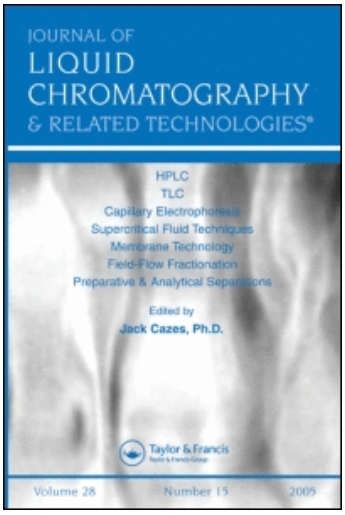

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Use of Quality by the Design for the Modelling of Chromatographic Separations

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Abstract: A desired goal of the PAT framework is to design and develop well understood processes that will consistently ensure a predefined quality at the end of the manufacturing process.[1] Achieving this goal will reduce the time and cost of process development and ensure development of robust processes that can handle variability and deliver a predefined quality as regards yield, purity and productivity within the defined design space. This communication will focus on a set of scientific principles supporting process understanding and process design of chromatographic separations.

Keywords: Association, Isotherm, Model, Preparative chromatography, Protein, Quality by design

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THE SCIENTIFIC PRINCIPLES AND TOOLS

The scientific theory of processes is a prerequisite of good process understanding, and a gauge of process understanding is the ability to comprehend and apply the scientific principles to develop, model, and design robust processes. Process robustness is defined as the ability of a process to demonstrate acceptable quality and performance and tolerate variability in inputs at the same time.[2] The scientific principles include chromatographic theory in general, models of adsorption isotherms and mass-transfer.

Pharmaceutical development can benefit from computer-aided process design and process simulation to support process development and optimisation of manufacturing. Process simulation has been successfully used in the chemical and oil industries since the early 1960s to expedite development and optimise the design and operation of integrated processes. Similar benefits can be expected from the application of computer-aided process design and simulation in the pharmaceutical and biopharmaceutical industries. At present, the utility of computer-aided process design and process simulation in drug product manufacturing operations is limited. The use of computer-aided process design and process simulation should result in more robust processes developed faster and at a lower cost, resulting in higher quality products[2] The limiting factors are only computational effort, the quality of the applied models and accessibility to experimental data for model parameter estimation. If the models are of high quality, they will put one in a position to utilise a model-based simulation tool to develop, design and optimise the processes, to identify critical process parameters and critical sources of variability, to develop a strategy for managing process variability, and to analyse aberrations.

The be-all and end-all in simulation of a chromatographic separation process is the quality of the adsorption equilibrium model. Therefore, this paper will focus on the application of a recent model,[3] named the self-association (SAS) isotherm, that has been developed to deal with protein-protein association in adsorption chromatography. A protein that associates to the ligands of the stationary phase can act as a ligand for another protein molecule and thus form a dimer in the adsorbed state. This phenomenon, which gives rise to a sigmoid isotherm, has been observed with β-lactoglobulin A on a Source 30Q adsorbent[4] and it is also observed in reversed phase chromatography.[3]

PROCESS DEVELOPMENT

The conventional process development is to a great extent an experimental trial and error based approach. An approach for the reduction of experimental efforts, materials consumption, and costs is automation and

miniaturisation of the involved steps. This can be achieved applying a high throughput screening platform to determine static capacities and uptake kinetics.[5] However, if the goal is to go for a model assisted process development scheme, the platform is less suited for what turns out to be the most essential experiments, the isocratic retention volumes. These experiments are easy to perform, require very little material, and are used to determine the partition coefficients at low protein concentration. The parameters modelling the initial slope of an isotherm are determined from the partition coefficients. The mass-transfer coefficients can be determined from the second moment of isocratic elution chromatograms, from batch uptake measurements, or from breakthrough curves. Traditional static capacities determined from batch experiments are in general not sufficiently accurate to allow for determination of all model parameters for the isotherm and besides these experiments require more material than an experimental determination of isocratic retention volumes does.

If models of high quality are available, a model assisted development scheme can be drawn up. That is, the model parameters are estimated from isocratic retention data supplemented with a few capacity measurements, breakthrough experiments or chromatographic runs at high load to adjust the non-linear parameters of the isotherms. The simulator is used to design and develop the separation and define the design space, that is, to estimate acceptable values of process operating parameters, i.e., feed composition, load, pH, eluant compositions, gradient slopes and length, flow rate, etc. A sensitivity analysis should be performed to determine which input parameters have the greatest impact on the column performance. The input parameters could be either process operating parameters or model parameters. Highly sensitive process parameters influence the possible design space while highly sensitive model parameters should be measured as accurately as possible to improve the quality of the predictions. When designing and analysing a capture step sensitivity analysis as regards the dynamic binding capacity and HCP removal could be useful for resin selection. Similarly, model predictions could be especially useful for troubleshooting if experimental data are not available or scarce, as could be the case when analysing how to manage trace impurities. When a reasonable design space has been defined the model predictions must be validated experimentally. If the agreement is not satisfactory one can tune the model parameters to improve the agreement between simulated and experimental results. Scale-up is a trivial issue because, load, flow rates, gradient etc. are scaled to the column volume, that is, one has to keep identical residence times in the small scale as well in the large scale column.[6,7] This paper deals with thermodynamic modelling aspects. Other equally important aspects are cleaning, sanitisation and validation. An introduction to these subjects are analysed by Hagel et al.[8]

MODELLING PROTEIN ADSORPTION

In adsorption chromatography, a protein molecule adsorbs by forming an association complex with a number of ligands that are covalently bonded to the stationary phase. In the adsorption model it is assumed that the ligands are homogeneously distributed in the pore volume. When the protein associates with the ligands it either displaces the counter-ions associated with charged ligands or forms a complex by non-polar interactions with hydrophobic ligands. Hydrophobic chromatography includes hydrophobic interaction chromatography (HIC) and reversed phase chromatography (RPC). In the simple case where there is no protein-protein self-association in the adsorbed state, all isotherms are convex isotherms.[3] The general expression for the convex isotherm is given in Eq. (1). When m protein species adsorb the equilibrium is calculable by solving the equations, $i = 1, \ldots, m$

$$\frac{q_i}{c_i} = A_i \left(1 - \sum_{j=1}^{m} \frac{q_j}{q_j^{max}} \right)^{\nu_i} \tag{1}$$

where c_i and q_i are the protein concentrations of free and of associated species in the pores of the adsorbent, respectively. At low protein concentration A_i become identical to the initial slope of the isotherms, ν_i are the stoichiometric coefficients, and q_j^{max} are the saturation capacities of the individual species. The term $1 - \sum_{j=1}^{m} q_j/q_j^{max}$ is the fraction of free ligands. The model derived for A_i depends on the chromatographic technique.[3,4] The models are

Hydrophobic interactions, HIC and PRC

$$A_i = \left(\frac{\Lambda}{c} \right)^{\nu_i} \widehat{K}_{H,i} \tilde{\gamma}_i \tag{2}$$

Ion-exchange chromatography, IEC

$$A_i = \left(\frac{\Lambda}{c_s z_s} \right)^{\nu_i} \widehat{K}_{E,i} \tilde{\gamma}_i \tag{3}$$

Λ is the density of ligands (mol/L pore volume), c is the molar density of the fluid in the pores, c_s is the molar counter-ion concentration in the pores, z_s is the charge number of the counter-ion, $\widehat{K}_{E,i}$ and $\widehat{K}_{H,i}$ are the practical thermodynamic equilibrium constants, and finally, $\tilde{\gamma}_i$ are the asymmetric activity coefficients of the solutes and depend on the concentrations of solvents, modulators, buffer, solutes, and adsorbates.

Proteins in solution can self-associate to form dimers and an adsorbed protein can also act as a ligand and associate with a free protein molecule to form a double layer of proteins on the adsorbent. This modifies the shape of the isotherm and gives rise to a sigmoid isotherm that at low concentration is concave and at high concentration becomes convex.[3] When the isotherm is concave, the fronts of the eluting peaks will coincide, and when the isotherm is convex, the rear edges of the eluting peaks will coincide.[9] The model for the self-association (SAS) isotherm is an extension of the model shown in Eq. (1). The SAS model is analysed in the Appendix.

Figure 1 shows an overlay of elution profiles of a biopharmaceutical protein that self-associates in the adsorbed state. The isotherm is characterised by the parameters $\Lambda = 0.3\,\mathrm{M}$, $\widehat{\mathbf{K}}_{\mathrm{E},1} = 0.00459$, $\nu_1 = 3.66$, $\mathrm{q}_1^{\max} = 0.0392\,\mathrm{M}, \mathrm{c} = 55.5\,\mathrm{M}$, and the self-association equilibrium constant $\widehat{\mathbf{K}}_{\mathrm{D},1} = 500000$. The load varies from 0.00002 to 0.001 mol/L column volume (CV). The asymmetric activity coefficient is assumed to be unity. The elution is from 0.0132 to 0.0705 M NaCl in the eluant over 10 CV on a column packed with Source 30Q adsorbent. The differential material balance[6,9] was solved using Comsol Multiphysics™. At low load we observe that the leading edges of the peaks coincide whereas the trailing edges coincide at high load.

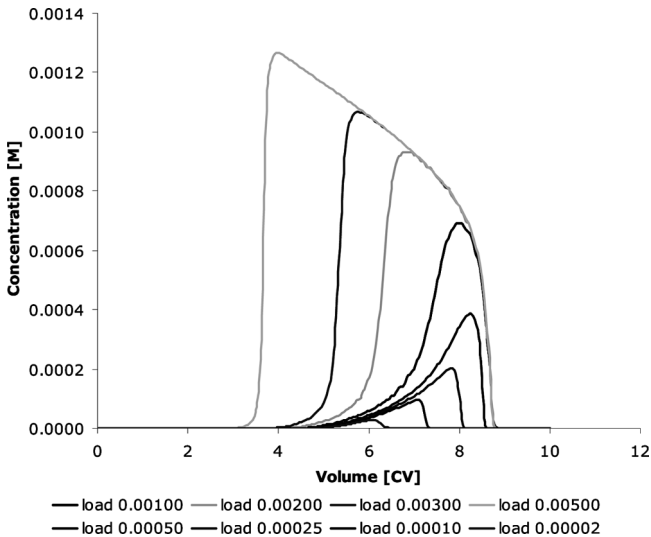

Figure 1. An overlay of the elution profiles of a protein that self-associates in the adsorbed state. The load varies from 0.00002 to 0.001 mol/L column volume (CV). The elution is from 0.0132 to 0.0705 M NaCl in the eluant over 10 CV on a column with Source 30Q adsorbent.

ESTIMATION OF MODEL PARAMETERS FROM PARTITION COEFFICIENTS

The partition coefficients A_i are determined form isocratic retention volume measurements. These experiments are easy to perform, require little material, but provide a lot of useful information about the adsorptive behaviour. The retention volume model is[10]

$$V_{R,i} = V_c(\varepsilon + (1-\varepsilon)\varepsilon_p k_{d,i}(1 + A_i)) \tag{4}$$

V_c is the bed volume, ε is the interstitial bed porosity, ε_p is the particle porosity and $k_{d,i}$ is an exclusion factor that represents the fraction of the pore volume in the stationary phase which is available for diffusion of species *i*. If no adsorption (NA) takes place, i.e., when $A_i = 0$, the species will be retained in the porous structure of the adsorbent because the molecules can diffuse into some of the openings. Small molecules can diffuse into a larger fraction of the openings than large molecules and large molecules are thus retained less than the small molecules. This separation mechanism is utilised in size exclusion chromatography. The model for this retention volume is derived from Eq. (4) by putting $A_i = 0$

$$V_{NA,i} = V_c(\varepsilon + (1-\varepsilon)\varepsilon_p k_{d,i}) \tag{5}$$

Since $k_{d,i}$ by definition is zero for common salts, it can for a protein be estimated from the difference between the salt retention volume and the retention volume of the protein under non-binding conditions. In IEC this is done using an eluant with high salt concentration. Subtraction of Eq. (5) from Eq. (4) enables estimation of A_i from isocratic experiments. The isocratic experiments are performed at low protein concentration where the self-association, if present, has very little influence on the retention and thus, the models Eq. (2) or (3) can be utilised as models for the partition coefficients in the linear range of the isotherms. Equation (2) applies to HIC and RPC and Eq. (3) applies to ion-exchange.

When the technique is ion-exchange chromatography combining Eqs. (3–5) shows that the model parameters in Eq. (3) are calculable from the equation

$$\ln(V_{R,i} - V_{NA,i}) = \ln \widehat{K}_{E,i} + \nu_i \ln \frac{\Lambda}{z_s} + \ln V_c(1-\varepsilon)\varepsilon_P k_{d,i} + \ln \tilde{\gamma}_i^{\infty} - \nu_i \ln c_s \tag{6}$$

The quantity of material injected for an isocratic experiment is very low, wherefore the asymmetric activity coefficient is equal to the

asymmetric activity coefficient at infinite dilution $\tilde{\gamma}_i^{\infty}$. An asymmetric activity coefficient at infinite dilution depends on the solvent and modulator compositions, and it is unity in the pure reference solvent but not in a mixed solvent. Analysis of Eq. (6) shows that a plot of $\ln(V_{R,i} - V_{NA,i})$ versus the logarithm of the counter-ion concentration displays a straight-line plot provided the term $\ln \tilde{\gamma}_i^{\infty}$ is of minor importance. If the plot is a straight-line plot, the slope of the straight line is $-\nu_i$ and $\hat{K}_{E,i}$ can be determined from the value of the ordinate of the line at $\ln c_s = 0$.

When the parameters for A_i have been determined, one has to estimate the value of the maximum available capacities q_i^{max} and if self-association takes place one must also estimate the equilibrium constants for the self-association. These parameters are estimated from either capacity measurements or by an adjustment of the parameters in order to get a good agreement between the experimental chromatograms and the simulations.

EXPERIMENTAL

All experiments were performed on an ÄKTA explorer 100 using a Source 30Q adsorbent and the eluant is a NaCl/TRIS pH 8.0 (20°C) buffer system. The column volume was 11.8 mL (length 15.0 cm). The eluate comprises three proteins, a major component and two impurities.

RESULTS

The result of analysis of the measured isocratic retention volumes are shown in Figure 2. The figure shows a double-logarithmic plot of the adsorptive isocratic retention volumes ($V_R - V_{NA}$) of the three components versus the counter-ion concentration. The stoichiometric coefficients, ν_i, are estimated from the slopes of the straight lines and the practical equilibrium constants, $\hat{K}_{E,i}$, are estimated from the intersections of the straight lines with the ordinate axis at $\ln c_s = 0$. The values are given in Table 1. The particle porosity ε_p is 0.57, the bed porosity ε is 0.45, the exclusion factors $k_{d,i}$ are unity, and the ligand density Λ is 0.3 M.

Several runs at preparative load were performed and $\hat{K}_{D,2}$ was estimated by fitting the model to the preparative runs. It is reasonable to assume that self-association only plays a role for the major component because the concentrations of the other components are much lower. The mass-transfer resistance is represented by a Stanton number which was estimated to 4,800 for all components. The Stanton number is the timescale of flow divided by the timescale of equilibration[6] and a large Stanton number indicates that the equilibration is fast compared to the

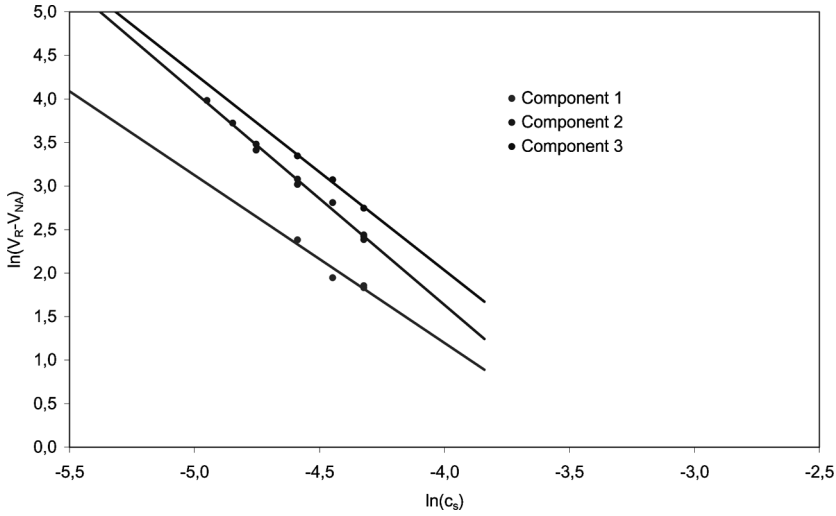

Figure 2. A double-logarithmic plot of the adsorptive isocratic retention volumes ($V_R - V_{NA}$) of the three components versus the counter-ion concentration. The stoichiometric coefficients ν_i are estimated from the slopes of the lines and the practical equilibrium constants $\widehat{K}_{E,i}$ are estimated from the values of the ordinates of the straight lines at $\ln c_s = 0$.

flow rate. The estimated number for the self-association equilibrium constant $\widehat{K}_{D,2}$ is $1.8 \cdot 10^5$. The estimated numbers for q_j^{max} are given in Table 1. These numbers are calculated from Eq. (XI) in the Appendix using a hindrance factor $\sigma = 2.1$ for all components.

Figures 3–5 show a comparison of the results of modelling of the major peaks of three experimental chromatograms resulting from a gradient elution. The loads are 2.9, 5.8, and 11 g/L adsorbent, respectively. The experimental chromatograms of the major component show that the peaks display fronting. This is an indication of a concave behaviour of the isotherm caused by self-association of the protein on the adsorbent. The results of modelling of the elution profiles of the major component show a satisfactory agreement between model and experiment. The corresponding isotherm is shown in Figure 6. The

Table 1. Estimated model parameters

Component	ν	$\widehat{K}_{E,i}$	$\widehat{K}_{D,i}$	q_j^{max} [M]
1	1.93	0.04833	0	0.074
2	2.44	0.01763	$1.8 \cdot 10^5$	0.066
3	2.26	0.04436	0	0.069

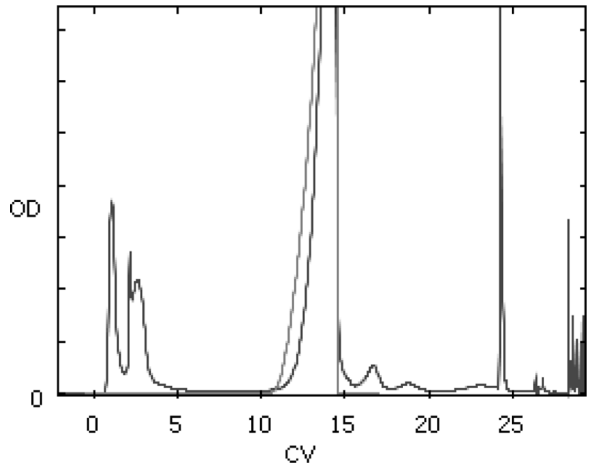

Figure 3. Elution peaks resulting from a gradient elution of the sample at a load of 2.9 g/L adsorbent on a Source 30Q adsorbent. The result of simulation of the major peak is shown using the SAS isotherm.

abscissa is the concentration in the solution and the ordinate to the left in the figure shows the adsorption capacity. The ordinate to the right in the figure shows the slope of the cord q/c which elucidates the sigmoid nature of the isotherm.

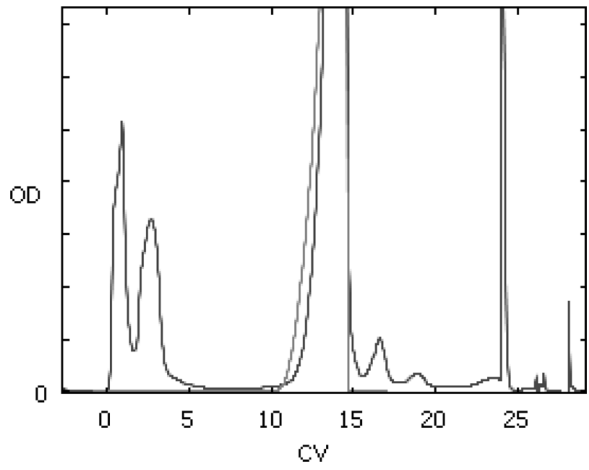

Figure 4. Elution peaks resulting from a gradient elution of the sample at a load of 5.8 g/L adsorbent on a Source 30Q adsorbent. The result of simulation of the major peak is shown using the SAS isotherm.

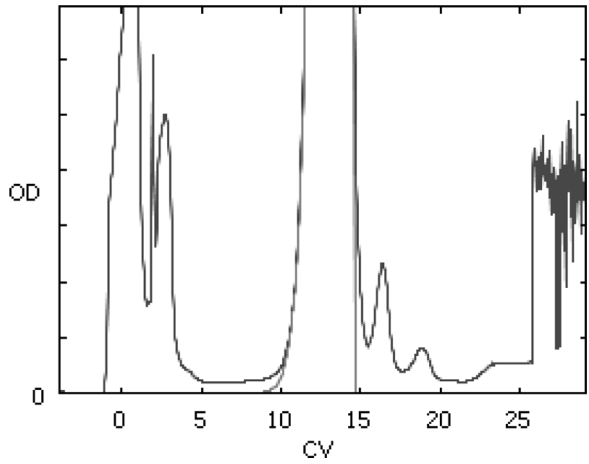

Figure 5. Elution peaks resulting from a gradient elution of the sample at a load of 11 g/L adsorbent on a Source 30Q adsorbent. The result of simulation of the major peak is shown using the SAS isotherm.

DISCUSSION AND CONCLUSIONS

Related industrial applications of the approach and the models outlined above to IEC are shown by Mollerup et al.[6,11] The key-plot is the

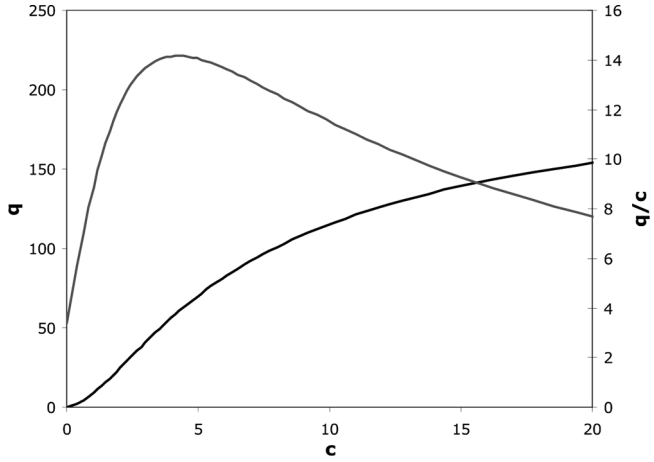

Figure 6. The SAS isotherm of component 2 on a Source 30Q adsorbent. The abscissa is the concentration in the solution and the scale on the ordinate to the left shows the adsorption capacity in g/L and the scale to the right shows the slope of the cord q/c.

double-logarithmic plot, Figure 2. A similar plot for four components is shown in Figure 3 in Ref. [11] and in this study the curvature of the isotherm was established from a few capacity measurements as shown in Figure 4 in Ref. [11]. The investigations show examples of comparison of simulated and experimental chromatograms to validate the quality of the simulations as well as examples of application of simulations for improving the productivity of a process and for analysing an aberration.

Comparing a Kinetic and a Thermodynamic Approach

In a recent publication McCue et al.[12] examined the separation of a monomer and an aggregate protein mixture using Phenyl Sepharose FF. Mass-transfer was described by a homogeneous diffusion model and a competitive binary Langmuir isotherm was used to model the adsorption and desorption. The model was derived from a kinetic approach where at equilibrium the rate of adsorption is equal to the rate of desorption. The chromatographic model was formulated and used to predict the separation of the monomer and aggregate species. The experimental studies showed a fraction of the aggregate species bound irreversibly to the adsorbent, which was a major factor governing the separation of the species. Inclusion of irreversible binding in the adsorption model greatly improved the model predictions over a range of operating conditions.

The experimental procedures by McCue et al. are distinct from those used above, but more in line with the classical procedures. The mechanisms of protein adsorption, desorption, and diffusion of the two species were evaluated using several experimental approaches. Adsorption equilibrium data for the monomer and aggregate forms of the fusion protein were determined from batch contact experiments. The parameters for the binary Langmuir model were determined from these experiments. Breakthrough and desorption curves were measured and values for the effective diffusivities during the adsorption and desorption processes for both species were determined by minimizing the difference between the model and the experimental results.

A model parameter sensitivity analysis was performed and their conclusions are very important. The results of the parameter sensitivity studies showed that small changes to the elution adsorption isotherm could result in a significant change to the separation performance of the examined protein and the adsorbent. Thus, values for the adsorption isotherm parameters should be measured as accurately as possible to minimize uncertainty in the model predictions. It was observed that the model predictions were insensitive to changes in the effective diffusivities. This suggests that extremely accurate values for rates of the mass-transfer are not required.

A comparison of the isotherm expression used by McCue et al. and the model presented in Eqs. (1) and (2) will elucidate why a thermodynamic approach is preferable to a kinetic approach. In the case where no irreversible binding occurs for either species the single component isotherm model used by McCue et al. reduces to the classical Langmuir adsorption isotherm

$$\frac{q_1}{c_1} = \frac{K_1}{q_1^{max}} \left(1 - \frac{q_1}{q_1^{max}}\right) \tag{7}$$

where K_1 is the ratio of the adsorption and the desorption rate constants and therefore it is not identical with the equilibrium constant in Eq. (2). When the stoichiometric coefficient is unity the corresponding single component isotherm model derived from Eqs. (1) and (2) is

$$\frac{q_1}{c_1} = \left(\frac{\Lambda}{c}\right)\hat{K}_{H,1}\tilde{\gamma}_1\left(1 - \frac{q_1}{q_1^{max}}\right) = A_{H,1}^0\tilde{\gamma}_1\left(1 - \frac{q_1}{q_1^{max}}\right) \tag{8}$$

$\hat{K}_{H,1}$ is the practical equilibrium constant of the hydrophobic association. The model shows that the retention is controlled by the magnitude of the asymmetric activity coefficient, $\tilde{\gamma}_1$, of the solute. Analysis of Eq. (8) demonstrates that the partition coefficient at very low protein concentration where $q_1 << q_1^{max}$ is $A_{H,1}^0\tilde{\gamma}_1^{\infty}$. The partition coefficients can be determined from isocratic experiments. The model for IEC is shown in Eq. (6) and the equivalent equation for HIC is

$$\ln(V_{R,1} - V_{NA,1}) = \ln A_{H,1}^0 + \ln V_c(1-\varepsilon)\varepsilon_P k_{d,1} + \ln\tilde{\gamma}_1^{\infty} \tag{9}$$

This equation shows that a semi-logarithmic plot of $\ln(V_{R,1} - V_{NA,1})$ versus the modulator concentration will demonstrate how $\ln\tilde{\gamma}_1^{\infty}$ depends on the modulator concentration. The experiments will most likely show that $\ln\tilde{\gamma}_1^{\infty}$ turns out to be a linear function of the modulator concentration as observed by Mollerup et al.[11] Chen et al.[13] determined retention parameters for 20 proteins on Butyl Sepharose 4 FF at pH 7 with 0.2–1.4 M ammonium sulphate in the eluant and fitted the data to an equation similar to Eq. (9). The regressed R^2 values for each linear fit were consistently greater than 0.95. A kinetic approach will never reveal how K_1 in Eq. (7) depends on the thermodynamic properties of the solution.

Robustness and Sensitivity Analysis—Design of Experiments Studies

When a chromatographic operation is prepared for validation before commercial production, numerous tests have to be performed to establish the relative importance of each operating parameter to define its future

role and importance in the framework of in-process controls. This prioritisation process is usually performed using a purely empirical approach.

Rathore et al.[14] presented a case study involving design of a process analytical technology based control scheme for an ion-exchange chromatography step. They conducted a design of experiments study with purity of the load material, start collect, and stop collect as variables. Pool purity and step yield were monitored for each experiment. An empirical model was developed and used to calculate the pool purity for all the experiments that were performed and a comparison of pool purities that were calculated using the model was made with the measured pool purity. It was observed that the two numbers correlate very well and support feasibility of the PAT based control scheme. Their example highlights the usefulness of an empirical model, but there are significant limitations of this approach. Since the empirical model is not based on fundamental chromatographic theory its application is limited to the ranges of data that the model is based on. An extensive summary is provided by Kaltenbrunner et al.[15]

Kaltenbrunner et al.[15,16] investigated relative importance of operating parameters like changes in ionic strength, pH, resin ligand density, bed height, elution flow rate, and gradient slope on a specific chromatographic situation. They compared the use of a chromatographic model to rank process parameters to the traditional statistical experimental design approach. They performed both the typical screening of parameters in a fractional factorial experimental design and, independently, developed a theoretical model for a particular ion-exchange chromatography operation for the separation of closely related protein species. The theoretical model is limited to linear isotherms but can be applied to all types of ion-exchange operations. There is a good agreement between the results of the parameter screening of the rational approach and the empirical approach. Both rank the ionic strength at the beginning of the elution gradient, pH, and resin ligand density as the parameters that have the highest effect on separation behaviour. Similarly, both methods identify gradient slope and flow rate as parameters that have a lesser effect on the separation behaviour. The results of their investigation indicate that the modelling approach can be applied for the initial screening of operational parameters during process characterisation. This demonstrates that the application of a model based approach has the potential to accelerate the evaluation and significantly reduce the amount of analytical testing needed. It is stated that such a modelling approach can be used to reduce a screening matrix of 10 parameters to a matrix of 4.

The detection of variability in the feed can be made offline, but online measurements offer the possibility of making real time decisions. Rathore et al.[17] examined the feasibility of using a commercially available online HPLC system for real-time process decisions. Experimental data from the feasibility studies are modelled using a second order polynomial equation

and predictions of the model are compared to actual experimental data. It is shown that the application is feasible and thus its implementation is likely to result in more consistent product quality. However, the authors remark that achieving this on the manufacturing floor would require a higher level of process understanding for designing such an approach and increased operational complexity. It is pointed out that in certain cases, column performance may deteriorate over multiple cycles and then the model would need to account for column age and a system for detection of this would be necessary. Rathore et al.[17] conclude that, in general, it is evident that adoption of control schemes such as the one presented will require changes in our approaches towards process and analytical development, manufacturing, quality assurance, and regulatory filings.

LIST OF SYMBOLS AND DESCRIPTION

A_i	parameter in the isotherm
A_i^0	$A_i/\tilde{\gamma}_i$
a_i	activity; $a_i = x_i\gamma_i = c_i\gamma_i/c$
$\hat{a}_i$	activity of an associated component; $\hat{a}_i = y_i\hat{\gamma}_i = q_i\hat{\gamma}_i/c$
c	molar density of the solution in the pore volume
c_i	molar concentration of a solute in the pore volume
G	Gibbs energy
G^E	excess Gibbs energy; $G^E = RT\sum_1^N n_i \ln \gamma_i$
$\overline{G}_i$	partial molar Gibbs energy; $\overline{G}_i = \mu_i$
$\overline{G}_i^E$	partial molar excess Gibbs energy; $\overline{G}_i^E = RT \ln \gamma_i$
ΔG^0	standard Gibbs energy change of association (adsorption), eqn. (III)
$\Delta\widehat{G}^0$	practical Gibbs energy change of association (adsorption), Section A3
K	thermodynamic equilibrium constant defined in eqn. (III)
$\widehat{K}$	practical equilibrium constant defined in Section A3
$k_{d,i}$	the fraction of the column pore volume into which a solute can penetrate
m	number of associating components
N	number of components
n_i	mole number
P	pressure
q_i	molar concentration of an associated component
q_j^{max}	maximum available capacity
R	gas constant, 8.31451 J/(mol K)
SAS	Self-Association
T	temperature

V_c	bed volume
$V_{NA,i}$	retention volume when no association takes place
$V_{R,i}$	retention volume
x_i	mole fraction; $x_i = c_i/c$
y_i	mole fraction of an associated component; $y_i = q_i/c$
z_i	charge number of an ion

Greek letters

γ_i	activity coefficient; $\gamma_i = a_i/x_i$
$\hat{\gamma}_i$	activity coefficient of an associated component; $\hat{\gamma}_i = \hat{a}_i/y_i$
γ_i^∞	activity coefficient at infinite dilution in a picked reference solvent
$\tilde{\gamma}_i$	asymmetric activity coefficient; $\tilde{\gamma}_i = \gamma_i/\gamma_i^\infty$
ε	bed porosity
ε_p	porosity of the adsorbent
Λ	molar concentration of ligands in the pore volume
μ_i	chemical potential
$\hat{\mu}_i$	chemical potential of an associated component
μ_i^0	reference chemical potential
$\tilde{\mu}_i^0$	asymmetric reference chemical potential, Section A1
ν_i	stoichiometric coefficient
σ_j	steric hindrance factor

Subscripts

D	self-association
E	ion-exchange
H	hydrophobic
L	ligand
i, j	index of component i or j, respectively
s	counter-ion

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APPENDIX: THE SELF-ASSOCIATION (SAS) ISOTHERM

A1. The Equilibrium Association Schemes

A protein molecule adsorbs by forming an association complex with a number of ligands that are covalently bonded to the stationary phase. In ion-exchange chromatography, the protein displaces the counter-ions associated with the ligands. When we assume that the ligand L has unit charge, the general scheme is

$$P_i^{z_i} + \nu_i L_{z_s} S^{z_s} \rightleftharpoons \nu_i S^{z_s} + P_i^{z_i} L_{z_i} \qquad \text{(I)}$$

Here z_i is the number charge on the protein P_i that binds to the ligands, z_s is the charge number of the counter-ion S, and $\nu_i = z_i/z_s$ is the stoichiometric coefficient. In the exchange scheme we have disregarded all ions that do not take place in the exchange. If the protein molecule can associate with an adsorbed protein molecule the association scheme is

$$P_i + P_i L_{\nu_i} \rightleftharpoons P_{2,i} L_{\nu_i} \qquad \text{(II)}$$

Here $P_{2,i}$ is the dimer adsorbent.

Since $\Delta G = \Sigma \nu_i \mu_i = 0$ at equilibrium, it implies that the equilibrium constant K is calculable from the equation

$$RT \ln K \equiv RT \sum \nu_i \ln a_i = -\sum \nu_i \mu_i^0(T, P) = -\Delta G^0 \qquad \text{(III)}$$

because the activities a_i are defined by the equation

$$RT \ln a_i \equiv \mu_i - \mu_i^0(T, P) \qquad \Leftrightarrow \qquad \mu_i = \mu_i^0(T, P) + RT \ln a_i \qquad \text{(IVa)}$$

where μ_i are the chemical potentials in the mixture and μ_i^0 are the corresponding reference potentials taken as states of pure species at the temperature and the pressure of the mixture. The thermodynamic equilibrium constant K is independent of the composition of the mixture because the reference chemical potentials, μ_i^0, are taken as states of pure species. Similarly, the activities of pure substances are unity.

The activities of ideal mixtures are by definition equal to the mole fractions x_i. Therefore it is convenient to define a quantity that accounts for the deviation from ideal mixture behaviour. This quantity, the activity coefficient of species *i*, γ_i, is defined as the ratio of the activity of species *i* to the mole fraction of species *i*, that is, the equivalent of eqn. (IVa) is

$$\mu_i = \mu_i^0(T, P) + RT \ln x_i \gamma_i \qquad \text{(IVb)}$$

The activity coefficient of a pure substance *i*, γ_i, is unity because the reference potential is taken as a state of pure *i* at the temperature and the pressure of the mixture.

It is sometimes convenient to apply an activity coefficient that becomes unity at infinite dilution in a pure reference solvent. This activity coefficient is denoted an asymmetric activity coefficient and defined as the ratio of the activity coefficient to the activity coefficient at infinite dilution in a pure solvent, that is, $\tilde{\gamma}_i \equiv \gamma_i/\gamma_i^\infty$ where γ_i^∞ are the activity coefficients at infinite dilution in the pure reference solvent. The asymmetric activity coefficients at infinite dilution, $\tilde{\gamma}_i^\infty$, are only unity in the pure reference solvent. Since the potentials μ_i are partial molar Gibbs energies, they must be independent of how one defines an activity coefficient. Therefore, the asymmetric reference potentials must include the partial molar excess Gibbs energies at infinite dilution, that is, $\tilde{\mu}_i^0 = \mu_i^0 + RT \ln \gamma_i^\infty$, wherefore

$$\mu_i = \tilde{\mu}_i^0 + RT \ln x_i \tilde{\gamma}_i \qquad \text{(IVc)}$$

Note that Eqs. (IVa), (IVb), and (IVc) are equivalent. One must also note that the reference potentials in eqn. (IVc), $\tilde{\mu}_i^0$, depend on nature of the substance *i* and the picked reference solvent.

A2. Simplifying Assumptions

In order to derive the expression for the self-association isotherm we will make a number of convenient assumptions in order to reduce the number of parameters.

a. The activity coefficients of the adsorbates, $\hat{\gamma}_i$, are constant. An associated molecule is at a fixed position and less flexible than a molecule in solution and that is the reason to assume that the activity coefficients of the adsorbates be independent of the adsorbate concentration. Furthermore, it is assumed that the activity coefficients of adsorbates are not influenced by moderate changes in the composition of the eluant.
b. The ratio of the activity coefficient of a counter-ion in solution, γ_s, to the activity coefficient of an associated counter-ion, $\hat{\gamma}_s$, is independent of the counter-ion concentration.
c. The maximum available capacity of the monomer is twice the maximum capacity of the dimer, that is $q_{monomer}^{max} = 2q_{dimer}^{max} = q_i^{max}$.

Assumption one is convenient because little is known about the excess properties in the adsorbed state. The justification of the second

assumption is mainly based on experience with sodium chloride in the eluant and it may not be appropriate if other salts are applied, ammonium sulphate for example. The third assumption is made in order to reduce the number of parameters [3], and because it is easy to estimate the over-all protein concentration in the adsorbed state, q_i, whereas an estimation of the monomer and the dimer concentrations can be difficult. The over-all concentration is, $q_i = q_{monomer} + 2q_{dimer} = q_{1,i} + 2q_{2,i}$.

Due to the assumption a) and b), the equilibrium constants will be modified to include the 'constant' activity coefficients. The true equilibrium constants are defined in Eq. (III).

A3. The Practical Equilibrium Constants

Ion-Exchange

$$\widehat{K}_{E,i} = K_{E,i} \frac{\gamma_i^{\infty}}{\hat{\gamma}_i} \left(\frac{\hat{\gamma}_s}{\gamma_s} \right)^{\nu_i} \tag{V}$$

Here $K_{E,i}$ are the true equilibrium constants for ion-exchange scheme in Eq. (I) whereas $\widehat{K}_{E,i}$ are the practical equilibrium constants. Likewise, the standard Gibbs energy changes of ion-exchange processes, $\Delta G^0_{E,i}$, are also modified to include contributions from $(\gamma_i^{\infty}/\hat{\gamma}_i)(\hat{\gamma}_s/\gamma_s)^{\nu_i}$. That is

$$\begin{aligned} -RT \ln \widehat{K}_{E,i} &= \Delta \widehat{G}^0_{E,i} \\ &= (\Delta G^0_i - RT \ln(\gamma_i^{\infty}/\hat{\gamma}_i)) - \nu_i (\Delta G^0_s - RT \ln(\gamma_s/\hat{\gamma}_s)) \\ &= \Delta \hat{G}^0_i - \nu_i \Delta \hat{G}^0_s \end{aligned} \tag{VI}$$

Self-Association

$$\widehat{K}_{D,i} = K_{D,i} \frac{\hat{\gamma}_{1,i} \gamma_i^{\infty}}{\hat{\gamma}_{2,i}} \tag{VII}$$

Here, $K_{D,i}$ are the true equilibrium constants for the self-association whereas $\widehat{K}_{D,i}$ are the practical equilibrium constants. $\hat{\gamma}_{1,i}$ are the activity coefficients of monomer adsorbates, $\hat{\gamma}_{2,i}$ are the activity coefficients of dimerous adsorbates. Likewise, the standard Gibbs energy changes of self-associations, $\Delta G^0_{D,i}$, are also modified to include contributions from $\gamma_i^{\infty} \hat{\gamma}_{1,i}/\hat{\gamma}_{2,i}$. That is,

$$-RT \ln \widehat{K}_{D,i} = \Delta \widehat{G}^0_{D,i} = \Delta G^0_{D,i} - RT \ln(\hat{\gamma}_{1,i} \gamma_i^{\infty}/\hat{\gamma}_{2,i}) \tag{VIII}$$

A4. The Self-Association Isotherm–Ion-Exchange

The SAS isotherm[3] model is

$$\frac{q_i}{c_i} = \widehat{K}_{E,i}\left(\frac{\Lambda}{c_s z_s}\right)^{\nu_i}\left(1 - \sum_j^m \frac{q_j}{q_j^{max}}\right)^{\nu_i}\left(1 + 2\frac{\widehat{K}_{D,i}}{c}c_i\tilde{\gamma}_i\right)\tilde{\gamma}_i \qquad \text{(IX)}$$

Here, Λ is the charge density of ligands, c_s is the molar concentration of the counter-ion, z_s is the charge number of the counter-ion, ν_i are the stoichiometric coefficients, c_i are the molar concentrations of the solutes, q_i are the molar concentrations of the adsorbates, and $\tilde{\gamma}_i$ are the asymmetric activity coefficients of the solutes and depend on the concentrations of solvents, modulators, buffer, solutes, and adsorbates. The molar density in the pore volume, c, is the sum of the molar concentrations including solvents, modulators, buffer, solutes, and adsorbates. All concentrations have the unit (mol/L pore volume).

The stoichiometric coefficients depend on the pH of the solution. A suitable correlation is $\nu_i = a_i \ln(pH) + b_i$ where a_i and b_i are two adjustable constants.[3] In an aqueous electrolyte solution one can in many cases use the approximation that the logarithms of the asymmetric activity coefficients of proteins in solution are linear functions of the molar concentrations, that is

$$\ln\tilde{\gamma}_i = \alpha_i c_{salt} + \beta_i c_i \qquad \text{(X)}$$

where α_i and β_i are constants that are specific for the salt-protein pair, c_{salt} is the salt concentration and c_i is the protein concentration. The activity coefficients of proteins in salt solutions parallel the Hofmeister series as discussed in Ref. [4].

A5. The Maximum Capacity

The maximum available capacity of a single protein in IEC is theoretically speaking Λ/z_i, but due to steric hindrance and electrostatic exclusion one will often realise that it is of the order of magnitude 50% or less for large molecules.[9] Brooks and Cramer[18] suggested the correlation

$$q_j^{max} = \frac{\Lambda}{(\sigma_j + \nu_j)z_s} \qquad \text{(XI)}$$

where σ_i is an empirical steric hindrance factor.

A6. The Self-Association Isotherm-Hydrophobic Interactions

The association scheme for the association of the monomer is

$$P + \nu_i L \rightleftharpoons PL_{\nu_i} \tag{XII}$$

If only monomers associate with the ligands, all hydrophobic isotherms are convex and calculable from Eqs. (1) and (2). Thus, in view of Eqs. (1), (2), and (3), the conversion of the SAS ion-exchange isotherm, Eq. (IX), to a SAS hydrophobic interaction isotherm is straight-forward. The eventual result is

$$\frac{q_i}{c_i} = \widehat{K}_{H,i}\left(\frac{\Lambda}{c}\right)^{\nu_i}\left(1 - \sum_{j=1}^{m}\frac{q_j}{q_j^{max}}\right)^{\nu_i}\left(1 + 2\frac{\widehat{K}_{D,i}}{c}c_i\tilde{\gamma}_i\right)\tilde{\gamma}_i \tag{XIII}$$

where $\widehat{K}_{H,i}$ are the practical equilibrium constants for the association of the monomers with the ligands

$$\widehat{K}_{H,i} = K_{H,i}\frac{\hat{\gamma}_L^{\nu_i}\gamma_i^{\infty}}{\hat{\gamma}_i} \tag{XIV}$$

In this equation $K_{H,i}$ are the true equilibrium constants for the association scheme Eq. (XII), $\hat{\gamma}_L$ is the activity coefficient of the ligand, $\hat{\gamma}_i$ are the activity coefficients of monomer adsorbates, and finally γ_i^{∞} is the activity coefficient of solute *i* at infinite dilution in the picked reference solvent.

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