optimal point, but it is not a very robust operating point. The variations in an operating point determined by a model-based approach consist of two parts. The first part is the error that is incorporated into the parameter estimation, and was accounted for by determining the 95% confidence interval for the model parameters in the present study. The second part has its origin in the normal variability of the process parameters, i.e. variations in flow rate, conductivity and so on, which is defined by the equipment used for the chromatographic separation.

A calibrated model can provide information about the severity of the impact of the variations in model and process parameters. The effect of parameter variation is related to a performance function that reflects the quality of the product, for example, the purity of the product. The variation analysis is conducted by simulating the impact on the performance function when the model and process parameters are simulated at the edge of their intervals. To determine the sensitivity of the process with respect to a certain parameter, the model response is simulated for a small variation in the parameter, for example 0.1%, and the impact of the performance function, for example the purity, is divided by the variation itself, see Eq. (9),

$$\text{Sensitivity} = \frac{\Delta \text{PF}(M)}{\Delta P} \tag{9}$$

where ΔPF is the difference in the performance function, PF, calculated from the model, M, caused by the parameter variation, ΔP.

To investigate whether any of the parameters has a non-linear impact on the product quality the variation at the edges of the parameter intervals can be calculated by linear extrapolation from the sensitivity calculation and compared with the model response at the edge of the parameter intervals. In this way it is possible to see which parameters are important in the model and to identify the process parameters that must be strictly controlled, as well as those that can be excluded from an experimental study.

2.4. Simulation technique

The model was implemented using a modelling and simulation tool called gPROMS developed by Process System Enterprise (London, UK) [23]. The column was simulated using a finite-difference approximation and a fourth-order approximation for the linear solver of the resulting set of differential equations. The number of grid points in the column was set to 100 to ensure that there was no numerical broadening in the column. The parameters were estimated the gEST entity in gPROMS using a least squares fitting procedure. The optimisation procedure was carried out using a "toolbox" developed at the Department of Chemical Engineering at Lund University, based on MATLAB [24].

3. Materials and methods

3.1. Materials

The columns used in the hydrophobic interaction chromatography experiments was a Phenyl Sepharose High Performance (HP), 1 ml pre-packed column (diameter 7 mm, length 25 mm), supplied by Amersham Biosciences (Uppsala, Sweden), with a mean bead diameter of 34 μm, and a Resource 15 Phenyl 1 ml pre-packed column with 15 μm bead diameter (diameter 6.4 mm, length 30 mm), also supplied by Amersham Biosciences. The column used in the gel filtration experiments was an SKW-23030 from Toso-Haas (Tokyo, Japan).

Two proteins were used in the experiments: bovine serum albumin (BSA) (A-1900, Lot no. 75H9305) from Sigma (Steinheim, Germany) and polyclonal IgG, kindly provided by Biovitrum AB (Stockholm, Sweden). The latter protein solution consisted of four different types of IgG (>99% pure) and had a concentration of 15.7% (w/w). Ammonium sulphate, sodium phosphate and potassium phosphate for the buffer preparation were obtained from Sigma. All samples and buffers were filtered through a 0.2 mm membrane filters from Schleicher & Schuell (Dassel Germany) prior to use.

The chromatography experiments were carried out on an ÄKTA purifier 100 system from GE Healthcare (Uppsala, Sweden).

3.2. Methods

3.2.1. Experiments to determine the dead volume of the chromatography system

The ÄKTA Purifier system has a relatively small dead volume for the sample when using a 2 ml loop or a superloop in the injection of the sample. The dead volume in the ÄKTA Purifier system was found to be 0.14 ml [12], and the dead volume between the UV detector and the conductivity cell was found to be 0.4 ml. The dead volume for the buffers in the experimental set-up was taken into account by using a tank series model. The model and the resulting parameters have been described by Jakobsson et al. [12].

3.3. Screening experiments to determine initial operating conditions

The screening was conducted using a factorial experimental design, in which two columns (Resource 15 Phenyl and Phenyl Sepharose High Performance), two different salts (ammonium sulphate and potassium phosphate) at two different concentrations in the loading step (1 and 1.2 M salt) and two levels of pH (6.5 and 7.5) were investigated. The result was a full factorial experiment with four factors and three centre points (Phenyl Sepharose High Performance, ammonium sulphate 1.1 M and pH 7). The choice of optimal conditions was made based on gradient elution experiments. The column was loaded with 2 ml protein sample (1 mg/ml IgG and 2 mg/ml BSA) in a buffer with high salt content. The column was washed with 8.5 ml loading buffer and the bound material was eluted with a negative gra-

dient with 10 column volumes (CVs) slope. The upper limit of salt concentration was chosen so as to be approximately 10% under the solubility limit for IgG in the sample. The experiments were evaluated by calculating a performance quotient from the resulting chromatogram from each experiment, see Eq. (10),

$$\text{Performance quotient} = \frac{\text{peak position IgG} - \text{peak position BSA}}{\text{peak width IgG} + \text{peak width BSA}} \times \text{yield of IgG} \quad (10)$$

where the yield of IgG is determined by dividing the peak area of the IgG peak in each experiment by the area of the largest peak in the entire screening experiment. The experiment with the highest quotient was considered to give the best operating point and was the basis for model calibration, optimisation and robustness analysis in the present study.

The results were also evaluated using MODDE 7 from Umetrics (Umeå, Sweden) [25]. This statistical tool was used to fit a statistical model to the results of the experiments.

3.3.1. Gradient elution experiments to determine the linear parameters and shape of the elution peaks

A minimum of three gradients is needed to fit the linear parameters (K_{eq} and γ) in the Langmuir MPM model [18,26]. The inlet concentrations used were 1 mg/ml IgG and 2 mg/ml BSA in 40 mM sodium phosphate buffer containing 1.2 M ammonium sulphate at pH 7.5. The elution buffer was 40 mM sodium phosphate buffer at pH 7.5. The flow rate for all gradient elution experiments was 1 ml/min (linear velocity 156 cm/h). The conductivity during the loading step was about 155 mS/cm and about 5.4 mS/cm at the end of the gradient elution. The loading step lasted 2 CVs and the column was washed with 8.5 CVs of buffer. The linear gradients used for parameter estimation were 7.5, 10, 12.5, 15 and 20 CVs. The experimental results were compensated for dead volumes in the system to isolate the behaviour due to the column.

3.3.2. Column load experiment to determine the capacity, q_{max}

The protein concentrations at the inlet were 1 mg/ml IgG and 2 mg/ml BSA. The salt concentration in the sample was 1.2 M ammonium sulphate. The flow rate was 1 ml/min and the buffer was 40 mM sodium phosphate, pH 7.5. A sample of 46.3 ml was loaded onto the column and the column was washed with 5.3 ml buffer; the slope of the gradient elution was 10 CVs. Fractions were collected and analysed using gel filtration to determine the composition at the outlet of the column at different parts of the chromatography cycle.

3.3.3. Optimisation

In this work, the objective of was to achieve maximum productivity while ensuring a minimum purity of 99% IgG. The productivity is defined as mg IgG/h, see Eq. (11),

$$\text{Pr} = \frac{\int_{t_1}^{t_2} FC_{\text{outlet,IgG}}\,\text{d}t}{\text{Cycle time}} \quad (11)$$

where F is the flow rate (ml/h), $C_{\text{outlet,IgG}}$ the concentration of IgG leaving the column, t_1 the starting time of the elution and t_2 the end point of the elution.

In the optimisation procedure the conductivity in the loading step, loading time, washing time and elution gradient were the decision variables. In the present study, the conductivities in the loading and washing steps were the same, and limited by setting that the maximum allowed conductivity to 155 mS/cm, which is about 7% below the solubility limit at 166 mS/cm. Optimisation was conducted using a simplex method with 0.1% tolerance for both the objective function and the decision variables.

3.3.4. Robustness analysis

Once the optimal running conditions had been determined, the sensitivity of the optimal point to fluctuations in the process and errors in the parameter estimation was determined. The most important factor in most chromatography steps in the pharmaceutical industry is the purity of the product, and therefore, the robustness analysis was conducted with respect to the purity of IgG. The variations in the model were represented by the 95% confidence interval for each model parameter. The variations in the process parameters were defined with respect to the normal variations in a pharmaceutical production plant. The flow rate was varied by ±10%, while the conductivity in the loading, and washing steps was varied by ±3%. The loading, washing and elution volumes were allowed to vary by ±5%.

4. Results and discussion

4.1. Screening experiments

The performance quotient was measured for each experiment and the experimental conditions that provided the highest value was considered to define the best operating point for the separation. The results of the experiments are given in Table 1. The results were evaluated using the statistics program MODDE 7 in order to identify the most important factors in the experiment. The resulting model includes all interaction coefficients but, for many of the interactions, the effect fell within the confidence interval and was thus not significant. The model was reduced so that only the main effects and the interaction terms that were significant were included. The height of the bars in the coefficient plot (see Fig. 1) shows that the most important factor is the choice of column, followed by pH, salt concentration in the sample and type of salt. The only significant interaction was between pH and type of column. The coefficient plot also shows rather large confidence intervals for the coefficients. The broad confidence intervals are probably not due to variations in the experimental equipment as the three centre points show little variance, but rather to non-linear behaviour of the investigated system that the statistical model cannot describe. The most advantageous operating conditions were found to be at 1.2 mol/l NH_4SO_4

Table 1
The experimental design and results of factorial screening experiments used to determine the most suitable conditions for the chromatography step, see Eq. (10)

Run no.	Salt concentration (mol/l)	Column	pH	Salt	Performance quotient
1	1.0	HP	6.5	$(NH4)_2SO_4$	0.20
2	1.2	HP	6.5	$(NH4)_2SO_4$	0.20
3	1.0	Source	6.5	$(NH4)_2SO_4$	0.20
4	1.2	Source	6.5	$(NH4)_2SO_4$	0.06
5	1.0	HP	7.5	$(NH4)_2SO_4$	0.22
6	1.2	HP	7.5	$(NH4)_2SO_4$	0.24
7	1.0	Source	7.5	$(NH4)_2SO_4$	0.06
8	1.2	Source	7.5	$(NH4)_2SO_4$	0.07
9	1.0	HP	6.5	KPO_4	0.17
10	1.2	HP	6.5	KPO_4	0.22
11	1.0	Source	6.5	KPO_4	0.05
12	1.2	Source	6.5	KPO_4	0.10
13	1.0	HP	7.5	KPO_4	0.22
14	1.2	HP	7.5	KPO_4	0.23
15	1.0	Source	7.5	KPO_4	0.06
16	1.2	Source	7.5	KPO_4	0.03
17	1.1	HP	7.0	$(NH4)_2SO_4$	0.22
18	1.1	HP	7.0	$(NH4)_2SO_4$	0.21
19	1.1	HP	7.0	$(NH4)_2SO_4$	0.23

at pH 7.5 and using the Phenyl Sepharose HP column (see Table 1).

4.2. Model calibration

4.2.1. UV response and void fraction of the column

The simulated breakthrough and elution experiments are obtained using concentrations expressed in mol/m^3, which can be converted into mg/ml using the molecular mass. Experienced scientists working with chromatography are used to observing breakthrough curves and elution peaks in terms of UV absorption. Therefore, experiments to determine the UV absorption for each protein at different concentrations were performed, and a linear relation was derived between UV absorption in the ÄKTA purifier UV cell and protein concentration for each protein. The conversion factors were 308 ml mg/ma.u. for IgG and 127 ml mg/ma.u. for BSA. The UV response is used in all figures when comparing simulated and experimental data, assuming that the total UV response is strictly additive for the components included in the simulation.

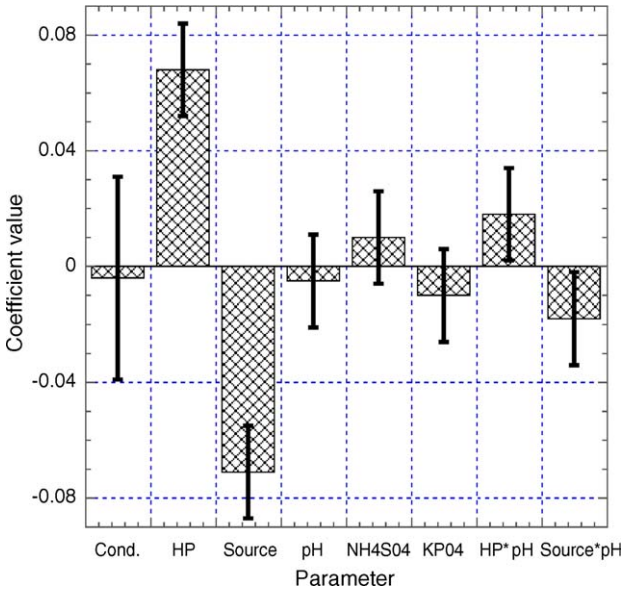

Fig. 1. The coefficient plot resulting from the statistical analysis of the screening experiments. Each coefficient is scaled and centred and the height and direction of the bars show the relative importance of each factor.

The column void was not measured experimentally. The column void fraction was set to 0.34 in the model. This is a relatively low value but it was considered reasonable as the column was industrially packed [14].

4.2.2. Calculation of salt concentration in the parameter estimation

The buffers used in the gradient elution experiments and in the breakthrough experiments had different conductivities. The conductivity of the sample and of the loading and elution buffers was measured. The 40 mM sodium phosphate buffer itself has a conductivity of 5.4 mS/cm, while the conductivity of the phosphate buffer with 1.2 M ammonium sulphate was 153 mS/cm. In the parameter estimation a linear relationship was assumed between conductivity and salt concentration.

4.3. Column dispersion

The Peclet number, *Pe* (see Eq. (8)), was set to 0.33 [13,14]. The axial dispersion coefficient in the column was calculated to be 6.1×10^{-7} m^2/s for a flow rate of 1 ml/min.

4.3.1. Determining peak position (K_{eq}, γ) and peak shape (k_{des0})

At low protein concentration and low column load only γ_i and $K_{\mathrm{eq},i}$ affect the peak position in the gradient elution. The interaction rate parameter, $k_{\mathrm{des0},i}$, was adjusted to give an accurate peak shape for the elution peaks. Fig. 2 shows that the peak shapes and positions are estimated with good accuracy at 10

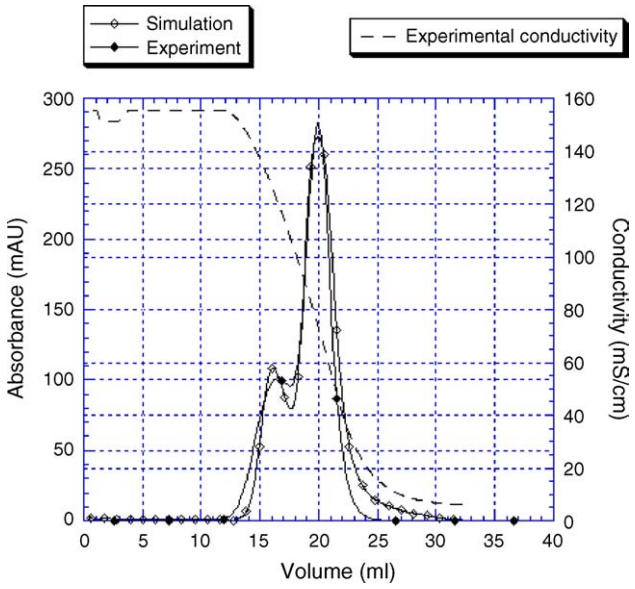

Fig. 2. Comparison between simulation and experiment at 10 CVs gradient elution with a 2 ml load of protein solution.

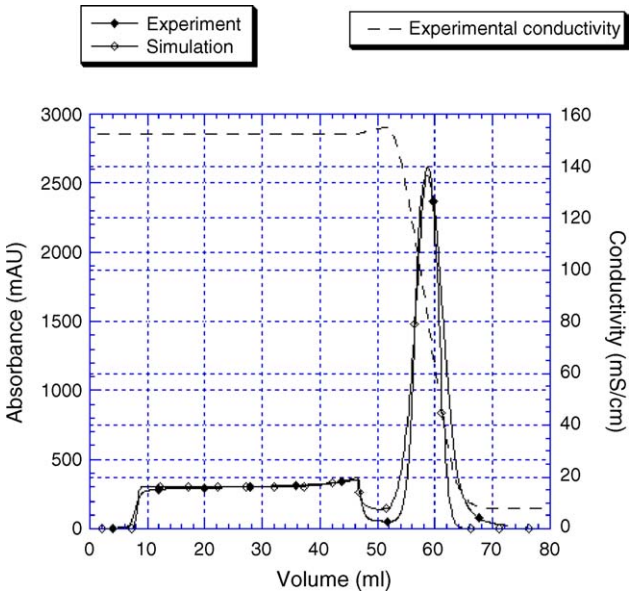

Fig. 3. Comparison between experiment and simulation in the column load experiment where the breakthrough of each component is used to estimate the maximum capacity for each protein.

CVs gradient elution. The parameter estimation resulted in very good agreement between the experimental peak position and the simulated chromatogram. The mean error in peak position for both proteins is less than 1%, and the resulting parameter values with their 95% confidence intervals are given in Table 2. For BSA the capacity parameter, q_{max}, has no impact on the peak position, but for IgG it does. This problem was solved by determining K_{eq} and γ from the gradient elution experiments and q_{max} from the column load experiment simultaneously in an iterative procedure.

When the linear parameters are determined from the peak position the entire UV curve from the experiment at 10 CVs gradient elution was used to fit the kinetic parameter k_{des0} in the interaction model to give an accurate peak shape, see Fig. 2. The final values for k_{des0} for BSA and IgG are given with 95% confidence interval in Table 2.

4.3.2. Determination of the capacity (q_{max})

The capacity parameter, q_{max}, of the two proteins was determined by running a column load experiment where the column was loaded until breakthrough was observed for both proteins and maximum capacity was adjusted for both proteins to fit the experimental breakthrough. With this procedure, q_{max}, was determined using a parameter estimation procedure, in this case adjusting the parameters until the simulated and experimental UV curves fitted as closely as possible. The components compete with each other and affect each other's breakthrough, and it is therefore necessary to fit the capacity for both proteins simultaneously. Single-component experiments are often impossible to conduct in industry, at least for the impurities and one advantage of the approach used in this work is that single-component experiments are avoided.

For the system investigated in the present study the normal competitive Langmuir expression, where K_{eq} and γ are estimated from gradient elution experiments showed too strong competition compared with the column load experiment. Therefore, the model with two types of adsorption sites, described above was applied to obtain the correct competitive behaviour.

Figs. 2 and 3 show that the model fits the experimental results very well, and the simulated breakthrough and the elution peaks are in good agreement with the experimental results.

Table 2
The mean values of all parameters in the model that are subject to parameter estimation together with the limits of their 95% confidence intervals

	Lower	Mean	Upper
BSA			
K_{eq} (m^3/mol)	5.53	5.44	5.58
γ (m^3/mol)	2.53×10^{-3}	2.54×10^{-3}	2.55×10^{-3}
q_{max} (mol/m^3)	0.491	0.512	0.533
k_{des0}	0.0394	0.0450	0.0506
IgG			
K_{eq} (m^3/mol)	1.94	2.02	2.1
γ (m^3/mol)	3.24×10^{-3}	3.31×10^{-3}	3.38×10^{-3}
$q_{max,1}$ (mol/m^3)	0.491	0.512	0.533
$q_{max,2}$ (mol/m^3)	0.919	0.918	0.944
k_{des0}	0.0458	0.0520	0.0582

4.3.3. Optimisation

The optimisation procedure was started by adjusting the column load, the conductivity in the loading step, the washing volume and the elution gradient. Early on in the optimisation process it was found that the conductivity in the loading step should be as high as possible with respect to protein solubility and that it was advantageous to use as steep a gradient as possible, i.e. step elution. In order to obtain the correct shape of the step elution in the experimental equipment the external volume for the buffers was modelled [12]. In the further optimisation process only the column load and the washing volume were adjusted to reach maximum productivity while maintaining

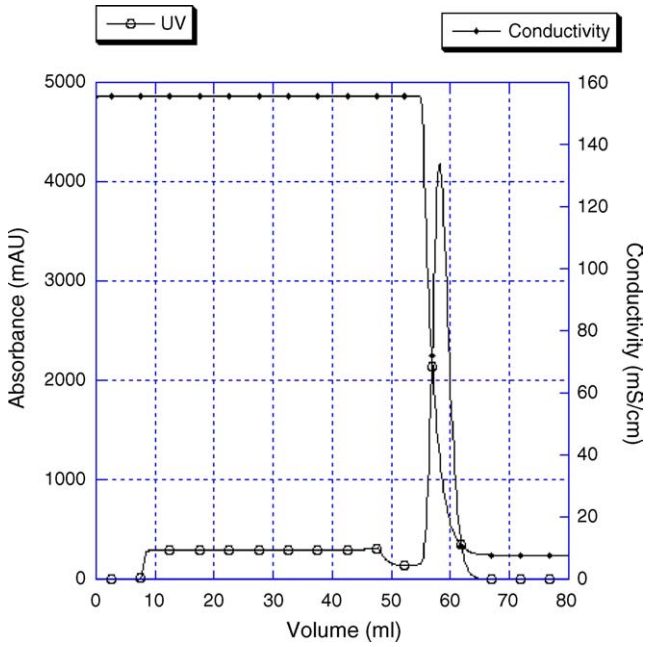

Fig. 4. Simulated results at the optimal point.

99% purity. The optimal point was found to be at a load volume of 49.4 CVs and a wash volume of 7.3 CVs using the maximum conductivity, 155 mS/cm, in the loading step and step elution. The maximum productivity was 38.8 mg IgG/(h ml) stationary phase, while maintaining 99% purity, resulting in a 95% yield of IgG. The simulated chromatography cycle is shown in Fig. 4.

4.3.4. Robustness analysis

The initial part of the robustness analysis involved calculating the sensitivity of each parameter in the model and of each process parameter to separate the parameters from their degree of variation. Sensitivity analysis allows the different parameters to be ranked according to their effect on the purity of IgG. The values given in Table 3 show that the conductivity in the loading step and γ for IgG are the most sensitive parameters in this study. Ordering the parameters in this way can give valuable insight into which parameters must be strictly controlled. The sensitivity calculation also provides information about the model parameters and points out the parameters that need to be estimated with good accuracy. The results in Table 3 also indicate in which direction the parameter variation causes a lower purity.

The sensitivity of each parameter together with its variation provides information on how much the purity of the product is affected by variations in that parameter. Simulations at the limits of the intervals of the model and process parameters, give the impact of each parameter on the purity of IgG in the elution pool. Table 3 shows that the most important parameter variations are; conductivity in the loading step, conductivity in the washing step, Load volume and the hydrophobic coefficient, γ, for IgG.

To investigate whether there is any non-linear impact of the variation of the parameters within their intervals sensitivity calculations can be used to predict the expected impact on purity if the result is linearly extrapolated. A comparison between columns 3 and 4 of Table 3 shows that there is significant non-linear behaviour for variations in the conductivity in the loading and washing step, as well as for variations in γ for IgG. This non-linear behaviour is expected as the adsorption coefficient $k_{\mathrm{ads},i}$ was modelled with an exponential dependency on salt concentration and γ, see Eqs. (5) and (6).

The final part of the robustness analysis is to consider the co-variation of the most important parameters. The co-variation effects considered are loaded volume, conductivity in the loading step, conductivity in the washing step and γ for IgG. The co-variation effects were studied by simulation of a full factorial experiment with four factors. Table 4 shows the results of this co-variation. The table also shows in which direction the parameters varied to cause these critical points for the purity of IgG.

In this case, the lowest expected purity at the optimal point would be 97.1%, which should be compared with the 99% specified in the optimisation. Variations that result in a purity below 98.5% are considered to be critical points. As guidance for an experimental study, this analysis points out five process parameter variations that need to be investigated experimentally (runs 1, 2, 6, 13 and 15). Runs 3 and 7 also result in purity below 98.5%

Table 3
The sensitivity of each model and process parameter, the impact on purity, obtained by linear extrapolation, the impact of simulation at the limits of the parameter variations and the direction of variation that causes lower purity

Parameter	Sensitivity	Impact on purity (%) linear extrapolation	Impact on purity (%) model response	Direction
Load volume	1.9	0.065	0.097	–
Flow	0.20	0.019	0.020	+
Conductivity, loading step	8.7	0.27	0.61	–
Washing volume	0.80	0.037	0.040	–
Conductivity, washing step	2.2	0.13	0.15	+
$k_{\mathrm{des0,IgG}}$	0.052	0.0054	0.0061	–
γ_{IgG}	6.1	0.078	0.13	–
$K_{\mathrm{eq,IgG}}$	1.2	0.035	0.050	–
$q_{\mathrm{max,IgG}}$	1.2	0.013	0.015	+
$k_{\mathrm{des0,BSA}}$	0.14	0.015	0.017	–
γ_{BSA}	1.7	0.0066	0.0066	+
$K_{\mathrm{eq,BSA}}$	0.54	0.014	0.014	+
$q_{\mathrm{max,BSA}}$	0.84	0.033	0.034	+

Table 4
The results of the co-variation analysis

Run	Load volume	Conductivity, loading step	Conductivity, washing step	γ_{IgG}	Purity (%)
1	–	–	–	–	97.7
2	+	–	–	–	97.7
3	–	+	–	–	99.0
4	+	+	–	–	99.5
5	–	–	+	–	97.1
6	+	–	+	–	97.2
7	–	+	+	–	98.4
8	+	+	+	–	99.2
9	–	–	–	+	98.8
10	+	–	–	+	99.1
11	–	+	–	+	98.6
12	+	+	–	+	99.5
13	–	–	+	+	98.2
14	+	–	+	+	98.8
15	–	+	+	+	98.0
16	+	+	+	+	99.1

but differ from runs 13 and 15 only in the value of γ_{IgG}. These five critical points should be investigated experimentally and if the resulting purities are acceptable, the rest of the points should also fall within the specification. If the experiments result in purities that do not fall within the specifications, another operating point must be chosen or the control of the critical parameters must be improved.

4.3.5. Limitations in the methodology

The methodology presented in this work should be useful in the pharmaceutical industry with an appropriate scale up procedure from laboratory to pilot and production scale columns. The accuracy of the resulting model is taken into account by using the confidence intervals of the parameters to calculate the lowest expected purity. One limitation when using this methodology is that it has to be possible to find a model and a calibration procedure that predicts the experimental results with good accuracy and reasonably narrow confidence intervals in order to get useful predictions.

5. Conclusions

The method presented in this paper constitutes a calibrated model that succeeds in predicting the separation of IgG from BSA, and can, with reasonable accuracy, predict the behaviour in the hydrophobic interaction chromatography column. It is thus reasonable to claim that the methodology can be used to find the optimal operating conditions and provide insight into the sensitivity of process performance with respect to variations in the parameters of the model and the sensitivity with respect to process variations.

The methodology presented in this work reduces the number of variables that must be included in the robustness analysis from five to three process parameters which, for a factorial experimental design reduces the number of experiments necessary from 32 to 16. The model also identifies the five most crucial points within the 16 combinations further reducing the experimental work. In addition, the model can be used to find the optimal running conditions without any additional experimental effort.

6. Nomenclature

c_i	concentration of component i in the mobile phase (mol/m^3)
$c_{inlet,i}$	inlet concentration of component i in the mobile phase (mol/m^3)
$C_{outlet,IgG}$	concentration at the outlet of the column (mg/ml)
d_p	bead diameter (m)
D_{ax}	dispersion coefficient (m^2/s)
$k_{ads,i}$	adsorption coefficient, Langmuir MPM (m^3/mol s)
$k_{ads0,i}$	modulator constant (m^3/mol s)
$k_{des,i}$	desorption coefficient, Langmuir MPM (s^{-1})
$k_{des0,i}$	modulator constant (m^3/mol s)
$K_{eq,i}$	equilibrium constant, Langmuir MPM
L	length of the column (m)
M	model
P	parameter
PF	performance function
Pr	productivity (mg IgG/h)
$q_{max,i}$	maximum concentration in the stationary phase of component i (mol/m^3 gel)
q_i	concentration in the stationary phase of component i (mol/m^3 gel)
S	concentration of the elution component (mol/m^3)
t	time (s)
t_1	starting time for the step elution (h)
v_{int}	interstitial velocity (m/s)
x	axial coordinate along the column (m)
β_i	constant describing the IEC characteristic
γ_i	constant describing the HIC characteristic (m^3/mol)
ε_c	void fraction in the column (m^3 mobile phase/m^3 column)

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