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Optimization of Lactoperoxidase and Lactoferrin Separation on an Ion-Exchange Chromatography Step

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Abstract

Lactoperoxidase (LP), which is a high-value minor whey protein, has recently drawn extensive attention from research scientists and industry due to its multi-function and potential therapeutic applications. In this study, the separation and optimization of two similar-sized proteins, LP and lactoferrin (LF) were investigated using strong cation exchange column chromatography. Optimization was started with central composite design based experiments to characterize the importance of different decision variables. The three variables used in the optimization were flow rate, length of gradient and final salt concentration in the linear elution gradient step. The obtained empiric functional model represented the effect of the significant factors on the yield as the objective function. Afterwards, the calibrated mechanistic model was employed to predict accurate optimal set of variables. The optimal operating points were found and the results were compared with validation experiments. Predictions respecting yield confirmed a very good agreement with experimental results while keeping purity, a product quality characteristic, equal or above to a predefined value.

Keywords: cation exchange chromatography; minor milk protein; response surface modeling; simulation; steric mass action (SMA); optimization

1. Introduction

Today, the purification of high-value proteins from waste food streams has attracted a great attention. Due to the high number of potential applications of protein isolates, a few chromatographic processes have been developed to isolate high-purity protein fractions [1] . Ion-exchange chromatography (IEC) is one of the most powerful techniques to overcome biomolecules purifying challenges and is commonly applied in downstream processes.

Milk whey is a mixture of a variety of proteins. The mixture displays a wide range of chemical, physical, and functional properties [2]. Whey proteins have been adequately separated into different fractions; the isolation of the major and minor proteins [3–7]; however, the efficient purification of high-value minor proteins of similar molecular weights such as lactoperoxidase (LP) and lactoferrin (LF) still remains as a challenge. LP has an approximate molecular weight of 77.5 kDa [8] with an isoelectric point approaching 9.5. Molecular weight of LF is 78.0 kDa with an isoelectric point around 8.7. In addition, proteins exhibit susceptible structure that alters their functionality, thus these macromolecules should be processed as quickly as possible and in as few steps as possible. In biotechnology industry, yield and bioactivity are directly associated to efficient processing [9,10]. Despite of the significant effort that has been applied toward developing downstream processes, there are still issues that need further considerations before an industrial application will be viable. Some of these challenges include labor-intensive experimental work, rule of thumbs and consequently optimization of downstream processes can cover up to 50–80% of total production costs [11]. Growing demands for high quality products result in more complexity of processes and analytics, thus increasing the costs for product work-up. Process analytical technology (PAT) [12], introduced in the guidelines of the US Food and Drug Administration, emphasizes on better understanding of a process, as well as sensitivity and robustness analyses that ultimately leads to reduce overall process development cost and time from laboratory to production scale.

This is the main motivation for extreme attempts to assist the development and optimize chromatography processes, mostly industrial preparative processes, aiming for higher productivity, yield, and purity of the protein of interest. Successful approaches to the optimization of chromatographic separations always include a detailed consideration of the physicochemical properties of involved components as well as interactions between the proteins

and the adsorbent phase. To the best of our knowledge, no model-integrated approach has been applied to optimize minor milk proteins separation in IEC, making it a topic worthy of investigation. The present work aims to study optimization of method development to maximize yield of lactoperoxidase with respect to a constraint on purity. A statistical study was first conducted to examine the influence of the operation variables (concentration of salt at the end of gradient, length of gradient and velocity). Subsequently productive information was used to assist the mechanistic model to gain more insight into the optimization of IEC. In this work, purity was defined as a nonlinear constraint in the optimization procedure to meet equal or above to a pre-set requisite at the operating point.

2. Theory

2.1 Response surface modeling and design of experiments

Response surface methodology (RSM), a collection of mathematical-statistical technique based on design of experiments (DoE), has been successfully used for optimization studies of different bio-separation processes [13]. RSM has been widely adopted to investigate the effects of several design factors influencing a response by varying them simultaneously in a limited set of experiments. The concept of DoE-techniques outlines various statistical approaches to maximize specific information in an experimental planning and after all determine the most favorable direction to move in order to find a true optimum. Central composite design (CCD) is an ideal choice as a symmetrical experimental design for sequential experimentation and allows reasonable information to calculate model lack of fit while reducing the number of design points. In general, CCD is the more known class of quadratic design that consists of: (1) a factorial (or cubic) design; (2) an additional design often a star design with all points set to an equal distance from the center and (3) at least one center point [14]. Therefore, full uniformly routable central composite designs involve the total number of $N=k^2+2k+c_p$ points, where k is the number of the factors, and c_p is the number of the replicate runs performed at the center point. In this design, the distance α from the center point depends on the number of the factors and can be calculated by $\alpha=2^{(k-p)/4}$. All factors have to be adjusted at five levels ($-\alpha,-1,0,+1,+\alpha$) [14]. The quadratic polynomial model for the measured values of the results from experiments variable, Y with k factors is given by:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where x_i and x_j are the design factors in coded values, β_0 is the constant parameter, β_i , β_{ij} ,and β_{ii} are the coefficient of the linear, interaction, and quadratic terms of the model, respectively. The coefficients of Eq. (1) are estimated using statistical software packages (e.g., Minitab, Design Expert, SPSS).

2.2 Mechanistic modeling of chromatography

A mechanistic model is used to describe the physical phenomena based on a set of mathematical equations. Two types of physical phenomena dominate chromatography; movement of solutes through the packed bed of porous particles via mass transfer mechanisms, and adsorption based on the fundamental thermodynamic interactions between migrating solutes and the stationary phase. The general system of equations used to describe the mass transfer phenomena consist of two sets of partial differential mass conservation equations. The general rate model for a chromatographic process includes convective and diffusive flows through porous particles on the column level and imitates mass transfer resistances and surface interactions on particle level. In IEC, an external film surrounding adsorbent particles is commonly presumed to model the movement of components from column to particle level; the sorption of protein on the particle surface can be described by the steric mass-action (SMA) model, developed by Iyer et al. [15] and generally used for the modeling of salt gradient elution in IEC, for example in [16].

On column level, concentration change for the i^{th} component with respect to the time and position, is described by:

$$\frac{\partial c_i}{\partial t} = -u_{\text{int}} \frac{\partial c_i}{\partial x} + D_{ax} \frac{\partial^2 c_i}{\partial x^2} - \frac{1-\varepsilon_c}{\varepsilon_c} \cdot \frac{3}{r_p} k_{eff,i} \left[c_i - c_{p,i} \right] \quad (2)$$

The first term on the right hand side represents the convective transport through the column while the second and third term represents respectively the dispersive transport and the mass transfer to the particle surface. The symbol u_{int} indicates the interstitial velocity, ε_c the column voidage, r_p the particle radius, D_{ax} the axial dispersion representing combined effect of dispersion and diffusive processes, and $k_{eff,i}$ epitomize combined effect of both the internal and

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external mass transfer resistances in one lumped film diffusion coefficient. Analogously, on particle level, concentration change for the i^{th} component is expressed by:

$$\frac{\partial c_{p,i}}{\partial t} = \frac{3}{\varepsilon_p r_p} k_{eff,i}[c_i - c_{p,i}] - \frac{1-\varepsilon_p}{\varepsilon_p}\frac{\partial q_i}{\partial t} \tag{3}$$

where q_i denotes the concentration of component i within the particle and ε_p the particle voidage. The first term on right hand side describes adsorption and desorption processes on particle level, i.e. the interaction between mobile and particle bound phase.

For the description of sorption kinetics, the steric mass-action (SMA) isotherm developed by Brooks and Cramer [17] was embedded into the mechanistic model. It has been successfully used to describe protein adsorption in IEC. Based upon the stoichiometric exchange of charges and steric hindrance of binding sites, the isotherm can be described by the reaction:

$$c_i + \nu_i \bar{q}_{salt} \Leftrightarrow q_i + \nu_i c_{salt}$$

The parameter ν_i is the characteristic charge of protein, which represents the average number of ligands, or binding sites, interacting during adsorption. $\bar{q}_{salt}$ is the concentration of adsorbed salt counter-ions that are available for exchange, c_{salt} is the salt concentration of the bulk.

$$\bar{q}_{salt} = \Lambda - \sum_{i=2}^{n+1}(\nu_i + \sigma_i)q_i \tag{4}$$

where the parameter Λ is the ionic capacity of the adsorbent and σ_i is the steric factor, which represents the average number of counter-ions shielded per adsorbed protein molecule.

SMA isotherm in its kinetic form can be expressed as:

$$\frac{\partial q_i}{\partial t} = k_{ads,i}\left[\Lambda - \sum_{j=1}^{k}\left(\nu_j - \sigma_j\right)q_j\right]^{\upsilon_i} c_i - k_{des,i} c_{salt}^{\nu_i} q_i \tag{5}$$

$k_{ads,i}$ and $k_{des,i}$ respectively denote the adsorption and desorption rates.

2.3 Optimization

Optimization maximizes/minimizes an objective function, by varying one or more variables, called decision variables to obtain an optimal purification step. A chromatographic purification step involves of many decision variables such as loading, washing and elution times, salt concentration, the flow rate in the different steps, column length, column diameter, and the gradient in the elution step. The decision variables can be narrowed down by including lower and upper boundaries. To limit the optimization further, constraints can also be added as equality or inequality functions. The most typical objective functions used in a preparative chromatographic purification step are production rate and yield. In this work, the objective function studied was the yield, and the inequality constraints set on the optimization was purity.

The yield is calculated as the fraction of the target component captured:

$$Y = \frac{\int c_{captured,i} dV}{V_{load} \times C_{load}} \quad (6)$$

where $c_{captured,i}$ is the concentration of the target component i leaving the column during the elution.

The purity is defined as

$$Pu_i = \frac{m_{captured,i}}{\sum_j m_{captured,j}} \quad (7)$$

where $m_{captured,i}$ is the amount of substance captured of component i.

3. Materials and methods

3.1 Materials, column and software

The study aims at an optimal separation of lactoperoxidase on the adsorbent SP Sepharose FF by linear gradients. Lactoperoxidase from bovine milk, ≥ 90% pure and Lactoferrin from bovine

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milk, approximately 95% pure were used. Sodium monobasic phosphate, sodium dibasic phosphate and sodium chloride for buffers preparation were purchased from Sigma-Aldrich (Oakville, Canada). NaOH was used for pH-adjustment. The running buffer in all experiments was 0.02 M sodium phosphate buffer at pH 6.7. The buffer for elution purposes contained additional NaCl. Proteins were diluted into phosphate buffers by slowly stirring to prevent any foam formation. Afterward, the protein solution was filtered through a 0.2 µm hydrophilic polypropylene membrane filter to remove any fine particles. The chromatographic setup consisted of a prepacked HiScreen™ SP Sepharose FF 4.7 ml column (10.0 cm length, 0.77 cm ID) and an ÄKTA purifier 100 system, both purchased from GE Healthcare (Mississauga, Canada). The software Minitab®17 (State College, Pennsylvania, USA) was used as a statistical tool for handling response surface methodology. The software MATLAB R2014a was used to execute the mechanistic model.

3.2 Experimental methods

In all experimental setups the column was at first equilibrated with running buffer for 5 column volumes (CV). This step was followed by an automated sample load of 2 mL protein mixture. Then the column was flushed for another 2 CV to remove unbound proteins, before initiating a linear elution gradient. The elution gradient was applied from 0% to 100% high salt elution buffer, followed by a 5 CV high salt wash step and regenerated and re-equilibrated with 1 M NaOH and running buffer respectively. Conductivity and UV-absorbance were measured online at column outlet. The data collected from these measurements was further analyzed and taken into account to estimate SMA parameter by the inverse method. Three design factors were employed to describe the gradient profile:

- Final concentration of salt in elution step [M]
- Length of linear elution gradient [CV]
- Superficial velocity [cm/min]

The experimental ranges used for these factors included (0.35-1.35 M) for final concentration of salt in elution step, (1.11-5.98 cm/min) for flow velocity, and (9.88–35.1 CV) for length of gradient. All the buffers were prepared using ultra-pure water (Barnstead easy-pure RODI, Fisher Scientific), filtered with a 0.45 µm membrane and degassed prior to use.

3.2.1 Protein quantification

Lactoperoxidase concentration was determined by Lowry protein assay. The collected fractions of lactoperoxidase were analyzed using measurement of absorbance at 280 nm (extinction coefficient of lactoperoxidase $\varepsilon_{1\%} = 14.9$ [18]) and 412 nm. Lactoperoxidase absorbs radiation at 280 nm as well as 412 nm; it has maximum absorbance at 412 nm [19] and its purity is estimated as a ratio of A_{412}/A_{280}. The BCA (bicinchoninic acid) protein assay kit from Sigma (Oakville, Canada) with BSA protein standard was used to further analysis of protein fractions from ÄKTA.

3.3 Mathematical method

3.3.1 Screening experiments to determine importance of design factors

Concentration of salt was studied over a broad range as for the elution step a lower salt concentration can result in a lower purity for lactoperoxidase and less lactoperoxidase will elute in the elution step. On the other hand, a higher salt concentration in the elution step is less critical as lactoferrin is far from eluting at lower conductivities. Flow rate is remarked to be easy to control and was investigated over a narrower range, with upper bound recommended by the manufacturer. Initial operating conditions to maximize chromatography performance were determined by means of a functional relationship between the experimental designs combined with response surface modeling. To assure the consistency of prediction error, the value (α) was adjusted as 1.68 ($\sqrt[4]{8}$), and it was assumed all points with the equal distance from the design center have the constant prediction variance. Table 1 shows design factors in their coded and un-coded (experimental) forms, in an experimental design performed in a random order to avoid systematic error. As seen in Table 1, the design experiments had 20 runs in total. The analysis of variance (ANOVA) was used to test the significance of the fit of the empiric model. The significance level of 0.05 was chosen to establish the statistical significance in all results.

Table 1 Coded and un-coded values of the process factors in screening experiments of central composite

Run	Coded			Un-coded		
	Salt	Length of gradient	Flow velocity	Salt (M)	Length of gradient (CV)	Flow velocity (cm/min)
1	-1	-1	-1	0.35	15	2.1
2	1	-1	-1	1.1	15	2.1
3	-1	1	-1	0.35	30	2.1
4	1	1	-1	1.1	30	2.1
5	-1	-1	1	0.35	15	4.998
6	1	-1	1	1.1	15	4.998
7	-1	1	1	0.35	30	4.998
8	1	1	1	1.1	30	4.998
9	-1.681	0	0	0.094	22.5	3.549
10	1.681	0	0	1.355	22.5	3.549
11	0	-1.681	0	0.725	9.88	3.549
12	0	1.681	0	0.725	35.1	3.549
13	0	0	-1.681	0.725	22.5	1.112
14	0	0	1.681	0.725	22.5	5.985
15	0	0	0	0.725	22.5	3.549
16	0	0	0	0.725	22.5	3.549
17	0	0	0	0.725	22.5	3.549
18	0	0	0	0.725	22.5	3.549
19	0	0	0	0.725	22.5	3.549
20	0	0	0	0.725	22.5	3.549

3.3.2 SMA model calibration and validation

The inverse method [20] was used to calibrate the model parameters to experimental data. SMA parameters were estimated based on 20 chromatograms seeking to achieve a best fit between model response and measured chromatogram data. The optimal set of the parameter values, $\{v, k_{ads}, k_{des}, \sigma\}$, can be generated by minimizing the error function $F(p)$ defined as following:

$$F(p) = \min_{\theta} \sum_{i=1}^{p} (c_{i,ex} - c_{i,m})^2, \qquad \theta = \{\nu, k_{ads}, k_{des}, \sigma\} \tag{8}$$

where p is the total number of the experimental data. The minimization of Eq. (8) was performed with the MATLAB function *lsqnonlin*.

Method of lines (MoL) [21] was used to discretize the column in space dimension. The boundary conditions of the column were Danckwert's boundary conditions [22]. The discretized model was solved with a stiff ordinary differential equation solver (*ode15s*) using variable-order method in MATLAB [23].

For estimating the dispersion coefficient's dependency on flow rate, the mean particle Péclet number (*Pe*) estimated to be 0.5 [24], the dispersion coefficient was calculated from:

$$D_{ax} = \frac{u_{\text{int}} d_p}{Pe} \tag{9}$$

The film mass transfer coefficient k_f can be estimated from the correlation [25]:

$$Sh = (7 - 10\varepsilon_c + 5\varepsilon_c^2)(1 + 0.7\,\text{Re}^{0.2}\,Sc^{1/3}) + (1.33 - 2.4\varepsilon_c + 1.2\varepsilon_c^2)\,\text{Re}^{0.7}\,Sc^{1/3} \tag{10}$$

where $Sh = k_{eff}\, d_p / D_m$ is the Sherwood number, $Re = u_0 \rho d_p / \eta$ is the Reynolds number and $Sc = \eta / \rho D_m$ is the Schmidt number.

To ascertain that the model is correct, the confidence interval of the parameters calculated from the Jacobian and the residual. The relative residual error is plotted against the cumulative distribution to show how well the model fits the data. The model is then compared to the validation experiments, and if the simulation fits the experiments, the model is valid.

3.3.3 Optimization method

The calibrated mechanistic model was then employed for optimization with respect to an objective function. In this study, yield as the fraction of target protein eluted was defined the objective function. The purity of lactoperoxidase was used as the nonlinear inequality constraint with the requirement of 85%. The parameters such as concentration of the feed and pH are

determined by the composition of natural milk. Other parameters such as buffer, eluting salt and stationary phase material were kept the same during the optimization step. The main decision variables were determined according to the results of the DoE-RSM; length of gradient has no significant effect on yield thus the response to variation of the final salt concentration in the elution and mobile phase flow velocity was studied by computer simulation. The optimization problem with respect to the objective was solved using *fmincon* in MATLAB. *fmincon* finds the optimum of an objective function Y with defined lower and upper boundaries on the decision variables. The optimization problem can be defined as:

$Y_{max} = \max(Y)$

Decision variables, $x =$ [*velocity, salt concentration at the end of gradient*]

$$LB \leq x \leq UB$$

subject to purity ≥ purity requirement

4. Results and discussion

4.1 Results of Response surface modeling

In this study, the best-fitting response surface provided a functional relationship between the objective function yield and the gradient explanatory factors salt concentration, length of gradient and flow velocity. The normal probability plot of the residuals from the analysis was normally distributed; indicating no evidence of non-normality, skewness, outliers, or unidentified variables exist. An R^2 of 0.89 is probably due to the variations in the experiments at the six center points.

According to the analysis of variance (ANOVA), the calculated probability (p-value) of a test statistic was less than 0.005 for the term final concentration of salt which translates its significance within 95% confidence interval; whereas the p-values of gradient length and flow velocity justifies that the coefficients are zero. The mixed effects/interaction terms of each two factors were also quantified and revealed no considerable effect in the response model. The coefficient plot (Figure 1) shows the scaled and centered coefficients for the most important factors and their influence on the objective function. The height and direction of the bars illustrate the corresponding significance of each factor. In addition, the coefficient plot displays

relatively large confidence intervals for the coefficients. The broad confidence intervals are probably due to variations in the experimental equipment as the six center points show fairly large variance. Another explanation could be the fact that the investigated system reveals non-linear performance that the statistical model cannot describe.

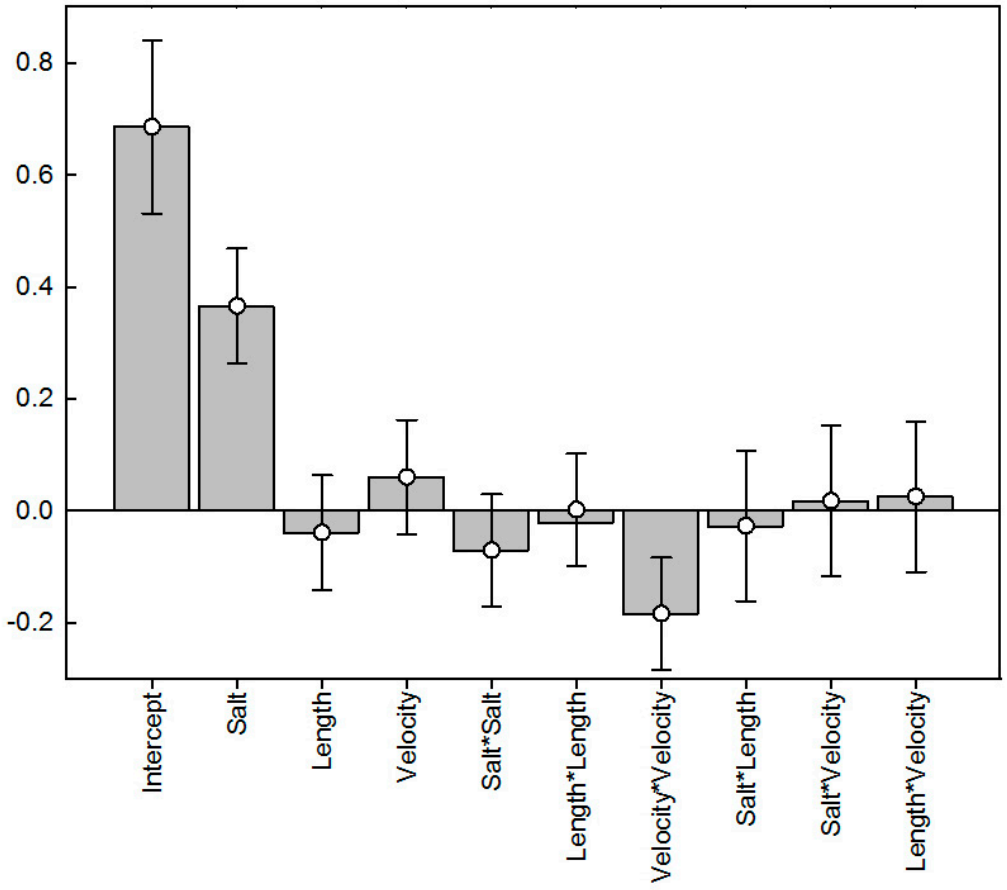

Figure 1 The coefficient plot resulting from the response surface regression of the screening experiments. The coefficient are scaled and centered and the height and direction of the bars show the relative importance of each factor.

Analysis of the model response shows that gradient final salt concentration is an important factor for yield. This is to be expected as salt will displace protein as elution proceeds, and as a result more protein will be present in the collected fractions. It suggests that gradients with a high salt concentration at gradient end were most successful with respect to the separation problem.

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The variation in the two factors gradient length and flow velocity were not high enough to cause an effect on the separation of lactoperoxidase in the process. It assures that at higher flow rates, there is no broadening effect or leakage in the loading step. The significance of the coefficient for velocity×velocity term with regard to the p-value predicted by the model indicates that the hypersurface exhibits a curvature meaning that there is a maximum/or minimum somewhere in the direction of flow velocity (Figure 2).

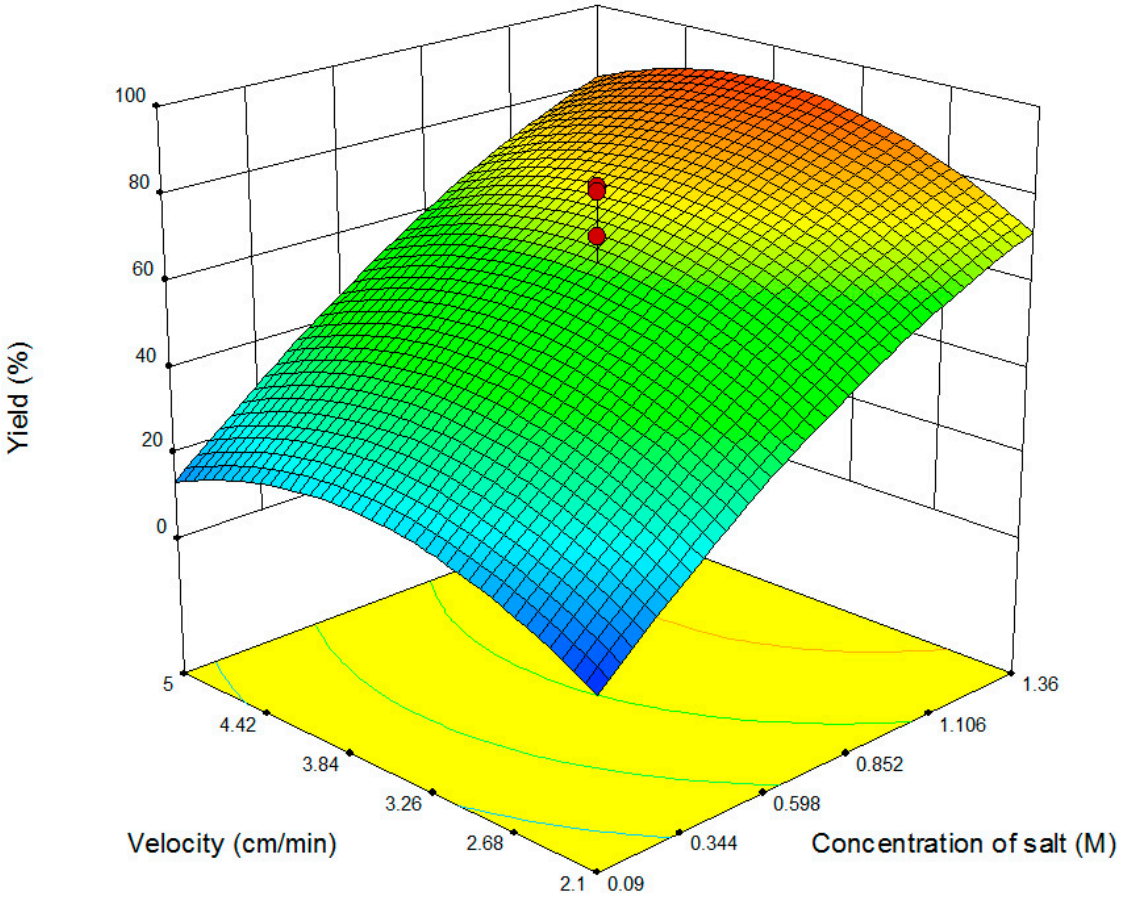
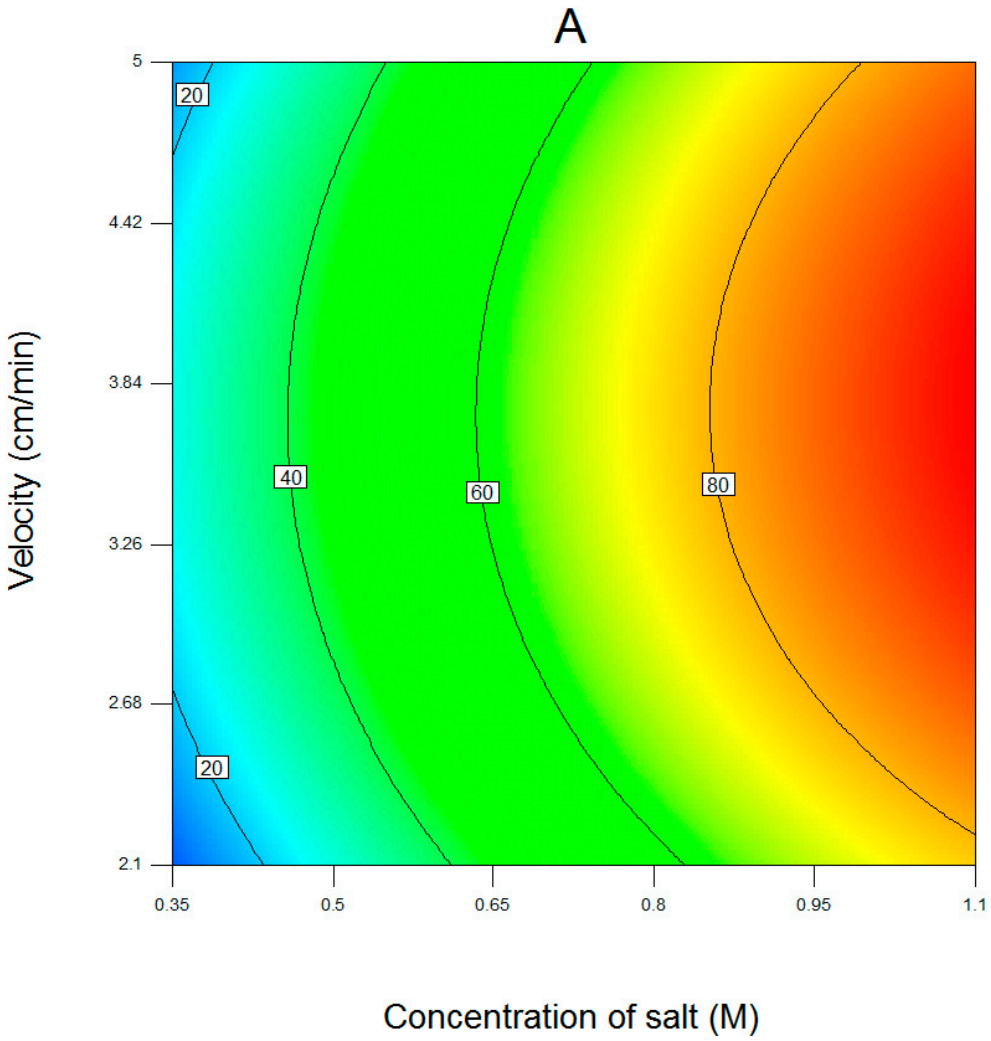
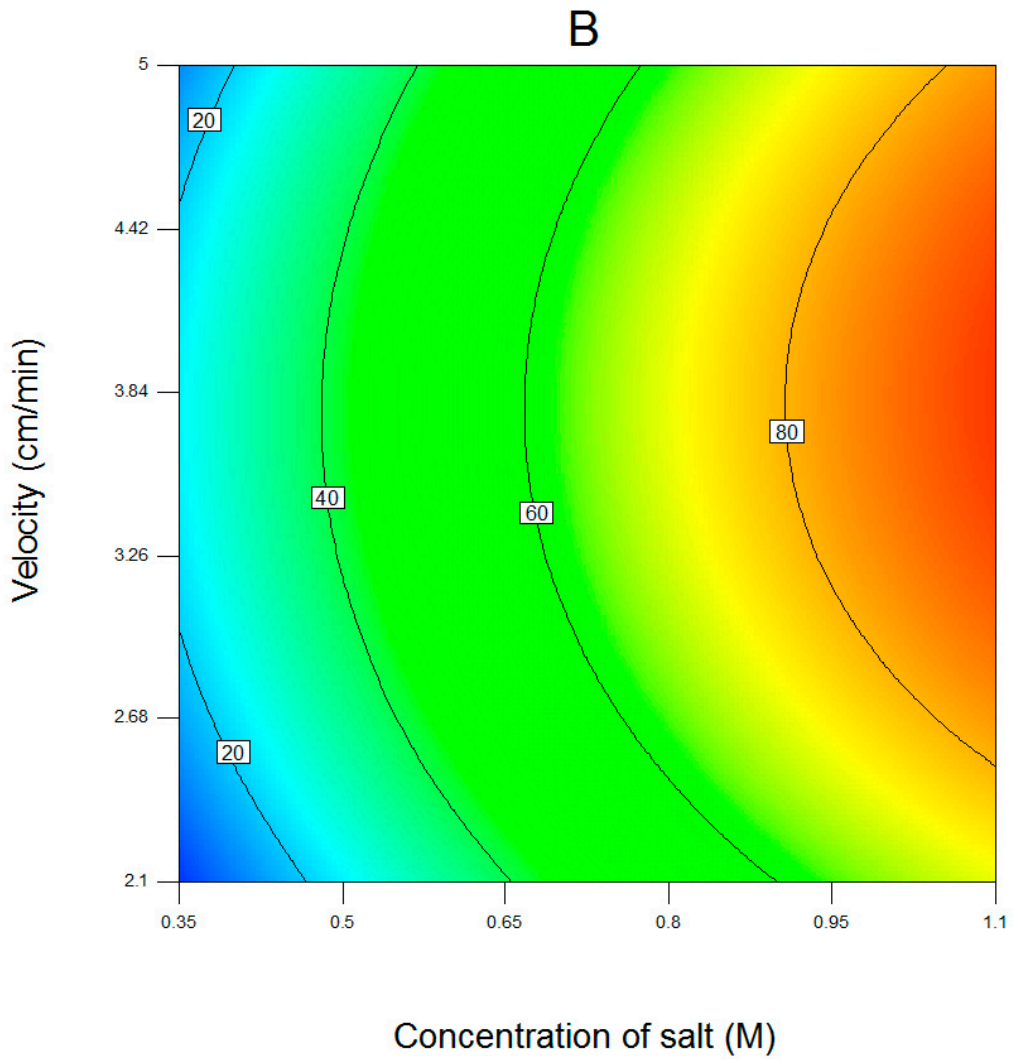


Figure 2 3D surface plot based on the CCD method

The Figure 3 illustrates the projection of the response surface as a two dimensional plane for three levels of the factor length of gradient 15, 22.5 and 30 CV. As can be seen in Figure 3, the maximum velocity of around 3.8 cm/min with highest concentration of salt in elution buffer is predicted to achieve maximum protein yield to for all gradient lengths. The optimal region is quiet large and the concentration of salt within the contour plot is steep. This indicates that small

changes in the concentration of salt in elution buffer will have significant effects on the product quality of the investigated process.





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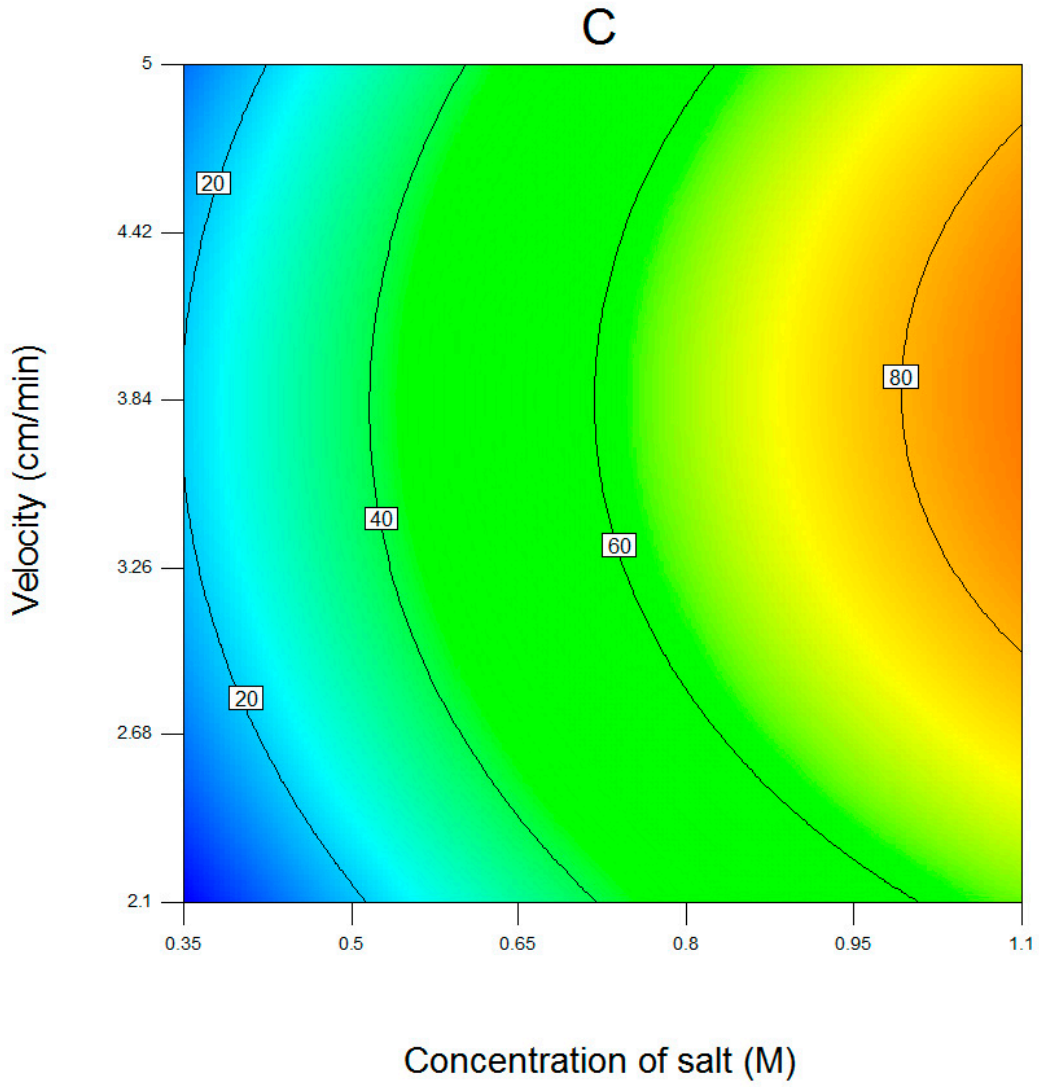

Figure 3 Surface contour plots based on 20 experiments. The factors Final concentration of salt and Flow velocity span the space, the factor Length is depicted in three levels: 15, 22.5, 30 CV. The contour lines illustrate the predicted values for the yield of lactoperoxidase

Based on this empiric model function and the modeling surfaces, the factor setups for maximum qualities of separation with respect to yield were predicted (see Table 2), experiments to evaluate the model predicted results were performed. The comparison between experimental results and the empiric model predictions shows low predictability of the RSM model. As it can be seen in Figure 4, the predicted coefficient of determination, pred-R^2 of about 0.66, indicates a narrow predictability. In other words, quadratic RSM can only describe 66% of the variety in the experimental data.

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Table 2 Optimum factor set for maximum yield of lactoperoxidase based on the DoE–RSM approach

Concentration of salt (M)	1.10
Flow velocity (cm/min)	3.77
Gradient length (CV)	15
Predicted yield (%)	96.41
Experimental yield (%)	78.04

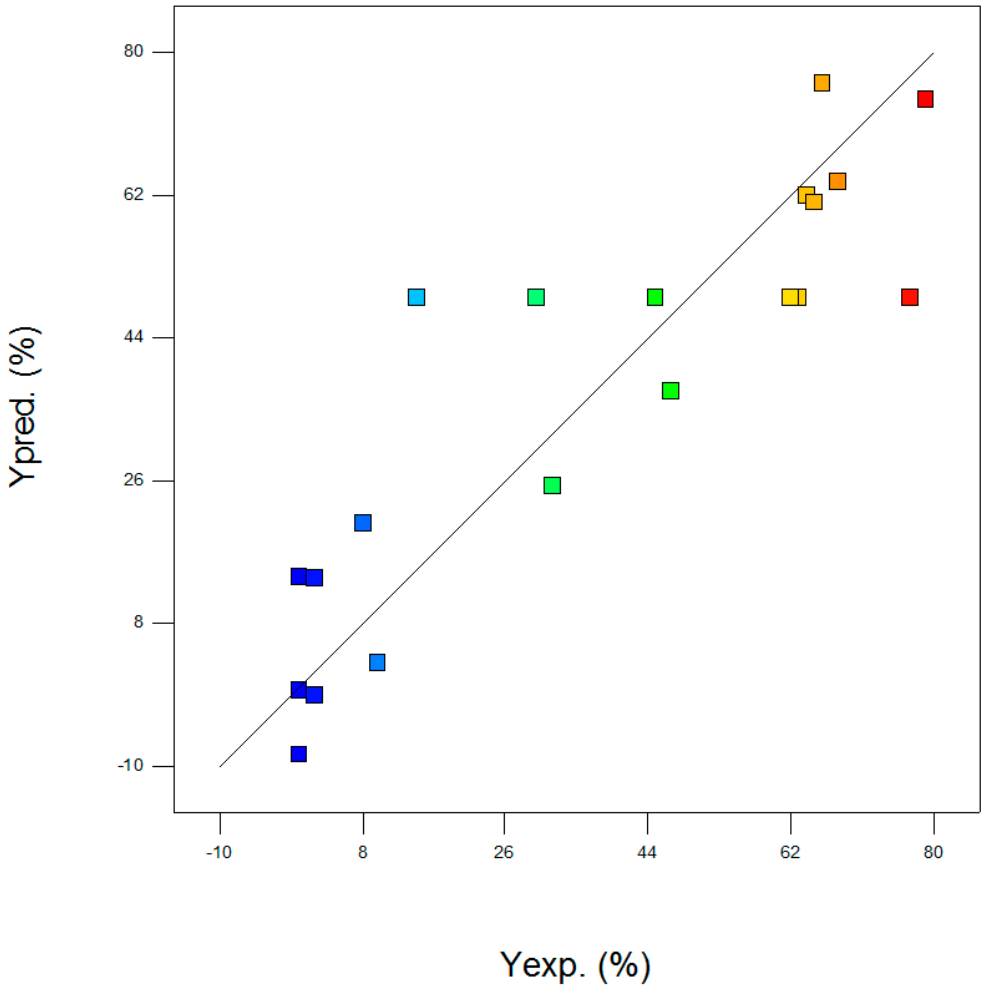

Figure 4 Experimental and predicted yield of lactoperoxidase. The predicted IEC yield was obtained using empirical functional relation

4.2 Results of the mechanistic model

4.2.1 Model calibration and validation

In this section, the bed parameters of a 4.7 mL prepacked HiScreen™ SP Sepharose FF column characterized in [26] were set into the mechanistic model. The adsorption parameters with their confidence intervals are presented in Table 3. The parameters were estimated based on DoE experiments in section 4.1 to keep the predictions over the similar design space. The steric factor (σ) had negligible influence on the fitting result; thus it was fixed during the optimization of Eq. (8) to previously calibrated values in [26]. Broad statistical range in the determination of σ has been reported before in [15,16].

Table 3 SMA parameters for lactoperoxidase and lactoferrin on HiScreen™ SP FF column

	k_{eq}	ν	k_{des}	σ
lactoperoxidase	0.22±0.005	3.07±0.014	19.89±0.071	1283
lactoferrin	11.65±0.003	2.73±0.006	0.98±0.22	0.98

The successful predictions based on the calibrated and verified mechanistic model is a key associated to a model-integrated process development. The validation experiments were carried out as a gradient elution experiment with higher protein concentrations that were not used in the model calibration. The model fitted the validation experiment with relatively good accuracy; in Figure 5 an example chromatogram for the gradient elution experiments with 3.55 cm/min flow velocity an elution gradient volume of 22.5 CV at 0.26 and 1.47 mg/ml for LP and LF concentrations is shown. The elution gradient with started after 50 minutes and continued for around 50 minutes, at which point 100% high salt buffer with a salt concentration of 0.725M NaCl was reached. The first breakthrough is LP and the second is LF. In the washing step, some of the LP and LF were washed out; the model did not capture this amount of protein loss, causing the lack of fit in the elution step at the chromatogram.

The cumulative distribution of the error residual, including both the calibration and the validation experiments, is plotted in Figure 6. The 90% of the error residual is below a relative error of 4.8 and 99% of the error residual is below 14% which may seem high; at high protein loading (validation experiments) a small deviation in the retention volume will result to a large residual

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error. However, these results confirm that the model is capable of describing the problem. If a more accurate solution is sought, the model should be recalibrated based on a new set of DoE experiments in this region.

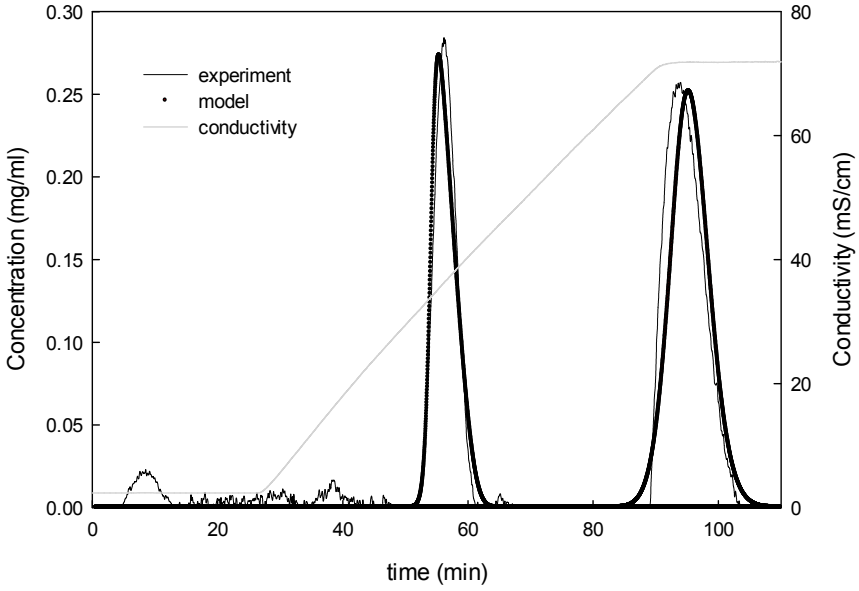

Figure 5 The validation experiment with an elution gradient of 22.5 CV

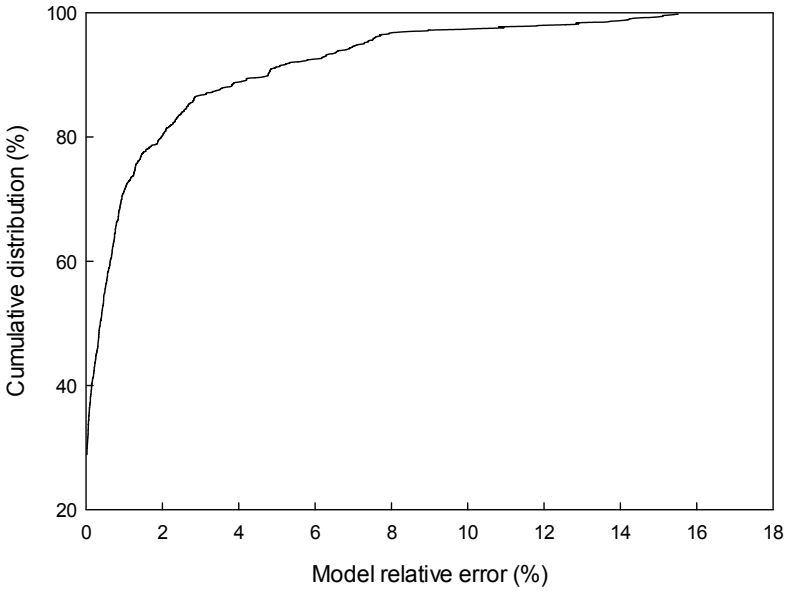

Figure 6 The cumulative distribution for the calibration and validation experiments plotted against the relative residual error

4.2.2 Optimization predictions based on the mechanistic model

The elution gradient was numerically optimized with respect to the objective function. On Table 4, the prediction for the optimal gradient based on SMA parameters derived from chromatograms of the 4.7 mL column on the ÄKTA system is given in numbers. To quantitatively evaluate the optimizing performance of the calibrated model, the experimental validation was performed with the corresponding data; chromatogram was beforehand transformed from the UV absorbance measurements on the ÄKTA system to mg/ml of protein using extinction coefficient. However, the experimental yield is close to the predictions with a good precision and describes the mechanistic model to be successful and predictive.

Table 4 Optimum factor sets for maximum yield of lactoperoxidase based on the mechanistic modeling approach

Concentration of salt (M)	0.82
Flow velocity (cm/min)	4.32
Gradient length (CV)	16.28
Predicted yield (%)	89.92
Experimental yield (%)	86.73

The predictions for the yield of lactoperoxidase with respect to optimal operating conditions deviate slightly; one explanation for this deviation can be the dynamic effects that the model cannot handle; for instance pH-variations caused by increasing high salt buffer in the system as suggested in [17]. However, as the constraint on purity (≥85%) was acceptably satisfied in the optimization step, thus deviation in the yield of protein can be ignored. The lowest expected purity can be determined by means of the confidence intervals of the process parameters.

5. Conclusions

The method presented in this manuscript constitutes data from factorial design of experiment approach to quickly identify the significance of process parameters on the objective function as well as complexity of the system. The empirical multi-variate model revealed a low capability to

predict the behavior in the IEC column with respect to optimal operating settings for separation as a result of lack of fit and limitations in robustness of linear gradient elution process. Nevertheless, it was reasonably accurate to prove the significant factors. In the next step, mechanistic model was calibrated based on the DoE-planned experiments by the inverse method. The calibrated model with reduced number of variables was then applied to find the optimal operating conditions and provide insight into the knowledge of process performance with respect to yield of lactoperoxidase and more accurate predictions with respect to process variations. The optimal operation was successfully predicted, yet the prediction of yield was slightly deviates from the experimental results. It can be related to some effects such as random configuration of protein interacting with binding sites of the adsorbent or slight changes in pH during salt gradient elution step that the model was unable to take into account.

In summary, model-integrated process development proved to be efficient with regard to the objective for the optimization of process and led to find the true optimum process parameters of flow velocity and concentration of salt at the end of gradient.

Nomenclature

$c_{p,i}$	concentration of protein *i* in the pores of the adsorbent (M)
c_{salt}	salt concentration in the pores of the adsorbent (M)
c_i	protein concentration *i* in the mobile phase (M)
D_{ax}	axial dispersion coefficient (mm^2/s)
D_m	molecular diffusivity in mobile phase (mm^2/s)
$k_{eff,i}$	effective mass transfer coefficient of protein *i* (mm/s)
$k_{ads,i}$	adsorption coefficient of protein *i* in the SMA isotherm
$k_{des,i}$	desorption coefficient of protein *i* in the SMA isotherm
$k_{eq,i}$	equilibrium coefficient of protein *i*
L	length of the column (mm)
q_i	protein concentration *i* on the adsorbent phase (M)
r_p	particle radius (mm)
u_{int}	interstitial velocity of the fluid (mm/s)
Y	recovery yield
ε_c	column voidage

ε_p	particle voidage
ε_t	total voidage
$1-\varepsilon_t/\varepsilon_t$	phase ratio
Λ	total ionic capacity (M)
v_i	characteristic charge of protein *i* in SMA isotherm
η	mobile phase viscosity (Pa.s)
σ_i	steric factor of protein *i* in the SMA isotherm

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