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Determination of parameters for the steric mass action model—A comparison between two approaches

A. Osberghaus[a], S. Hepbildikler[b], S. Nath[b], M. Haindl[b], E. von Lieres[c], J. Hubbuch[a,*]

[a] Institute of Process Engineering in Life Sciences, Section IV: Biomolecular Separation Engineering, Karlsruhe Institute of Technology, Engler-Bunte-Ring 1, 76131 Karlsruhe, Germany
[b] Pharmaceutical Biotech Production, Roche Diagnostics GmbH, Nonnenwald 2, 82377 Penzberg, Germany
[c] Institute of Bio- and Geosciences 1, Research Center Jülich, 52425 Jülich, Germany

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ABSTRACT

The application of mechanistic modeling for the optimization of chromatographic steps increased recently due to time efficiency of algorithms and rising calculation power. In the modeling of ion exchange chromatography steps, the sorption processes occurring on adsorbent particle surfaces can be simulated with the steric mass action (SMA) model introduced by Brooks and Cramer (1992) [14]. In this paper, two approaches for the determination of SMA parameters will be carried out and discussed concerning their specific experimental effort, quality of results, method differences, reasons for uncertainties and consequences for SMA parameter determination: **Approach I**: estimation of SMA parameters based on gradient and frontal experiments according to instructions in Brooks and Cramer (1992) [14] and Shukla et al. (1998) [16]. **Approach II**: application of an inverse method for parameter estimation, resulting in SMA parameters that induce a best fit of chromatographic data to a mechanistic model for column chromatography. These approaches for SMA parameter determination were carried out for three proteins (ribonuclease A, cytochrome *c* and lysozyme) at pH 5 and pH 7. The results were comparable and the order of parameter values and their relations to the chromatographic data similar. Nevertheless, differences in the complexity and effort of methods as well as the parameter values themselves were observed. The comparison of methods demonstrated that discrepancies depend mainly on model sensitivities and additional parameters influencing the calculations. However, the discrepancies do not affect predictivity; predictivity is high in both approaches. The approach based on an inverse method and the mechanistic model has the advantage that not only retention times but also complete elution profiles can be predicted. Thus, the inverse method based on a mechanistic model for column chromatography is the most comfortable way to establish highly predictive SMA parameters lending themselves for the optimization of chromatography steps and process control.

1. Introduction

Ion exchange chromatography (IEC) is one of the key procedures in bioseparation processes. As chromatography steps cover up to 70% of the overall financial effort in biopharmaceutical production, the development of optimal and efficient chromatography processes is a central issue. This issue is even more urgent with respect to the concept of Quality by Design, launched by the US Food and Drug Administration, which requires additional attention to process robustness and reproducibility matters [1]. The search for a favorable and robust operating point of a separation process represents a complex multi-factor optimization problem. One way to tackle this problem is given by screenings for optimal factors in the design space based on design of experiments (DoE). This procedure is often complemented by empiric response surface modeling (RSM). The DoE-RSM-approach is quite established for chromatography optimization; a review on this approach is, for example, given in [2].

However, the application of mechanistic modeling for the optimization of chromatographic steps is on the rise due to increased time efficiency of algorithms and progresses in calculation power (cf. argumentation lines in [3–5]). Based on mechanistic modeling, highly precise predictions of chromatograms can be achieved, as was demonstrated in [6] for step gradients, for linear gradients in [7] and for displacement systems in [8]. In addition, mechanistic modeling lends itself for efficient robustness and sensitivity analyses, which was demonstrated in [9,10]. Thus, a simulation of chromatographic processes based on mechanistic modeling can, similar to the DoE-RSM approach, support and cheapen the search for optimal conditions and provides additionally troubleshooting and error diagnostic tools for process development.

* Corresponding author. Tel.: +49 721 608 42557; fax: +49 721 608 46240.
E-mail address: juergen.hubbuch@kit.edu (J. Hubbuch).

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Despite of some obvious advantages of mechanistic modeling, a main drawback, particularly in comparison with the DoE-RSM-approach, is given by the high effort for preliminary model calibration. A calibrated mechanistic model simulates the flow of the mobile phase through the column and imitates interactions between the mobile phase and the adsorbent surface; a most important but hardly monitorable piece of the whole IEC process. These simulations base on parameters that determine the chromatographic system on column and particle level. Numerous publications with proposals for most effective model calibration have been released, like for example [11], discussing mainly the determination of parameters characterizing the packed bed, [12], dealing with parameters of mass transfer kinetics and [13], where a specific set of experiments is proposed for the determination of all model parameters. These publications reveal that system parameters (dead volume, etc.) and bed characterizing parameters (axial dispersion, porosities, etc.) can be determined with a few basic pulse experiments. However, the determination of parameters for the steric mass action (SMA) model that describes the interaction between the proteins and the adsorbent surface in IEC [14] is based on time-consuming isocratic or gradiental experiments and material-consuming frontal experiments (cf. for example the instructions for SMA parameter determination in [14–17]). In this context, approaches for the determination of SMA parameters based on batch experiments, like in [18,19] are interesting but not yet fully established and validated.

A quite established alternative to the experimental determination of SMA parameters is given by model-based inverse methods. In these methods the parameters are calculated by the best fit between data and chromatography model response. Recently, this method was applied more often for sorption parameter determination, for example in [20,21,4], but so far no direct comparison between the results of the suggested methods in [14–16] and the results of approaches based on an inverse method has been given. It is expected that within the context of high throughput process development which has had an immense impact in the last couple of years within the field of industrial process development, new methods which might be less precise but quicker and more intuitive in their realization will pave the way of model based process development. Inverse modeling might, for example, be performed with historic data, data already existing from process development, process data etc. Thus it offers a by far more potent application tool than the other approaches. However, a precise determination of sorption parameters allowing a high predictivity is essential for model based process development, as ad- and desorption and adsorbent capacities remarkably decide on retention time and separation quality. Thus, only a direct comparison between different approaches can act as a background for discussion on the optimal determination of modeling parameters and influence of noise in chromatographic data on parameter estimation. Such a comparison should on the one hand discuss experimental effort and parameter qualities, on the other hand it should pay attention to prediction performance, chances, advantages and disadvantages of both approaches. It would thus be of interest, how both approaches differ and what consequences this has for the determination of sorption parameters and which approach is to be favored in future SMA parameter determinations. The latter might lead to a clear distinction if one is interested in the physical meaning of the underlying isothermal concept or simply aims towards a tool for modern process development schemes.

In this paper two approaches in SMA parameter determination will be experimentally executed and discussed concerning specific experimental effort, quality of results, method differences, reasons for uncertainties and consequences for the determination of sorption parameters:

Approach I: estimation of SMA parameters based on gradient and frontal experiments according to instructions in [14,16].
Approach II: application of an inverse method for parameter estimation resulting in SMA parameters that induce a best fit of chromatographic data to a mechanistic model for column chromatography.

Although the considered methods can as well be applied to protein mixtures with industrial importance, the determination of SMA parameters will be performed based on a case study for three proteins (lysozyme, ribonuclease A and cytochrome *c*) at pH 5 and pH 7 on a prepacked 1 ml column with the strong cation exchange adsorbent SP Sepharose FF. This case study guarantees a complete comparability of datasets and a comparison of methods based on a well known system of proteins, that highlights advantages and disadvantages of the methods. The discussion will be supported by a comparative literature review and a Monte-Carlo study on the influence of noise in data on the quality of the determined SMA parameters.

2. Theory

2.1. SMA model for sorption processes in IEC

For IEC processes, a highly regarded characterization of sorption is given by the steric mass action (SMA) model introduced by Brooks and Cramer [14], which accounts for the influence of charged modifiers and their rivalry with proteins for binding sites on the absorber surface. In the understanding of [14], in the SMA model every component interacting with the particle surface owns four characterizing parameters (three in case of a rapid equilibrium assumption):

- ν (characteristic charge): average number of binding sites of the component (under the assumption of a single charged counterion)
- σ (steric factor): average number of shielded/covered binding sites on adsorbent surface due to the 3D-structure of the protein components
- k_{ads} and k_{des} (ad- and desorption coefficient): the ratio of the ad- and desorption coefficients is lumped to a single parameter k_{eq} when a rapid equilibrium is assumed (compare with the following equations).

Based on these initial considerations, the time dependent change of the concentration of component i on the adsorbent surface ($\partial q_i/\partial t$) in the SMA model is given by:

$$\frac{\partial q_i}{\partial t} = k_{ads,i} c_i \overline{q_1}^{\nu_i} - k_{des,i} c_1^{\nu_i} q_i \quad i > 1 \tag{1}$$

$$\Lambda = q_1 + \sum_{i=2}^{n} \nu_i q_i \tag{2}$$

$$\overline{q_1} = q_1 - \sum_{i=2}^{n} \sigma_i q_i \tag{3}$$

with respect to n components ($n = 1$ [salt] + number of protein components). Λ, the parameter describing the ionic capacity of the adsorbent, limits the available binding places and displays the rivalry between salt ion concentration q_1 and the other bound components q_i with their characteristic charges ν_i (cf. Eq. (2)). $\overline{q_i}$, the concentration of bound salt ions available for exchange with the protein, is given by the total salt ion concentration q_1 less the shielded ions determined by the protein specific steric factors (σ_i) in Eq. (3). If the assumption of a rapid equilibrium is valid

($\partial q_i/\partial t = 0$), the equation for the SMA-isotherm can be derived from the above equations to be:

$$k_{i,eq} = \left(\frac{q_i}{c_i}\right)\left(\frac{c_1}{\Lambda - \sum_{i=2}^{n}(\nu_i + \sigma_i)q_i}\right)^{\nu_i} \quad i > 1 \tag{4}$$

The results of SMA parameter determination following instructions in [14,16] (**approach I**) will be compared to the estimation of SMA parameters by fitting a mechanistic model for IEC chromatography processes to the monitored and time resoluted concentrations of a protein component at column outlet.

2.2. Approach I: Determination of parameters for the SMA model according to instructions in [14,16]

The determination of the parameters ν and k_{eq} for the SMA model based on gradiental chromatographic experiments was performed based on an equation of Parente and Wetlaufer [22] modified by [16]:

$$V_R = \left(\left(c_{a,s}^{\nu+1} + \frac{V_d k_{eq}\varepsilon_c \Lambda^{\nu}(\nu+1)(c_{e,s} - c_{a,s})}{V_G}\right)^{1/(\nu+1)} - c_{a,s}\right) \tag{5}$$

The determination of the parameter σ based on the previously determined parameters ν and k_{eq} and additional frontal chromatographic experiments was carried out based on Eq. (6) given by [14]:

$$\sigma = \frac{\beta}{c_{prot}\vartheta}\left(\Lambda - c_{salt}\left(\frac{\vartheta}{\beta k_{eq}}\right)^{1/\nu}\right) - \nu \tag{6}$$

with

$$\vartheta = \left(\frac{V_B}{V_0} - 1\right)$$

Eq. (5) provides a correlation between gradient volume and elution volume, where ν and k_{eq} appear implicitly. Eq. (6), derived based on [14], poses an explicit expression for the steric factor σ based on the previously estimated parameters ν and k_{eq}. β describes the column phase ratio $(1-\varepsilon_t)/\varepsilon_t$. The necessary information for the solution of these equations can be divided into 'experiment-enclosed information' – information from the gradient and breakthrough experiments and 'external information' – supplementary parameters from other sources:

experiment-enclosed information

- V_R, V_G – retention time V_R with respect to a specific elution gradient volume V_G
- $c_{a,s}$, $c_{e,s}$ – salt concentration at gradient begin, respectively end
- c_{prot} – protein concentration in the stock solution (breakthrough)
- c_{salt} – salt concentration in the buffer (breakthrough)
- V_B – breakthrough volume at 10% of the complete breakthrough

external information

- Λ – ionic capacity of the adsorbent
- V_d – column dead volume
- ε_c – column porosity
- ε_t – total porosity
- V_0 – breakthrough volume at 10% of a nonretarded tracer

2.3. Approach II: Determination of parameters for the SMA model by an inverse method

2.3.1. Introduction to the employed mechanistic model for chromatography

A mechanistic model for chromatography consists of equations describing convective and dispersive transport, mass transfer resistances and equations describing sorption kinetics, for example the SMA model in IEC. Here, a short overview with respect to the employed model equations, a transport-dispersive model, is given, details on the equations and the implementation of their solution can for example be found in [23,24,5].

On column level, the time- and position-dependent change of concentration for the ith component, $\partial c_i/\partial t$, is described by:

$$\frac{\partial c_i}{\partial t} = -u_{int}\frac{\partial c_i}{\partial x} + D_{ax}\frac{\partial^2 c_i}{\partial x^2} - \frac{1-\varepsilon_c}{\varepsilon_c}\cdot\frac{3}{r_p}k_{eff,i}[c_i - c_{p,i}] \tag{7}$$

where the first term on the right hand side describes the convective transport through the column, the second term the dispersive transport and the third term the mass transfer to the particle surface, $k_{eff,i}$ representing the lumped film diffusion coefficient and r_p the particle radius. u_{int} denotes the interstitial velocity, ε_c the column porosity, D_{ax} displays the axial dispersion, more precisely, a combined effect of dispersion and diffusive processes, dispersion being eddies and all effects implied by three dimensionality.

Analogously, the time-dependent change of concentration on particle level for the ith component, $\partial c_{p,i}/\partial t$, is described by:

$$\frac{\partial c_{p,i}}{\partial t} = \frac{3}{\varepsilon_p r_p}k_{eff,i}[c_i - c_{p,i}] - \frac{1-\varepsilon_p}{\varepsilon_p}\frac{\partial q_i}{\partial t} \tag{8}$$

with q_i denoting the concentration of particle-bound component i and ε_p the particle porosity. The second term of Eq. (8) describes ad- and desorption processes on particle level, i.e. the interaction between mobile and bound phase. Thus, the expression $\partial q_i/\partial t$ is defined in Eq. (1) respectively, when a rapid equilibrium is assumed, in Eq. (4).

For the solution of the whole differential-algebraic equation system, Danckwert's boundary conditions were employed [25]. This model was solved in MatLab on a Dual Core Processor with 2.81 GHz in approximately 10 s with a density of 200 knots over the whole column length. That is a reasonable time span since the model has to be solved hundreds of times for the inverse method (approach II).

2.3.2. A model-based inverse method for the determination of SMA parameters

Let $c(t_j)$ be the chromatogram monitored at column outlet at the points in time $j = t_0, \cdots, t_{end}$, preprocessed to a concentration time series. Let $\hat{c}(t_j)$ be the solution of a mechanistic model for chromatography at the same location and points in time; $\hat{c}(t_j)$ can then be compared to the chromatograms. Let now θ_{fix} be the set of all model input parameters that are fixed on a constant value and θ_{est} the set of model input parameters that can be manipulated by the algorithm solving the inverse problem (for estimating the SMA parameters $\theta_{est} = \{\nu, k_{eq}, \sigma\}$). Then the inverse problem can be stated as an minimization of a least squares residual given by:

$$res(\theta_{est}) = \sum_{t=t_0}^{t_{end}}(\hat{c}(t_j, \theta_{fix}; \theta_{est}) - c(t_j))^2 \tag{9}$$

The minimization of Eq. (9) was in all cases performed with the Matlab procedure `lsqnonlin`.

Analogously to the determination of SMA parameters described in Section 2.2, the solution of the inverse method demands for 'experiment-enclosed information' provided by column

chromatographic data and 'external information' – additional parameters from other sources:

experiment-enclosed information

- the chromatogram
- $c_{a,s}$, $c_{e,s}$ – salt concentration at gradient begin, respectively end
- c_{prot} – protein concentration in the stock solution (breakthrough)
- c_{salt} – salt concentration in the buffer (breakthrough)

external information

- information characterizing the packed bed
  - Λ – ionic capacity of the adsorbent
  - D_{ax} – axial dispersion
  - u_{int} – interstitial velocity
  - ε_c – column porosity
  - ε_p – particle porosity
  - ε_t – total porosity
  - k_{eff} – lumped film diffusion coefficient
- parameters characterizing column geometry
  - L_C – column length
  - r_p – particle radius.

The determination of external information as referred to here and at the end of Section 2.2, will be briefly described in the next section. Detailed instructions can be found for example in [11].

3. Materials and methods

3.1. Materials

Blue dextran 2,000,000, cytochrome *c* (horse heart), lysozyme (chicken egg white) and ribonuclease A (bovine pancreas) were purchased from Sigma (St. Louis, MO, USA). The buffer substances, acetic acid, sodium monobasic phosphate, sodium dibasic phosphate and sodium hydroxide as well as phenolphthalein for titration were purchased from Merck KGaA (Darmstadt, Germany). Acetone and ethanol (99.8%) were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany) and sodium chloride from AppliChem (Darmstadt, Germany).

3.2. Apparatus and software

Prepacked HiTrap SP Sepharose FF 1 ml columns [0.025 m length, 0.7E−2 m ID] from GE Healthcare (Buckinghamshire, United Kingdom) were applied for all column experiments. The experiments were performed on an Ettan LC system from GE Healthcare with a system flow rate of 2.17×10^{-4} m/s [0.5 ml/min]. Tubing connections and all other LC system parameters were standardized and kept constant. The absorption at 280 nm and 528 nm was measured online in all experiments. Primary analyses and documentation of the chromatograms were performed with the control software Unicorn. All further data analysis as well as the solution of model equations and all applications connected to the model was performed with MatLab (The Mathworks, Natick, ME, USA).

3.3. Gradient elution experiments

The running buffer for all experiments at pH 5 was a 0.02 M acetate buffer. The running buffer for all experiments at pH 7 was a 0.02 M sodium phosphate buffer; the same buffers with additional 0.5 M NaCl served for elution purposes. The proteins were each dissolved in the running buffer to a concentration of 0.2 mM. All gradient elution experiments were performed similar to the instructions in [16]. At first the column was equilibrated with the running buffer for four column volumes (cv), then 20 μl of the protein solution were injected. This step was followed by another wash step with 4 cv running buffer. Afterwards, a linear gradient from 0% to 100% high salt buffer was set to elute the protein. This experimental setup was executed for five different gradient lengths: 5 cv, 10 cv, 30 cv, 60 cv and 120 cv.

3.4. Breakthrough experiments

Breakthrough experiments are necessary for the determination of the protein-specific steric factor σ_i (compare Eq. (6)). Thus, breakthrough experiments for ribonuclease A, cytochrome *c* and lysozyme at pH 5 and pH 7 were performed with the respective running buffers and stock solutions. After equilibrating the column for 10 cv with running buffer, it was isocraticly loaded with a system flow rate of 2.17×10^{-4} m/s until the breakthrough was complete.

3.5. Determination of parameters for 'external information'

For the determination of external information in approach I and approach II (ε_c, ε_p, ε_t, the column, particle and total porosity, Λ, the ionic capacity of the column, D_{ax}, the axial dispersion coefficient and V_0, the breakthrough volume of a non-retarded species), pulse and displacement experiments were performed according to instructions in [11,26]. All experiments were at least three times repeated for a check of reproducibility and variances. The lumped film diffusion coefficient k_{eff} was estimated to be 1.5×10^{-6} m/s by the inverse fit of the mechanistic model to the tracer peaks from the pulse experiments described in the next section.

3.5.1. Pulse experiments

Dextran blue was provided as nonbinding and nonpenetrating tracer; acetone (1% in deionized water) was used as nonbinding but penetrating tracer. The absorption at 280 nm was measured online and retention times were corrected with respect to system dead volume. Porosities were calculated with the method of central moments based on several repetitions for all employed columns. Based on the total column volume of 0.96×10^{-6} m^3, the total porosity ε_c was calculated to be 0.92 ± 0.025, the column porosity to be 0.36 ± 0.0009 and the particle porosity ε_p to be 0.85 ± 0.038. The axial dispersion coefficient was calculated according to Eq. (10):

$$D_{ax} = \frac{\sigma_{mom}^2}{\mu_{mom}^2} \frac{L_C u_{int}}{2} \tag{10}$$

with u_{int} being the interstitial velocity, L_C the column length and μ_{mom} and σ_{mom} the first respectively second central moment of the nonbinding and nonpenetrating tracer peaks. The result for axial dispersion was $1.574 times 10^{-10} m^2 s^{-1}$ with a relative standard deviation of about 1.75%.

3.5.2. Displacement experiments

The determination of total ionic capacity Λ was performed according to instructions in [26]. The packed column was equilibrated with deionized water and then isocraticly loaded with acetic acid. The system was washed for another ten column volumes with deionized water and then the acetic acid was eluted with 1 M KNO_3-solution. 10 μl of phenolphthalein solution (10 mg/ml in ethanol) were added to the eluate and the mixture was titrated with 0.01 M NaOH. The ionic capacity of the column was calculated by

$$\Lambda = \frac{c_{NaOH} V_{NaOH}}{V_C(1 - \varepsilon_t)} \tag{11}$$

with the column volume V_C and the concentration and volume of NaOH used for titration (c_{NaOH}, V_{NaOH}). Three determinations of the ionic capacity of the column were averaged resulting in Λ = 800 mM with a relative standard deviation of about 5%.

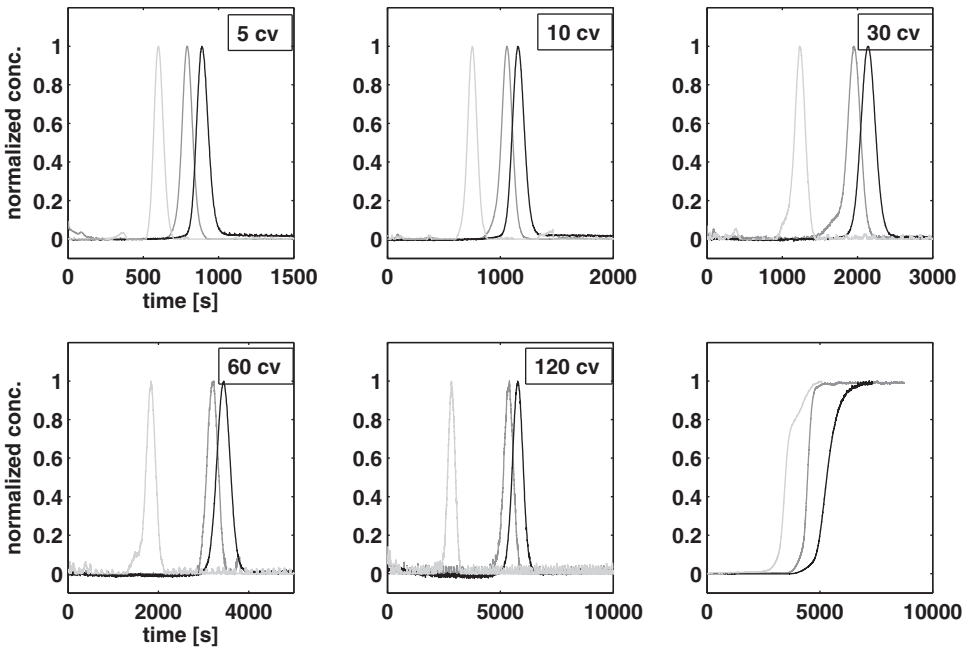

Fig. 1. Superimposed results of single gradient and frontal experiments at pH 7 (SP Sepharose FF, flow rate: 2.17×10^{-4} m/s, elution volumes: 5 cv, 10 cv, 30 cv, 60 cv and 120 cv). The gradient elution experiments are displayed with normalized concentration for better comparability [light gray continuous line: ribonuclease A, dark gray: cytochrome *c*, black: lysozyme].

3.6. Monte-Carlo method for the noise sensitivity of model-based SMA parameter determination

The inverse method described in Section 2.3 can as well be employed for sensitivity analyses of parameter determination. By Monte-Carlo simulations it is possible to quantify the effect of noise in chromatograms or of retention time shifts on the parameter estimation (more details on this method can be found in [27,28]). In short, 10,000 chromatograms were simulated based on the model equations given in Section 2.3.1 and then certain effects were attached to every single chromatogram, here, a normally distributed absolute noise or a normally distributed shift in time:

The influence of noise on chromatographic data was applicated with an absolute standard deviation on the data [in mM] in seven levels [$a = 0, 2, 4, 8, 16, 32, 50$] using the following equation:

$$data = data + a \times 10^{-7} \cdot randn \tag{12}$$

with `randn` a Matlab function providing normally distributed numbers with mean 0 and standard deviation 1. The influence of shift-noise in time was applicated as a normally distributed time-shift in the simulation in seven levels [$b = 0$, 2, 4, 8, 16, 32, 50, standard deviation of time shift in seconds] using the following equation:

$$time = time + b \cdot randn \tag{13}$$

Both noise applications were performed for three gradient lengths; 5 cv, 20 cv and 60 cv. Then, SMA parameters were re-estimated based on the deviation-afflicted chromatograms and the variances, correlations and distribution of the 10,000 estimation results analyzed.

4. Results

4.1. Gradient elution and breakthrough experiments

In both approaches for SMA parameter determination, chromatograms from gradient elution experiments and breakthrough data are the main source of information (cf. Sections 3.3 and 3.4). These experiments were performed for the proteins ribonuclease A, cytochrome *c* and lysozyme at pH 5 and pH 7. Example results for gradient elutions with five gradient lengths (5 cv, 10 cv, 30 cv, 60 cv and 120 cv) and breakthrough data at pH 7 are given in Fig. 1; for better comparability, the experimental results from the single component experiments are superimposed.

The first eluting component is ribonuclease A (light gray), followed by cytochrome *c* (dark gray) and lysozyme (black). The elution peaks of cytochrome *c* and lysozyme overlap. Obviously, an increase in elution gradient length increases the gap between the retention time of ribonuclease A and the other components. The frontal experiments show that the capacity of SP Sepharose FF for ribonuclease A is lower than for cytochrome *c* and lysozyme (highest capacity). Repetitions of gradient and frontal experiments showed a high reproducibility; absolute deviances in retention times determined by gradient experiments were always smaller than 57.725 s (0.5 ml), respectively smaller than 115.45 s (1 ml) for breakthrough volumes.

4.2. Determination of SMA parameters according to approach I

In Fig. 2 the correlation between retention time and gradient volume, given by Eq. (5), is exemplarily illustrated for cytochrome *c*.

The measurement points for cytochrome *c* at pH 5 are displayed by ∇-symbols, for pH 7 by Δ-symbols. The least squares fit of Eq. (5) to the data is displayed by the dotted line (pH 5), respectively the continuous line (pH 7), each fit having a coefficient of determination R^2 of 0.99. The correlation is positive proportional and slightly convex for both pH conditions. Retention times at pH 5 are generally larger and this effect even increases with increasing elution volume.

Based on Eqs. (5) and (6), on the five chromatograms at 5 cv, 10 cv, 30 cv, 60 cv and 120 cv and the breakthrough curve for every single protein, SMA parameters were determined for pH 5 and pH 7. The results for SMA parameters for ribonuclease A, cytochrome *c* and lysozyme at pH 5 and pH 7 are given in Table 1. The characteristic charges for the proteins at pH 5 are quite close

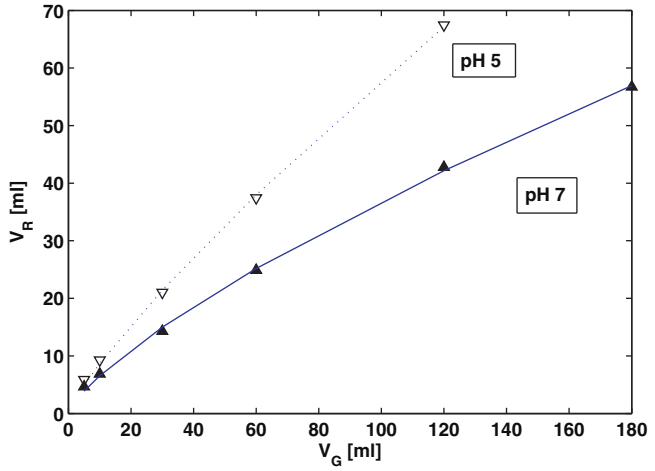

Fig. 2. The correlation between elution gradient volume V_G and retention time V_R of a protein is described by Eq. (5). This figure illustrates the correlation for cytochrome *c* at pH 5 (∇ – measurement points, dotted line – fit) and pH 7 (Δ – measurement points, continuous line – fit).

Table 1
SMA parameters determined according to instructions in [14,16] [**approach I**].

Parameter	pH	
	5	7
Ribonuclease A		
ν	5.11	2.39
k_{eq}	0.148	0.233
σ	28.88	29.34
Cytochrome *c*		
ν	5.0	3.31
k_{eq}	0.307	0.356
σ	28.7	40.8
Lysozyme		
ν	4.72	4.07
k_{eq}	0.441	0.17
σ	36.8	29.74

together in the limits from 4.72 to 5.11. The values are decreasing corresponding to the elution order of the proteins. For pH 7 the characteristic charges are more distinct (ribonuclease A: 2.39, cytochrome *c*: 3.31 and lysozyme: 4.07) and they are increasing corresponding to the elution order. The equilibrium coefficients are located between 0.148 (ribonuclease A at pH 5) and 0.441 (lysozyme at pH 5). At pH 5 they show an increasing trend corresponding to the elution order of the proteins. The steric factor of the proteins lies in the ranges between 28.7 (cytochrome *c* at pH 5) and 40.8 (cytochrome *c* at pH 7).

4.3. Determination of SMA parameters according to approach II

Example of simultaneous least-squares fits of the mechanistic model to the five chromatograms at 5 cv, 10 cv, 30 cv, 60 cv and 120 cv (cf. Section 2.3.2) for every single protein at pH 5 and pH 7 are shown for cytochrome *c* at pH 5 respectively at pH 7 in Figs. 3 and 4.

The continuous line displays the experimental data, whereas the dotted line shows the model response for simulations with SMA parameter estimations from Table 2. The fit of the model response to the gradiental elution data is for most of the datasets at both pH-conditions highly precise and was not corrupted by noise in the data, for example the small side-peak in the subfigure for 10 cv elution in Fig. 3. Only for short gradients with a length of 5 cv the model response slightly deviates from the data.

The SMA parameters that were estimated by the inverse method are given in Table 2. Structured multiple start guesses at the beginning of the optimization process (cf. Eq. (9)) provided the deviances in the optimization results that are given next to the estimated parameters in Table 2. These deviances show, that the estimation deviance is dependent on the considered parameter. Thus, the parameters ν and k_{eq} are more determined, when estimated based on gradient elution data and a breakthrough, than the steric factor – a fact, that was qualitatively shown earlier, for example in [17].

The characteristic charges for the proteins at pH 5 are again close together, now in the limits from 5.07 to 5.42. They are also

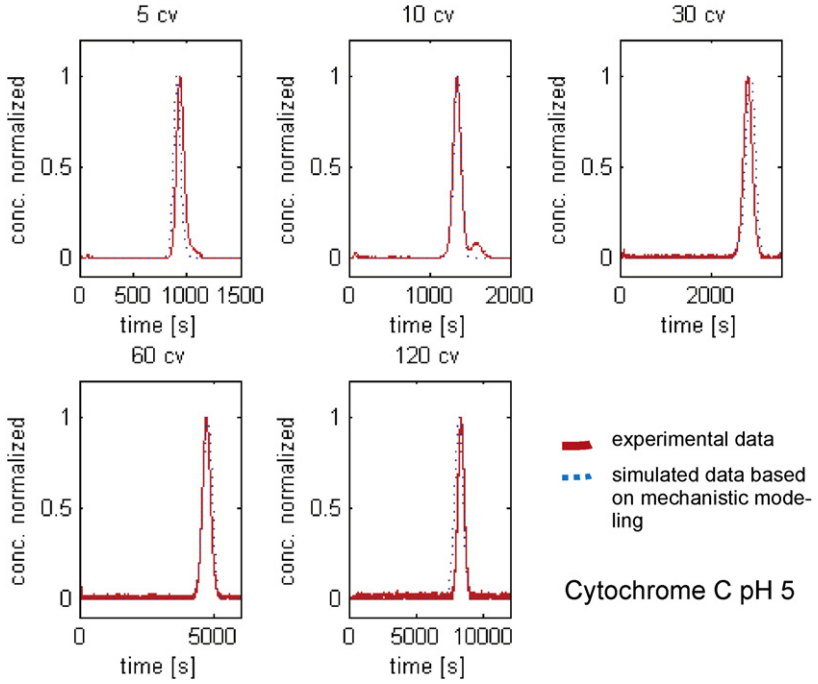

Fig. 3. Example of inverse method fits based on mechanistic modeling. The experimental data of cytochrome *c* at pH **5** is displayed by a continuous line and the dotted line shows the inverse model-based fit.

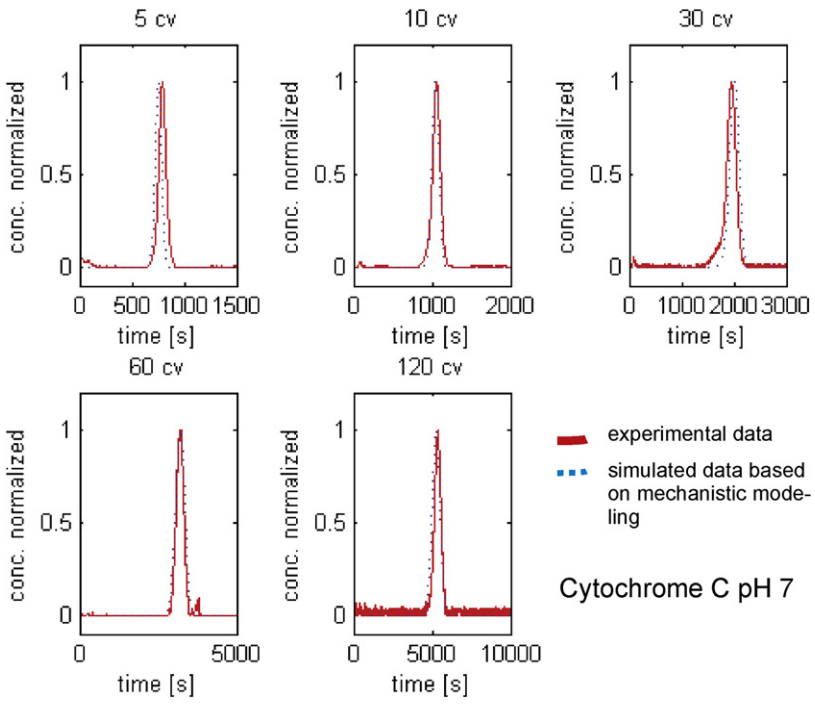

Fig. 4. Example of inverse method fits based on mechanistic modeling. The experimental data of cytochrome *c* at pH **7** is displayed by a continuous line and the dotted line shows the inverse model-based fit.

decreasing corresponding to the elution order of the proteins. For pH 7 the characteristic charges are again more distinct (ribonuclease A: 3.25, cytochrome *c*: 4.09, lysozyme: 4.72) and they are increasing corresponding to the elution order. The equilibrium coefficients lie between 0.013 (ribonuclease A at pH 7) and 0.118 (lysozyme at pH 5). At pH 5 and pH 7 they show an increasing trend corresponding to the elution order of the proteins. The steric factor of the proteins lies in the ranges between 28.5 (ribonuclease A at pH 5) and 57.6 (cytochrome *c* at pH 7). Apart from some outliers, the estimations for the steric factor and for the equilibrium coefficients show significantly higher mean relative deviations (about 0.5%) than the estimations for the characteristic charge (about 0.25%), but in general, the deviations on the estimated SMA parameters are very small.

4.4. Predictivity examinations on both approaches

For both approaches, retention times for gradient lengths of 25, 80 and 106 cv were predicted and the adequate experiments performed for every protein at pH 5 and pH 7. The predictions for approach I are based on interpolation [IP], the predictions in approach II are based on the solution of a system of differential equations [ODE]. No predictions outside the calibration range were examined, as the considered range with gradient lengths from 5 to 120 cv is very broad. Gradients outside this range might be of little use in practical applications.

In Table 3, the predictions and a posteriori experimentally determined retention volumes [in ml] for these gradient lengths are listed as well as the experimentally determined retention volumes [E]. The predictions for both approaches are very close to the experimental results; most of the deviances between prediction and validation are smaller than 1 ml, even for long elution gradients with 106 cv.

Examples for the prediction of chromatograms based on the SMA parameters estimated by an inverse method are shown in Fig. 5. For cytochrome *c* at pH 5 the three subfigures on the top of the

Table 2
SMA parameters determined based on mechanistic modeling (inverse method) [**approach II**]. The specific deviations on the estimations were determined by multiple start guesses.

Parameter	pH	
	5	7
Ribonuclease A		
ν	5.42 ± 0.09	3.25 ± 0.008
k_{eq}	0.037 ± 0.004	0.013 ± 0.0002
σ	28.5 ± 2.2	57.6 ± 0.24
Cytochrome *c*		
ν	5.3 ± 0.03	4.09 ± 0.009
k_{eq}	0.094 ± 0.006	0.041 ± 0.0002
σ	29.8 ± 0.15	53.8 ± 0.28
Lysozyme		
ν	5.07 ± 0.004	4.72 ± 0.01
k_{eq}	0.118 ± 0.0005	0.0372 ± 0.005
σ	31.2 ± 0.19	38.75 ± 0.74

Table 3
Predictions and validation results of retention volumes [ml] for salt elution gradients with the lengths of 25 cv, 80 cv and 106 cv. The predictions entitled with [IP] are based on the correlation given by Eq. (5). Predictions from the mechanistic model are entitled with [ODE] and results from experimental validation with [E].

	pH					
	5			7		
	IP	ODE	**E**	IP	ODE	**E**
Ribonuclease A						
25 cv	16.55	16.59	**16.45**	7.68	8.27	**7.32**
80 cv	43.23	44.31	**43.05**	16.52	18.65	**16.03**
106 cv	54.51	55.22	**55.45**	19.8	22.18	**19.27**
Cytochrome *c*						
25 cv	18.47	18.41	**19**	12.95	12.90	**12.34**
80 cv	48.12	50.83	**50.23**	31.32	32.73	**30.15**
106 cv	60.65	63.44	**64**	38.74	40.06	**37.87**
Lysozyme						
25 cv	18.85	18.64	**18.75**	13.75	13.87	**14**
80 cv	48.63	50.25	**49.32**	34.33	35.39	**34.8**
106 cv	61.14	62.6	**62.7**	42.8	42.94	**43.41**

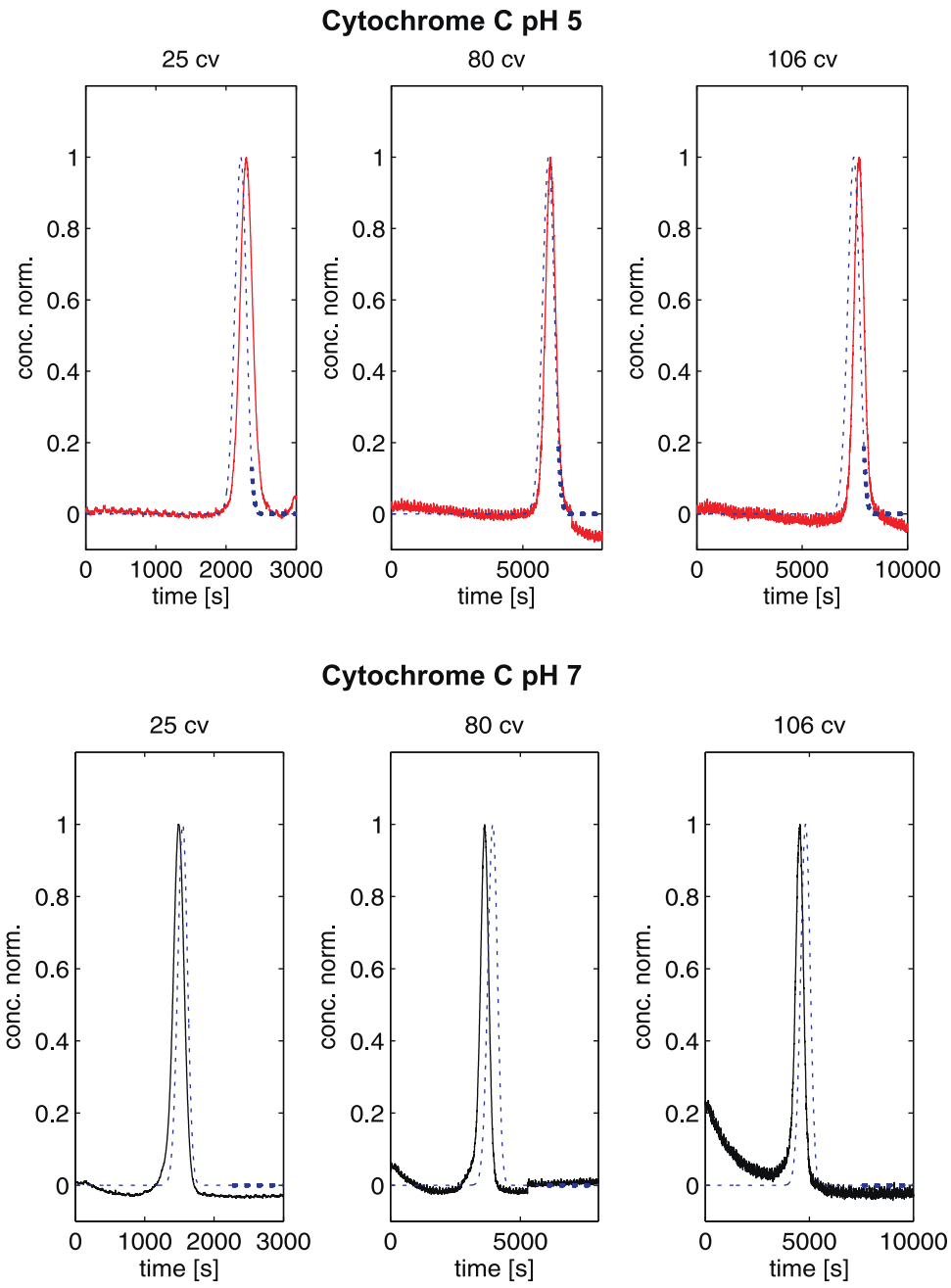

Fig. 5. Prediction and experimental results of gradient elution data. The model-based prediction for the elution of cytochrome *c* at pH 5 is shown in the three subfigures on the top (dotted line) and the experimental results superimposed the predictions with a continuous line. Results for cytochrome *c* at pH 7 are shown in the subfigures at the bottom.

figure show predictions for gradiental elutions with gradient volumes of 25 cv, 80 cv and 106 cv (dotted line) and the experimental validation results (continuous lines); results for cytochrome *c* at pH 7 are shown in the three subfigures beneath. Obviously, data quality decreased slightly with increasing elution volumes, mainly due to baseline drift and peak broadening. The prediction quality is very high for both, small and large elution volumes, thus, independent of the noise.

4.5. Sensitivities for SMA parameters in approach I

In approach I the parameters ν and k_{eq} are determined simultaneously based on a correlation equation for gradient and retention volume (cf. Eq. (5)). Though the results are unique, the parameters estimation of k_{eq} is significantly influenced, when ν is fixed to a defined value while the change in the coefficient of determination is negligible.

Fig. 6 shows the results for a single determination of parameter k_{eq}, when different values for ν in Eq. (5) are set to be fixed. All remaining parameters in Eq. (5) were valid for lysozyme at pH 7. The coefficient of determination R^2 was close to 0.99 in every estimation and the residual in every estimation comparably small with differences in the third decimal place. A negative correlation between the parameter estimations could be observed. Small positive changes in the value of the characteristic charge ν induce significant negative changes in the estimation value of the equilibrium coefficient k_{eq}. For example, the characteristic charge determined by approach I (4.07) implies an equilibrium coefficient of 0.17 whereas a

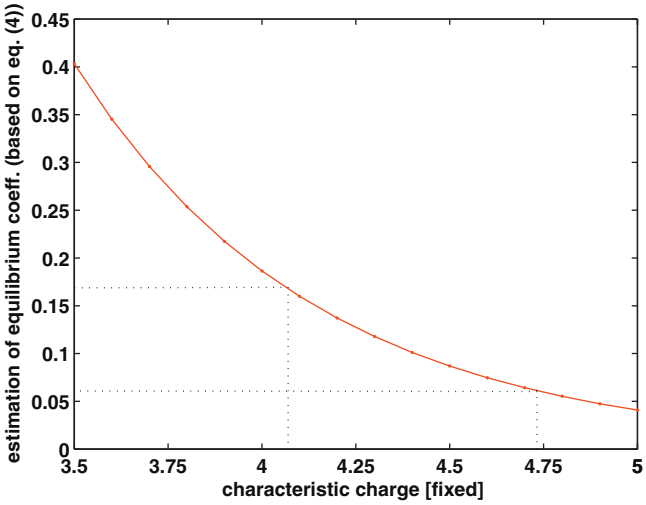

Fig. 6. Results for the determination of SMA parameter k_{eq} in Eq. (5) in case of a fixed SMA parameter ν (based on data for lysozyme pH 7). The correlation curve shows the high sensitivity of Eq. (5) towards changes in ν.

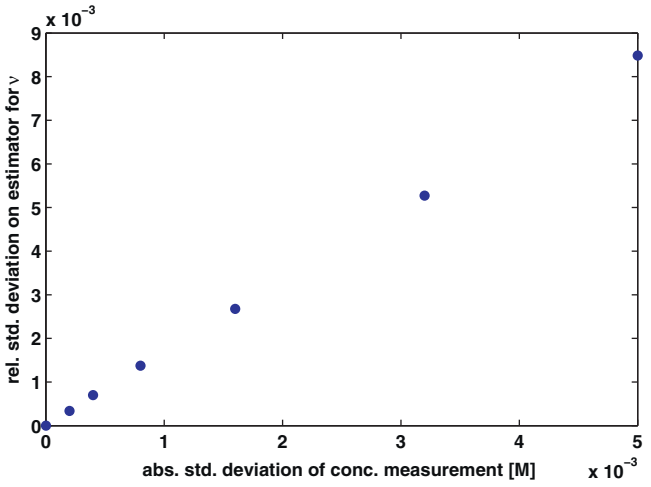

Fig. 7. Illustration of the effects of **absolute noise on concentration measurement** in chromatographic data on the estimation of the SMA parameter of characteristic charge. This effect was determined for various noise levels (x-axis) by Monte-Carlo simulations.

characteristic charge of 4.72, like it was determined by approach II, would result in an equilibrium coefficient estimation of about 0.06 (compare with the dotted lines in Fig. 6).

4.6. Sensitivities of SMA parameter estimation with respect to noisy data (approach II)

The sensitivities of SMA parameter determination due to absolute noise on chromatograms, respectively, noise shifts in time, were examined by Monte-Carlo simulations.

In Fig. 7 different levels of absolute noise on chromatogram concentration data are correlated with the relative deviation in the estimation of the steric parameter ν. The correlation is linear. It could be shown that even a strong absolute noise on chromatograms (±0.05 M, original initial concentration: 0.0002 M) leads only to deviances in the second position after decimal point in the estimation of the characteristic charge ν. Similar correlations were observed for all of the SMA parameters. Thus, the application of absolute noise to the chromatograms had nearly no influence on the SMA parameter estimations by the inverse method.

System-dependent noise (for example changes in tubing configurations) that leads to shifts in the retention time is more influential on the estimation of SMA parameters. The effects of this kind of noise on the estimation of SMA parameters ν, σ and k_{eq} are illustrated in Fig. 8. It becomes obvious that all SMA parameters are significantly more sensitive on retention time noise than on absolute noise on data measurements. ν (rel. std. deviation of 0.075 for peak shifts of about 50 s (0.43 ml)) is the least sensitive parameter to time-dependent shifts and σ (rel. std. deviation of almost 1.5 for peak shifts of about 50 s (0.43 ml)) the most sensitive parameter. These observations were valid for all three examined elution gradient lengths of 5 cv, 20 cv and 60 cv.

4.7. Literature review

This literature review is based on [17,29–32] and other publications cited in this paper. Table 4 shows specific publications including SMA parameters for the proteins ribonuclease A, cytochrome *c* and lysozyme.

The literature review on the determination of SMA parameters shows that absolute values for SMA parameters are not only dependent on the examined protein and the pH value, but in the same way on the adsorbent and column properties like bed geometry or porosity. This is also obvious regarding the 'external information' that is necessary for all approaches of determination and can nicely be demonstrated by a comparison of two SMA parameter sets for α-chymotrypsinogen A, both determined at pH 6 on 40-μm-Waters adsorbent (published by [33] [$\nu = 5.20 \pm 0.07$, $k_{eq} = 0.003$ and $\sigma = 45 \pm 3$] and [32] [$\nu = 4.8 \pm 0.17$, $k_{eq} = 0.0066$ and $\sigma = 52$]). These sets, although determined at the same pH-condition and the same adsorbent, deviate from each other for about 4% (values for ν) up

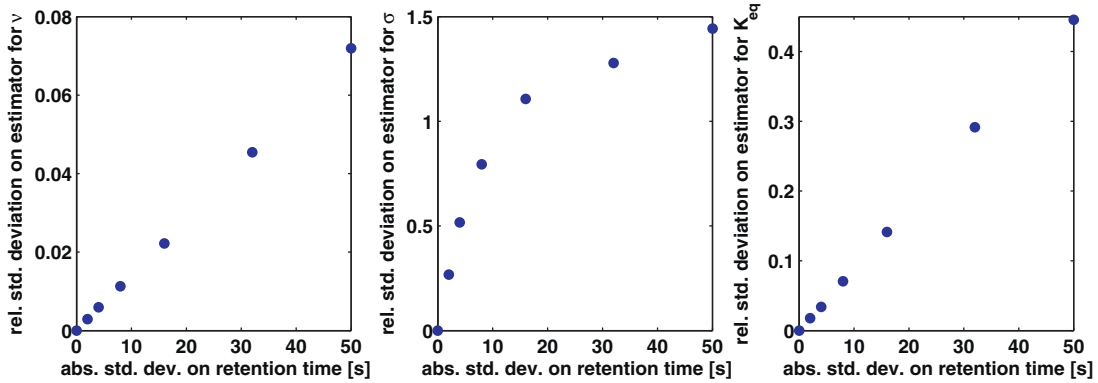

Fig. 8. Illustration of the effects of **absolute shift noise on retention time** in chromatograms on the estimation of SMA parameters. This effect was determined for various noise levels (x-axis) for the three SMA parameters ν, σ and k_{eq} by Monte-Carlo simulations.

Table 4
Literature overview – SMA parameters for the proteins ribonuclease A, cytochrome *c* and lysozyme in publications.

Publication	pH	Column	Proteins	
Gadam, S. et al. (1993). Characterization of non-linear adsorption properties of dextran-based polyelectrolyte displacers in ion-exchange systems, J. Chromatogr. 630, 37–52	6	Strong cation exchanger with sulfopropyl groups	Cytochrome *c*	$\nu = 6$ $k_{eq} = 0.0106$ $\sigma = 53.6$
			Lysozyme	$\nu = 5.3$ $k_{eq} = 0.0148$ $\sigma = 34$
Gallant, S. et al. (1995). Optimization of step gradient separations: Consideration of nonlinear adsorption. Biotechnol. Bioeng. 47 (3), 355–372	6	Strong cation exchanger with sulfopropyl groups	Cytochrome *c*	$\nu = 6.15$ $k_{eq} = 0.00637$ $\sigma = 53.4$
			Lysozyme	$\nu = 5.95$ $k_{eq} = 0.124$ $\sigma = 9.5$
Gallant, S. et al (1997). Productivity and operating regimes in protein chromatography using low-molecular-mass displacers. J. Chromatogr. A 771, 9–22	6	Source 15S	Cytochrome *c*	$\nu = 6$ $k_{eq} = 0.12$ $\sigma = 28$
			Lysozyme	$\nu = 5.5$ $k_{eq} = 1.1$ $\sigma = 14$
Ghose, S. et al. (2001). Characterization and modeling of monolithic stationary phases: application to preparative chromatography. J. Chromatogr. A 928 (1), 13–23	6	UNO S6, strong cation exchanger	Ribonuclease A	$\nu = 5.69$ $k_{eq} = 0.00335$ $\sigma = 118$
			Cytochrome *c*	$\nu = 6.08$ $k_{eq} = 0.01239$ $\sigma = 125$
Ladiwala, A. et al. (2005). A priori prediction of adsorption isotherm parameters and chromatographic behavior in ion-exchange systems. Proc. Natl. Acad. Sci. U.S.A. 102 (33)	5	SP Sepharose FF	Ribonuclease A	$\nu = 5.4$ $k_{eq} = 0.0296$ $\sigma = 17.2$
			Cytochrome *c*	$\nu = 5.9$ $k_{eq} = 0.0295$ $\sigma = 15.8$
			Lysozyme	$\nu = 5.6$ $k_{eq} = 0.0763$ $\sigma = 17$

to about 14% (values for σ). SMA parameters determined by different experimental approaches, deviate even more: for example in [7,16] the characteristic charges ν for lysozyme at pH 6 are 5.95 [isocratic elution experiments] respectively 4.97 ± 0.37 [gradiental elution experiments] and the values for k_{eq} 0.124 respectively 0.91 ± 0.16.

This shows that even though in [14] the meaning of the SMA parameters is described with respect to protein characteristics like binding sites and shielding, their real virtue comes forth in comparative analysis, what has been indirectly shown before by affinity rankings in [34]. These rankings illustrate a method for the prediction of elution order based on the comparison of SMA parameter relations that seem to be valid for specific pH and similar adsorbent-systems. The intern order in SMA-values of proteins, for example $\nu_{cytc} > \nu_{ribA} > \nu_{\alpha chyA}$ at pH 6, coincided in almost every publication given in Table 4 and the published SMA parameters for lysozyme, cytochrome *c* and ribonuclease A on cation exchange adsorbents, show mostly the same order relations to the parameters determined in this paper.

5. Discussion

The intention behind the work presented in this study was to simply apply the SMA isotherm relationship as an equation describing ad- and desorption within a mechanistic chromatographic model; the latter finding its way into current high throughput process development strategies. Thus the paper does not aim to mechanistically explain ad- and desorption processes, but to develop a strategy for calibration of a mechanistic model for model based process development in the light of industrial needs, habits and data available. The first step on this path is of course a comparison of established and newer methods for parameter estimation.

The gradiental and frontal experiments for ribonuclease A, cytochrome *c* and lysozyme at pH 5 and pH 7 show the typical behavior and elution order of these proteins on SP Sepharose FF, already examined for example in [35] (cf. Fig. 1). The experiments are qualified for the determination of SMA parameters with both approaches. In approach I, the parameters for the SMA-isotherm were determined along the instructions of [14,16]. A significant correlation between elution volume and retention volume can be observed ($R^2 = 0.99$, cf. Fig. 2) and the determined parameters (cf. Table 1) have reasonable results compared to literature values published in the papers, listed in Table 4. In approach II, the SMA parameters were determined by a model-based inverse method (cf. Table 2). The fit of the model response to the gradiental and breakthrough data is very precise (cf. Figs 3 and 4). The observed small deviations of the model fit to the data of elution gradients with 5 cv can be attributed to kinetic effects that were neglected in order to impossible the comparison of both approaches (only isothermal SMA parameters can be determined with approach I). The fit is significantly improved by fitting the data of gradients shorter than 30 cv to Eq. (1) (data not shown).

By the multistart method, deviances in parameter estimations based on the inverse method could be determined. The comparison of these deviances for different SMA parameters shows that the information for the estimation of the steric factor is not as precisely given in gradiental and frontal experiments as for the estimation of the characteristic charge. This observation very likely explains difficulties to determine the steric factor, respectively higher deviances of this parameter, for example reported in [17,33]. Two datasets (ribonuclease A at pH 5 and lysozyme at pH 7) show overall larger uncertainties for the parameter estimations. The reason for this is most probably higher deviances in the retention times in gradiental experiments, caused by time-lags

and lot-changes between the experiments. By Monte-Carlo-based sensitivity analysis it could be shown that a significant reason for uncertainty in parameter estimations are time-dependent shifts in chromatograms (cf. Figs. 7 and 8). These shifts may for example appear, when experimental runs are performed with interruptions or other irregularities. However, absolute noise in chromatographic data has only negligible influence on the parameter estimation quality.

The sensitivity analysis and the comparison of SMA parameters from both methods (Tables 1 and 2) shows that the influences of external information and the slightly different model structures lead to different parameter values. While the steric factor σ has very similar values in both approaches, the characteristic charge ν determined by the inverse method is always higher than the value determined by approach I. Conversely, the equilibrium coefficients determined by the model-based inverse method are about ten times smaller than the values based on approach I. These deviations are plausibly explained by the findings in the sensitivity analysis of Eq. (5), displayed in Fig. 6. The high sensitivity of the equilibrium coefficient considering a fixed characteristic charge is most probably due to the parameter's position in the exponent. This position of the parameter might be omitted by using isocratic elutions and a log–log-plot, like it is described in [14]. Still a transformation of the original equation is necessary and sensitivity seems to be influenced by this transformation: [22] describe, that small errors were amplified by the log–log nature of the ion-exchange isocratic retention model they employed. Thus, a thorough sensitivity assessment for both approaches would be desirable but was out of the focus of this manuscript, which was laid on parameter predictivity.

Despite of the differences in parameter values, the order relations between the parameters are conserved in both approaches, what can be observed by comparison of the results in Tables 1 and 2. These differences can not be algorithm-specific as the same algorithm (`lsqnonlin` from MatLab) was used for the solution of implicit equations (Eqs. (5) and (9)). This suggests that parameter **values** are dependent on the column characteristics and the employed model-specific equations, but the **order relations** of SMA parameters rely significantly on adsorbent type and pH-conditions. This assumption is confirmed by the literature review. Furthermore, comparing the two approaches, the quality of fit was very satisfying (cf. Figs. 2 and 4). Even more important is the fact that for both approaches it could be demonstrated that they provide parameters of comparable high predictivity (cf. Table 3). The model-based approach has the important advantage of predicting complete chromatograms (cf. Fig. 5).

6. Conclusion and outlook

It could be shown that SMA parameters with comparable internal relations and equal predictivity could be determined based on both methods, the experimental method according to [16] and an model-based inverse method. Considering the physical significance of SMA parameters, the intention for SMA parameter determination is the crucial argument. The authors do not deny the physical significance of the parameters and the good reasoning behind approach I and similar approaches based on [14]. Still, from the view of high qualitative predictions and model calibrations, the physical significance slightly shifts into background leaving place to the very important predictive power of the determined parameters that was very high for both approaches. In addition, the inverse method has the obvious advantage of predicting complete chromatograms and best fit between model response and data. This leads to the possibility of further usage of the mechanistic model as troubleshooting and error diagnostic tool for the process. Thus, based on the findings in this paper, the inverse method for SMA parameter determination is recommended for fast process development. However, a certain amount and quality of data has to be provided for the inverse method for a precise determination of parameters and reliable predictions. This issue has to be further examined and the design of experiments optimized. With optimal experimental design and an *a priori* analysis of already existent data of the system of interest, it should be possible, to find an efficient way to estimate SMA parameters of equal predictivity directly from process data with no or only few additional experiments. Monte-Carlo simulations might support the analyses, as was shown in this study.

Nomenclature

DoE	design of experiments
IEC	ion exchange chromatography
SMA	steric mass action
$c_{a,s}$	salt concentration at gradient begin
$c_{e,s}$	salt concentration at gradient end (M)
c_i	concentration of component i in the mobile phase on column level (M)
$c_{p,i}$	concentration of component i in the mobile phase on particle level (M)
c_{prot}	protein conc. in stock solution (M)
c_{salt}	salt concentration in buffer (M)
c_0	initial concentrations of salt and protein at column inlet (M)
D_{ax}	axial dispersion ($m^2\ s^{-1}$)
F	PDAE equation system
$k_{eff,i}$	effective film transfer coefficient for component i (m/s)
$k_{i,ads}$	adsorption coefficient of component is $mM^{-\nu}$
$k_{i,des}$	desorption coefficient of component i ($s\ mM^{-\nu}$)
$k_{i,eq}$	equilibrium coefficient of component i
L_C	column length (m)
q_i	concentration of component i bound on the particle surface (M)
r_p	particle radius (m)
R^2	coefficient of determination
u_{int}	interstitial flow rate (m/s)
V_0	breakthrough volume at 10% of a nonretarded tracer (ml)
V_B	breakthrough volume at 10% of the breakthrough (ml)
V_d	dead volume (ml)
V_C	column volume ($m^3\ s^{-1}$)
V_G	gradient volume (ml)
V_R	retention volume (ml)
β	phase ratio
ε_c	column porosity
ε_p	pore porosity
ε_t	total porosity
Λ	ionic capacity (mM)
μ_{mom}	first moment of a peak (ml)
ν_i	characteristic charge of component i
σ_i	steric factor of component i
σ_{mom}	square root of the second moment of a peak
θ_{est}	parameters that will be estimated in the least squares optimization solving the inverse problem
θ_{fix}	parameters that are fixed during the least squares optimization solving the inverse problem

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