Mechanistic Modeling of Reversed-Phase Chromatography of Insulins with Potassium Chloride and Ethanol as Mobile-Phase Modulators

Karolina Arkell,*† Martin P. Breil,‡ Søren S. Frederiksen,§ and Bernt Nilsson‡

1Department of Chemical Engineering, Faculty of Engineering, Lund University, P.O. Box 124, SE-21100 Lund, Sweden
2Modelling and Optimization and §Mathematical Modelling, Novo Nordisk A/S, Smørøsevej 17-19, DK-2880 Bagsværd, Denmark

ABSTRACT: The purpose of this study was to investigate the adsorption mechanism in reversed-phase chromatography (RPC) of proteins and to develop a model for the effect of dual mobile phase modulators—a salt and an organic solvent—on this process. Two different adsorption mechanisms were considered: (1) pure association of a protein molecule and one or more ligands and (2) displacement of the organic modulator, with which the adsorbent is saturated, by the protein upon association with one or more ligands. One model was then derived from each of the two considered mechanisms, combining thermodynamic theories on salting-in, RPC, and the solubility of proteins. The model was then applied to chromatographic data from an earlier report as well as supplementary data for solubility and vapor–liquid equilibria, and case-specific simplifications were made. We found that an adaptation of Kirkwood’s electrostatic theories to hydrophobic interaction chromatography describes the observed effect of KCl well. Combining chromatographic and solubility data for one of the insulins, we concluded that the variation in the activity coefficient of the insulin with respect to the concentration of ethanol alone cannot describe its effect on retention. Consequently, one or more other phenomena must affect the adsorption process. Our second model fits the retention data well, supporting the hypothesis that ethanol is directly involved in the adsorption mechanism in this case. Using additional experiments at a high-protein load, we extended the linear-range equilibrium model into a dynamic model for preparative conditions. This model shows good agreement with the high-load data for one of the insulin variants, without any additional effects of the modulator concentrations on the adsorption capacity.

1. INTRODUCTION

Hydrophobicity is a physical property of a compound that denotes the degree to which the compound repels water. Accordingly, the aggregation of nonpolar molecules in an aqueous solution is caused by an effect termed the hydrophobic effect. Although the definitions of hydrophobicity and the hydrophobic effect are clear and simple, the phenomena per se—especially their quantification—are complex.† The current, long-standing consensus‡ is that water and its displacement or rearrangement play an important role in the hydrophobic effect. There are several theories on the extent of this rearrangement, spanning from the creation of quasi-crystalline icebergs§ to a decrease in the volume in which hydrogen bonding can occur.† From these theories on hydrophobicity and the hydrophobic effect, different models that describe and predict the related phenomena—for example, partitioning between phases, aggregation in aqueous solvents, and adsorption of solutes to ligands‡—have been developed.

The last of the mentioned phenomena forms the basis of separation in reversed-phase chromatography (RPC) and hydrophobic interaction chromatography (HIC). Both HIC and RPC are frequently applied for chromatographic purification of biomolecules, such as peptides and proteins, primarily in the biopharmaceutical industry.©,† Although preparative HIC and RPC are used extensively in the downstream processing of these products, there is no broad consensus on the nature of the underlying mechanisms nor on which theory best describes these processes.

Some of the most widely recognized theories on retention in HIC are the adaptations of the solvophobic### and preferential interaction#### theories to HIC, as well as that of Kirkwood’s electrostatic theories on macromolecules in solution by Mollerup and co-workers.##### The corresponding theories for RPC are the adaptation of the solvophobic theory to RPC;#### the partition model that was developed by Dill and co-workers##### and the adsorption model by Scott and Kucera.##### A number of proposed theories have combined adsorption and partition models.##### Most of the theories and models mentioned above have been developed mainly for small molecules and/or for the linear adsorption range and are thus
not generally applicable to preparative chromatography of proteins and peptides. Additionally, some of them include parameters that are difficult to measure or relate to physical properties.

Models that are based on mechanisms and physical principles, rather than fitted polynomials or quantitative structure–property relationships, enable the use of supplementary physical-property data from nonchromatographic experiments, more reliable extrapolation, and possibly predictions about similar adsorbate(s)–adsorbent–modulator(s) systems, for example, the effect of changing the adsorbent. In a previous study, we examined the combined effects of ethanol and KCl on the retention of three insulin variants on two RPC adsorbents. One of our main findings was that a change of adsorbents or in the KCl concentration only caused a parallel shift of the curve, describing the effect of ethanol on linear-range retention of the insulin variants. This suggests that, with a suitable model, the outcome of chromatographic experiments under varying mobile phase conditions and on different adsorbents can be predicted from a very limited number of experiments.

In this study, we derived two different chromatographic models and applied these to the two RPC systems. The models are based on the two most likely adsorption mechanisms—(1) the association between a protein molecule and a number of ligands, without direct involvement of the modulators and (2) the association between a protein molecule and a number of ligands, accompanied by the displacement of molecules of the organic modulator from those ligands. In addition to this possible direct effect of ethanol, it affects the activity coefficient of the protein in the mobile phase. The influence of the ethanol concentration on the activity coefficient was estimated from Wilson’s equation, using independent data for the vapor–liquid equilibrium (VLE) of water and ethanol and for the solubility of insulin.

The HIC model, based on Kirkwood’s electrostatic theories of macromolecules, by Mollerup et al. was used to describe the effect of the salt concentration on the protein activity. The main aim of this particular study is to develop a first principle model for RPC of proteins with dual modulators and to evaluate this model for the RPC data from our previous study. This also requires an investigation of what adsorption mechanism is most likely. The overall goal of this series of studies is to develop a model that can be used for the development, analysis, and optimization of RPC separations of proteins, employing dual-modulator gradients.

1.1. Theoretical Basis. The derivation of the correlation for the protein retention is based on the adsorption equilibrium. For RPC, however, we must first make an assumption regarding the nature of the retention mechanism. Because the highly hydrophobic ligands of RPC adsorbents can have the properties of a separate liquid phase, the retention might be due to partitioning into that phase rather than adsorption onto the ligands. It has proved to be difficult to distinguish between these phenomena, prompting theories that combine the two mechanisms to be developed.

However, proteins and other adsorbates that are several orders of magnitude larger than the ligands on the adsorbent cannot be submerged in a potential hydrophobic liquid phase unless their morphology changes drastically, such that their peptide chains are elongated and interlaced with the ligands. This morphological change would entail the denaturing of the proteins and peptides, which, of course, is undesirable. Another possibility is the formation of an interfacial region with a higher ethanol content, between the ligands and the bulk liquid, which the adsorbates partition into. Considering that the diameter of insulin, which is a small protein, is around 10% of that of the particle pores for the adsorbents used in this study, we find this alternative possible but less likely than adsorption. We have thus decided to assume that the retention of the insulin variants is governed by an adsorption mechanism.

1.1.1. Adsorption Mechanisms. Adsorption can be caused by two main different mechanisms—reversible association of the protein (P) and ligands (L) into the complex PL (eq 1) or the displacement of νL adsorbed modulator molecules (M) by the protein when it binds reversibly to ν ligands (L) and forms the complex PLν (eq 2).

\[ P + νL \leftrightarrow PL_ν \]  
\[ P + νM_νL \leftrightarrow PL_ν + νξM \]  

The process given by eq 2 is equivalent to the generally accepted mechanism for ion-exchange chromatography, often described by the steric mass action model by Brooks and Cramer, but here with the presumption of hydrophobic interactions modulated by an organic solvent instead of electrostatic interactions modulated by a salt. The species displaced by the protein (M) might also be water, which commonly constitutes the larger part of the mobile phase in protein RPC, but with the ligands being highly hydrophobic, adsorption of the organic solvent used as modulator is more likely. In the case of such a displacement process, the stationary phase is assumed to be saturated with the modulator. The equilibrium constant for the adsorption mechanism without (eq 1), Ks, and with displacement (eq 2), Kd, is given by eqs 3 and 4, respectively. Indices a and d denote association and displacement, respectively.

\[ K_a = \frac{a_{PL}}{a_{P}a_{L}} = \frac{x_{PL}γ_{PL}}{x_{P}x_{L}γ_{P}γ_{L}} = \frac{q_p}{ξ_{ref,m}} \frac{γ_{PL}}{γ_{P}γ_{L}} \Rightarrow \frac{q_p}{ξ_{ref}} \]  
\[ K_d = \frac{a_{PL}}{a_{P}a_{M}a_{L}} = \frac{x_{PL}x_{M}ξ_{ν}γ_{PL}γ_{M}γ_{L}}{x_{P}x_{M}x_{L}γ_{P}γ_{M}γ_{L}} = \frac{q_p}{ξ_{ref,m}} \frac{γ_{PL}γ_{M}γ_{L}}{γ_{P}γ_{M}γ_{L}} \Rightarrow \frac{q_p}{ξ_{ref}} \]  

where a is the activity, x is the molar fraction, and γ is the activity coefficient. \( q_p [\text{mol/m}^3] \) is the concentration of the protein in the mobile phase, whereas \( q_p [\text{mol/m}^3 \text{ pore volume}] \) is the concentration of the adsorbed protein in the stationary phase. Reference concentrations for the pores (\( ξ_{ref,m} \)) and the bulk mobile phase (\( ξ_{ref,s} \)) are needed because of the change from molar fractions to concentrations. In this study, however, the retention is assumed to be caused by adsorption and not by partitioning. This assumption implies that the stationary and mobile phases are not two separate phases in the thermodynamic sense. Consequently, \( ξ_{ref,m} \) equals \( ξ_{ref,s} \) enabling the simplification in the last step in eqs 3 and 4.

The molar fraction of free ligands, xL, can be calculated from eq 5, where Λ is the ligand density, cL is the total molarity of the mobile phase and N is the total number of adsorbates. \( σ_ι \) is
the shielding factor for adsorbate \(i\), that is, the number of ligands that are sterically hindered from binding to other protein molecules but are not included in the complex \(PL\), which constitutes the hindrance.

\[
x_L = \frac{\Lambda}{c_{tot}} \left( 1 - \sum_{k=1}^{N} \left( \frac{\nu_k + \sigma_l}{\Lambda} q_k \right) \right) \Rightarrow \lim_{q \to 0} x_L = \frac{\Lambda}{c_{tot}} \left( 1 - \sum_{k=1}^{N} \left( \frac{\nu_k + \sigma_l}{\Lambda} q_k \right) \right) \frac{\Lambda}{c_{tot}} \quad (5)
\]

For the linear adsorption range, the concentration of adsorbed proteins is negligible, and the simplification to the right in eq 5 can be used. The equilibrium expression in eq 2 postulates that all ligands are occupied—with either proteins or solvent molecules. This means that \(x_{ML}^i\) in eq 4 can be replaced by the expression for \(x_L\) (eq 5).

There is a possibility that the ligands are not fully saturated with the solvent, resulting in a combination of the two mechanisms in eqs 1 and 2. According to a study by Scott and Simpson, this is, however, unlikely at ethanol levels above 20 wt %.

In the RPC processes used by Novo Nordisk A/S, Bagsværd, Denmark, the ethanol content is 20–50 wt %, and this is likely the case for most of the RPC processes for the purification of proteins or peptides in which ethanol is used as a modulator. Additionally, the existence of two different types of adsorption sites would result in fronting peaks—a phenomenon that has not been observed in the experiments. Another possibility is that the protein molecules are adsorbed onto the layer of adsorbed solvent molecules. This would result in the same mechanism as in eq 1 but with another value of the equilibrium constant in eq 3.

1.1.2. Thermodynamic Retention Factor. In this study, the thermodynamic retention factor, \(A\), was used as a measure of protein retention. \(A\) is defined as the initial slope of the adsorption isotherm and is thus equal to the ratio between \(q_P\) and \(c_P\) at a very low protein load—that is, within the linear adsorption range. \(A\) can be determined experimentally from the retention volume under isocratic conditions and a very low protein load (eq 6).

\[
A \equiv \lim_{q \to 0} q_P/c_P = \frac{V_R - V_{NR}}{V_{pore\text{-}access}} = \frac{V_R - V_{col}(\epsilon_c + (1 - \epsilon_c)\epsilon_P k_D)}{V_{col}(1 - \epsilon_c)\epsilon_P k_D} \quad (6)
\]

where \(V_R\) is the retention volume of the protein, \(V_{NR}\) is its residence volume under nonretaining conditions, and \(V_{pore\text{-}access}\) is the particle pore volume accessible to it. \(\epsilon_c\) and \(\epsilon_P\) are the external and pore porosities, respectively, and \(V_{col}\) is the total column volume. \(k_D\) is the exclusion factor for the protein on the adsorbent in question—that is, the fraction of the particle pore volume of the adsorbent that is accessible to the protein.

Mollerup and co-workers\(^{12}\) have suggested and shown, for some cases, that the ratio of the activity coefficients of the free ligands \(\gamma_P\) and protein–ligand complexes \(\gamma_{PL}^i\) is reasonably constant, despite changes in the mobile phase composition. This assumption results in the linear-range expression for \(A\) given by eqs 7 and 8 for the adsorption mechanism without (eqs 1 and 3) and with displacement (eqs 2 and 4), respectively.

\[
A = K x_L, \quad A = A_{0} \frac{\Lambda}{c_{tot}} \gamma_P \Rightarrow \ln(A)
\]

\[
\ln(A_{0}) + \nu \ln \left( \frac{\Lambda}{c_{tot}} \right) + \ln(\gamma_P)
\]

\[
A = A_{0} \frac{\Lambda}{c_{tot}^{\nu}} \gamma_P \Rightarrow \ln(A)
\]

\[
\ln(A_{0}) + \nu \ln \left( \frac{\Lambda}{c_{tot}^{\nu}} \gamma_P \right)
\]

where \(A_0\) is the constant for a certain protein–adsorbent–modulator(s) system. For the mobile-phase compositions commonly used in HIC, the total molarity \(c_{tot}\) does not deviate significantly from that of pure water, and it is often considered to be a constant.\(^{12}\) This might, however, not be the case in RPC, and it is thus not certain that \(c_{tot}\) can be included in \(A_0\).

1.1.3. Effect of the Modulator Salt on the Protein Activity. Mollerup et al.\(^{12}\) chose to estimate the variation in \(\gamma_P\) from the salting-in \(\mu_{si}\) and salting-out \(\mu_{so}\) potentials according to Kirkwood’s theories on the electrostatics of macromolecules in solution. At low salt concentrations, the protein solubility increases as the salt concentration increases (salting-in), whereas the opposite pattern is observed at high salt concentrations (salting-out). The latter phenomenon is the basis for HIC, in which the protein retention increases with increasing salt concentration as the solubility of the protein in the mobile phase decreases. The effect of the modulator salt on the activity coefficient of the protein, within the linear adsorption range, is given by eqs 9–11.

\[
\ln(\gamma_{P,salt}) = \frac{\mu_{si}}{RT} + \frac{\mu_{so}}{RT} \quad (9)
\]

\[
\frac{\mu_{si}}{RT} = - \frac{3N_A}{32\pi \epsilon_P k T} \left[ \eta_P \left( 1 - \frac{\ln(1 + r_k k_D)}{r_k k_D} \right) + \left( \frac{\epsilon_P F^2}{k} \right) \sum_{k=1}^{N} \epsilon_k q_k r_k \left( \frac{\ln(1 + r_k k_D)}{r_k k_D} - \frac{1}{1 + r_k k_D} \right) \right] \quad (10)
\]

\[
\frac{\mu_{so}}{RT} = \frac{3}{2} k_T^2 \tau_p \phi_P \quad (11)
\]

where \(R\) is the ideal gas constant, \(T\) is the absolute temperature, \(F\) is Faraday’s number, and \(N_A\) is Avogadro’s number. \(\eta_P\) is the permittivity of the mobile phase, which depends on the concentration of the organic modulator and \(\tau_P\) is the charge of the protein at the specified pH. \(N\) is the number of types of proteins present. \(k\) is the inverse of the Debye length, which is directly proportional to the ionic strength of the mobile phase \((I)\) and inversely proportional to \(T\) and \(\epsilon_P\). \(\eta_P\), \(\tau_P\), and \(\phi_P\) are the protein- and salt-specific parameters, related to the dipole moment and size of the protein, and they are also functions of the concentration of the organic modulator. Details are found in the previously mentioned paper by Mollerup et al.\(^{12}\)

1.1.4. Effect of the Organic Modulator on the Protein Activity. The organic modulator can have several different effects on protein retention. In the case of adsorption of the organic modulator and displacement of it by the protein, \(\ln(A)\)
is linearly dependent on the natural logarithm of the concentration and activity coefficient of the modulator (eq 8). Wilson’s equation\(^{27}\) was chosen to describe the effect of the mobile phase composition on the activity coefficient of the organic modulator (eq 12).

\[
\ln(y^o_i) = -\ln(x_M + E_{MW}x_W) + x_W\left(\frac{E_{MW}}{x_M + E_{MW}x_W}\right)
- \frac{E_{W,M}}{x_W + E_{W,M}x_M} + 1
\]

(12)

The index \(W\) refers to water, and the parameters \(E_{MW}\) and \(E_{W,M}\) are the binary interaction parameters for the organic modulator and water. Possible effects of the protein are neglected because the concentration is generally very low—in this case, below 0.003 mol/L even under high-load conditions. To include the effect of the modulator salt, an additional term is needed, for example, a Debye–Hückel-like term. We have assumed that the main effect of the salt is on the activity coefficient of the protein, given by the salting-in and salting-out potentials (eq 9) and thus neglected a possible Debye–Hückel-like term for the activity coefficient of the organic modulator. The molar fractions \(x\) in eq 12 are thus determined on a salt-free basis, that is, they differ from those in the expression for the adsorption equilibrium.

The concentration of the organic modulator can also affect the activity coefficient of the protein \(\gamma_p\). This effect can be described by Wilson’s equation for the system, organic modulator—water—protein. At very low protein concentrations, infinite dilution can be assumed, and Wilson’s equation for this ternary system is simplified to (eq 13):

\[
\ln(y^o_{p,aq}) = -\ln(x_WE_{WP} + x_ME_{MP}) - \frac{x_WE_{WP}}{x_W + x_ME_{MW}}
- \frac{x_ME_{PM}}{x_M + x_WE_{WM}} + 1
\]

(13)

This version of the equation has the same number of parameters as the original one, but the removal of the dependence on \(x_p\) enables further simplification, accompanied by a parameter reduction (eq 14).

\[
\ln(y^o_{p,aq}) = \ln(\omega) + \ln(\varphi + x_M)
+ \frac{ax_M + \beta}{\chi x_M^2 + \delta x_M + E_{WM}}
\]

(14a)

\[
\omega = (E_{MP} - E_{WP})\exp\left(\frac{\theta}{\chi} - 1\right)
\]

(14b)

\[
\theta = E_{PW}(E_{WM} - 1) + E_{WM}(E_{MP} - 1)
\]

\[
\varphi = \frac{E_{WP}}{E_{MP} - E_{WP}}\alpha = E_{PW}(1 - 2E_{WM}) + E_{PM} - \frac{\theta}{\chi}
\]

(14c)

\[
\beta = E_{WM}\left(E_{PW} - \frac{\theta}{\chi}\right)
\]

(14d)

\[
\chi = (E_{MW} - 1)(1 - E_{WM})\delta = 1 + E_{WM}(E_{MW} - 2)
\]

1.1.5. Protein Solubility. Information on the variations in the activity coefficient of a protein in solution can also be extracted from solubility data. Mollerup et al.\(^{16}\) have previously shown the connection between solubility and protein retention in HIC, as well as the applicability of the same model structure to both phenomena. The dissolution of a solid protein \(P\) in an aqueous solution can be described by the reversible process in eq 15, and the equilibrium constant \(K_{sol}\) of this process is given by eq 16.

\[
P(s) ⇌ P(aq)
\]

(15)

\[
K_{sol} = \frac{dP_{aq}}{dP_s} = x_{P,aq}y_{P,aq} ⇒ \ln(x_{P,aq}) = \ln(K_{sol}) - \ln(y_{P,aq})
\]

(16)

The activity of a pure compound, such as the solid protein, is unity per definition, which means that the product of the molar fraction and activity coefficient of the dissolved protein is constant and equal to \(K_{sol}\) (eq 16). Combination of the expressions for the solubility equilibrium (eq 16) and the activity coefficient at infinite dilutions (eq 14) gives a model of how \(x_{P,aq}\) varies with the composition of the solution.

1.1.6. Dynamic Chromatography Models. The linear-range equilibrium models can predict the retention as a function of the mobile phase composition at low protein loads, but for a chromatography model to be a useful tool for design, tuning, and analysis of preparative chromatography processes, it must also be able to predict capacity effects and process dynamics. When the adsorption kinetics are assumed to be slower than the mass transfer of the protein from the bulk of the mobile phase to the pore surface, the reaction-dispersive model\(^{30}\) is often applied. The transport of adsorbate \(i\) inside of the column packed with adsorbent \(j\) is given by eq 17. Inherent assumptions are radial homogeneity of the column packing and spatial homogeneity of the porosities. The numerator in front of the adsorption term corresponds to the pore volume accessible to adsorbate \(i\), which was chosen as a basis for the adsorption capacity, and the denominator is the apparent total porosity of the column for adsorbate \(i\) (eq 18).

\[
\frac{\partial c_{i,j}}{\partial t} = D_{ax,i,j}\frac{\partial^2 c_{i,j}}{\partial z^2} - \frac{v_{sup}}{\epsilon_{i,j}}\frac{\partial c_{i,j}}{\partial z} - \frac{(1 - \epsilon_{i,j})e_{p,j}\kappa_{D,i,j}}{\epsilon_{i,j}}\frac{\partial q_{i,j}}{\partial t}
\]

(17)

\[
e_{i,j} = e_{i,j} + (1 - e_{i,j})e_{p,j}\kappa_{D,i,j}
\]

(18)

where \(t\) is the time from the process start and \(z\) is the axial position counting from the inlet of the column. \(D_{ax}\) is the apparent axial dispersion coefficient and \(v_{sup}\) is the superficial linear velocity of the mobile phase. The adsorption dynamics are given by eqs 19 and 20, where \(A_i\) describes the superficial linear velocity of the mobile phase.
\[
\frac{\partial q_{ij}}{\partial t} = k_{uni,ij} \left( A_{ij} (Y_{\text{Av,M}})^{v_{ij}} \right) \left( 1 - \sum_{k=1}^{N} \left( \frac{v_{k,j} + \sigma_{k,j}}{A_{j}} q_{ij} \right)^{v_{ij}} \right)
\]

where \( k_{uni,ij} \) is the kinetic constant for adsorption, \( A_{i} \) is the ligand density, and \( N \) is the number of adsorbate types.

2. EXPERIMENTAL SECTION

2.1. Chromatography Experiments. All experimental data used for the calibration of the linear-range equilibrium model were generated in a previous study, and because the experimental procedure used in this study is very similar to that in the previous one, only a brief description of the method is presented here, with an emphasis on the differences between the studies.

The chromatography system used in this study was an ÄKTApure 25 from GE Healthcare (Uppsala, Sweden) with a 50 mL superloop from the same supplier. For the experimental data taken from the previous study, the corresponding equipment was an ÄKTAXplorer 10 chromatography system and an A-900 autosampler, both from GE Healthcare. Three insulin variants (insulin aspart, desB30 insulin, and an insulin ester) were kindly provided by Novo Nordisk A/S (Bagsværd, Denmark). All chromatographic runs were isocratic, and to avoid capacity effects on retention, the protein load was kept below 0.05 g/L column in the first study. In this study, three different protein loads were investigated for each mobile phase combination: approximately 0.1, 1.2, and 12 g/L total column volume.

Two RPC adsorbents were used: one with C18 ligands and one with C4 ligands, both of which have a silica backbone and were obtained from Novo Nordisk Pharmatech A/S (Køge, Denmark). The pore diameters are within the range 100–300 Å. For the first study, the adsorbents were packed in Tricorn 10/100 columns from GE Healthcare at Novo Nordisk; and for this study, the adsorbents were packed in stainless steel columns by Dr. Maisch GmbH (Ammerbuch, Germany). Experiments were performed at varying concentrations of KCl and ethanol. All other experimental conditions, such as pH, temperature, and flow rate, were fixed throughout the studies.

2.2. Solubility Study. Approximately 1.2 g of crystallized desB30 insulin was added to 10 mL of aqueous solutions with 0.4 mol KCl/kg, 0.02 mol Tris/kg, pH 7.5, and 16 different ethanol levels in the range of 23.8–30.7 wt %.

2.3. Modeling. 2.3.1. Assumptions, Correlations, and Literature Data. An interstitial column porosity of 0.35 was assumed for both columns used for the high-load experiments, that is, the stainless steel columns. Using the total porosity values for each column, estimated from pulse experiments with NaNO₃, the particle porosity for column \( j \) was calculated per eq 18, assuming that \( k_D = 1 \) for the salt. The two C₄ columns were assumed to have the same particle porosity, and the C₁₈ and C₄ columns from our previous study were assumed to have the same interstitial column porosity. The three insulin variants were assumed to have the same exclusion factor, which was estimated for each column in pulse experiments with desB30 insulin under nonretaining conditions, and calculated per eq 18.

As in our previous study, the density correlation for the water–ethanol–KCl system of Galleguillos et al. has been used to calculate concentrations, molar volumes, and other quantities that depend on the density of the solution. Owing to the lack of available data, the permittivity of the mobile phase was assumed to be independent of the KCl concentration. A linear function of the water volume fraction of ethanol was fitted to the experimental data by Åkerlöf and Edsall and Wyman.

The combination of eqs 7, 9, 14a, and 16 gives the structure of the linear-range adsorption model for pure adsorption (eq 21),...
whereas the combination of eqs 8, 9, 14a, and 16 gives that for the displacement of the organic modulator by the protein (eq 22).

\[
\ln(A_d) = \ln(A_{0,d};\omega) + \nu \ln \left( \frac{A}{c_{tot}} \right) + \frac{\mu_1}{RT} + \frac{\mu}{RT}
- \ln(\phi + x_m) - \frac{\alpha x_m + \beta}{x_M^2 + \delta x_m + E_{W,M}}
\]

(21)

\[
\ln(A_d) = \ln(A_{0,d};\omega) + \nu \ln \left( \frac{A}{c_{tot}} \right) + \frac{\mu_1}{RT} + \frac{\mu}{RT}
- \ln(\phi + x_m) - \frac{\alpha x_m + \beta}{x_M^2 + \delta x_m + E_{W,M}}
\]

(22)

All simplifications made so far are based on the assumed adsorption mechanisms and on the properties of proteins and peptides in general. Starting from eqs 21 and 22, further case-specific simplifications can be made based on the experimental and literature data. The simplifications presented below can be applicable to other cases, but their applicability must be evaluated for each case.

3.1. Comparison of Ethanol Effect on Retention and Solubility. If the retention volume of the insulin variants varies with the ethanol content of the mobile phase mainly because of changes in the activity coefficient of the insulin variants, then the ethanol content should have the same effect on the solubility of insulin. This is seen from eqs 7 and 16, where the ethanol dependence is given by its effect on the activity coefficient of the protein. The logarithm of the retention and of the solubility should thus be parallel curves. As shown in Figure 1, this was not observed in the experiments.

Figure 1. Natural logarithm of the retention factor (A) of desB30 insulin for the experimental set at 0.4 mol KCl/kg and of the molar fraction of the dissolved desB30 insulin (x_{desB30}) from the solubility study, plotted against the molar fraction of ethanol.

The slope of the curves for the logarithm of the retention factor (ln(A)) is approximately three times steeper than that of the curve for the logarithm of the molar fraction of the dissolved insulin (−ln(x_{desB30})). This suggests that one or more additional phenomena are involved in the adsorption process, for example, the displacement of ethanol. Simultaneous calibration of simplified versions of the pure adsorption model (eq 21) and the solubility model (eqs 14a and 16) for desB30 insulin was attempted, but as suspected based on the results in Figure 1, a satisfactory fit for both retention and solubility data could not be obtained. Given these results, the pure adsorption model was not further studied, and we concluded that the likely mechanism in this case is the one involving modulator displacement (eq 2).

Other possible explanations are that (1) the assumption of constant ratio between the activity coefficients of the species in the stationary phase is invalid; (2) the concentration of desB30 insulin in the solubility study was too high to assume infinite dilution; or (3) other phenomena, such as changes in the protein conformation, occur during the adsorption process. The first and third possibilities are very difficult to explore and would require extensive investigation of the interactions on the surface of the adsorbent pores, which exceeds the scope of the current study.

The second possibility would be somewhat easier to investigate, but this explanation is less likely. At saturation and within the interval of ethanol concentrations studied, the molar fraction of insulin in solution is less than 10^{-4}, that is, less than 1% of x_{Einh}. Thus, the insulin concentration has an insignificant effect on the molar fractions of water and ethanol in the solution; the values of the binary interaction parameters E_{W,P}, E_{P,M}, E_{W,P}, and E_{P,W} would have to be very high if the concentration of insulin should have a significant effect on its activity coefficient.

Optimally, the question about the adsorption mechanism should be answered by searching for a change in the ethanol concentration during the adsorption and/or desorption. However, simulations with the model developed in this study (eq 26) showed that breakthrough experiments would only give a change of 0.2 percentage points, which is probably in the same order of magnitude as the precision of most measurement methods. A total protein load of 120 g/L would, according to the simulations, give a change of more than one percentage point. It might, however, still be difficult to measure. A number of attempts with an in-line refractive-index detector were made, but the insulin had such a high impact on the signal that the potential change due to ethanol adsorption or desorption was undetectable.

3.1.1. Case-Specific Model Simplifications. The retention and solubility data (Figure 1) suggest an almost linear dependence of ln(A_d) and especially ln(x_{Einh}) on the molar fraction of ethanol in the solution. As a consequence, the flexibility of Wilson’s equation needed to be restrained. The second last term of eqs 14a, 21, and 22, ln(\phi + x_m), will become a constant if \phi \gg x_m. If \phi \ll x_m the term will add a variation of approximately 0.25 to ln(A_d) and ln(x_{Einh}), which is rather small in this context. ln(\phi + x_m) was thus assumed to be constant and was combined with ln(A_d) and ln(\omega). Similarly, the denominator in the term including the unknowns \alpha and \beta only changes by approximately 6%. Consequently, \beta/(\chi x_m^2 + \delta x_m + E_{W,M}) was also included in the constants. An even smaller variation, potentially changing ln(A_d) by ±0.01, was calculated for x_m. This variable is per se not problematic because its value can be calculated if the density is known, but both \nu and A_0 cannot be estimated from the linear-range retention data. The assumption that x_m varies insignificantly enables the combination of the factor (\Lambda/c_0)^{\phi} with A_d.

The salting-out potential in the HIC model by Mollerup et al (eq 11) was omitted because the experimental data does exhibit a salting-out effect. The second term of the salting-in potential (eq 10) is proportional to the protein concentrations and was thus assumed to be negligible within the linear range, but it was
included in $A_d$ for the dynamic adsorption model (eq 19) because this should be valid for the whole adsorption range. Furthermore, the salting-in terms of eq 10 were simplified using two Taylor expansions, resulting in a linear dependence on $\kappa^2$ (eq 23) and on the protein concentrations (eq 24), respectively. This also enabled a parameter reduction by introducing the combined parameter $(\eta^2)_{0,P}$, which is correlated to $(\eta^2)_{0,P}$, according to eq 25. $\epsilon_{ratio}$ is the ratio between the permittivity in the cavity inside of the protein, which should be that of the free space ($\epsilon_0$) and that of the mobile phase ($\epsilon_D$). Further details on $\eta$, $\tau$, and $\epsilon_{ratio}$ can be found in ref 12. The approximations in eqs 23 and 24 are only valid for very small values of $\tau \kappa^2$.

$$\frac{3N_A \eta_P}{32 \pi \epsilon_D RT} \left( 1 - \frac{\ln(1 + \tau_k \kappa^2)}{\tau_k \kappa^2} \right) \approx \frac{3N_A}{64 \pi \epsilon_D RT} \kappa^2 (\eta^2)_P$$

(23)

$$\frac{3N_A}{32 \pi \epsilon_D RT} \left( \frac{z_F^p}{\epsilon_D RT} \right)^2 \sum_{k=1}^{N} c_k \eta_k \left( \frac{\ln(1 + \tau_k \kappa^2)}{\tau_k \kappa^2} - \frac{1}{1 + \tau_k \kappa^2} \right)$$

$$\approx \frac{3N_A}{64 \pi} \left( \frac{z_F^p}{\epsilon_D RT} \right)^2 \sum_{k=1}^{N} c_k (\eta^2)_k$$

(24)

$$(\eta^2)_P = (\eta^2)_{0,P} \frac{4}{(2 + \epsilon_{ratio})^2}$$

(25)

The final form of the models for protein adsorption involving modulator displacement (eq 26) and for protein solubility (eq 27) is found below.

$$\ln(A_d) = \ln(A_{d,0}) - \nu \xi \ln(x_M \delta_M) - \frac{3N_A \kappa^2}{64 \pi \epsilon_D RT} (\eta^2)_P$$

- $\frac{\alpha x_M}{\chi x_M^2 + \delta x_M + E_{W,M}}$ (26)

$$\ln(x_{P,M}) = \ln(\omega') + \frac{\alpha x_M}{\chi x_M^2 + \delta x_M + E_{W,M}}$$

(27)

### 3.2. Combined Calibration of Models for Linear-Range Adsorption and Protein Solubility

Before calibration of the adsorption and solubility models, the two binary interaction parameters for the water–ethanol system were fitted to a set of VLE data, giving $E_{M,W} = 0.7380$ and $E_{W,M} = 0.2532$. Simultaneous calibration of the adsorption model including modulator displacement (eq 26) and the solubility model (eq 27) against experimental data for desB30 insulin gave satisfactory results (Figure 2).

Because the difference between the three insulin variants is small, they should be equally sized and have similar surface properties. It was thus assumed that ethanol has the same effect on the activity coefficient for all three adsorbates, and thus that the same value of $\alpha$ could be used for all three adsorbates. The results from the calibration of the adsorption model (eq 26) against the retention data for insulin aspart and the insulin ester are shown in Figure 3.

The model fit of the insulin ester is comparable to that of desB30 insulin, whereas that of insulin aspart is less satisfactory, especially at low KCl concentrations. With the relatively small effect of the KCl concentration for this adsorbate, resulting in only a slight difference between the two series at 0.1 and 0.2 mol KCl/kg, this effect is rather difficult to model. The calibrated values of the final parameter set for eqs 26 and 27, used for the graphs in Figures 2 and 3, are given in Table 1, together with 95% confidence intervals for each parameter. The 95% confidence intervals confirm that all parameters are significant, and that the model is rather sensitive to the values of $\xi$, $\eta^2(x_M \delta_M)$, and $\alpha$ and less sensitive to $\ln(A_{d,0})$ and $\omega'$.

Because $\tau_0$ and $\eta_{0,P}$ were combined into the parameter $(\eta^2)_{0,P}$, an estimation of $\eta_{0,P}$ is needed to assess the validity of the simplifications (eqs 23 and 24). The criterion is that $\tau_0 \kappa^2$ is small. Assuming that $\eta_{0,P}$ for the insulin variants is close to that for lysozyme, $\tau_0 \kappa^2 \approx 10^{-10}$ and $\tau_k \kappa^2 \approx 0.01$, which should be small enough. The previously estimated molecular radius of insulin and the calibrated value of $(\eta^2)_{0,P}$ result in a dipole moment of 42 D, compared with the experimentally determined values of 360 D$^{17}$ and 72 D$^{38}$ for insulin in solution. Although our estimated value is outside of this range, it is much closer to the lower one than the two are to each other, reflecting the difficulty of obtaining a good estimate.

It is difficult to discuss the actual values of $\alpha$ because it is not directly linked to any physical property. However, the excellent agreement with experimental data for both solubility and retention (Figure 2) suggests that the simplified model for the effect of ethanol on the activity coefficient of the insulin
variants describes the phenomenon well, irrespective of the theoretical validity of the simplifications.

The introduction of the term for modulator displacement (second term in eq 26) means that, theoretically, a change in adsorbents does not only cause a change in the value of ln(A), that is not only a parallel shift of the curve for ln(A) as a function of the modulator concentration. However, the effect of the modulators on the activity coefficients of the adsorbates is not affected by the type of adsorbent, even though the hypothesis about a parallel shift between the adsorbents has been refuted. There are thus two parameter values to adjust for a new adsorbent, A0 and ν, which theoretically requires only two experiments at different concentrations of the organic modulator. If the ligand densities of the adsorbents differ significantly, adjustment of σ might also be needed.

3.3. Simulations of High-Load Adsorption. In the dynamic adsorption model (eq 19), there are three adsorption capacity parameters—σ, ν, and Λ. These are not found in the simplified version of the linear-range equilibrium model (eq 26) because their effect is observed only at high-protein loads. Because it is difficult to determine either one of them from supplementary data, at least one must be estimated from literature data. Unfortunately, information on the ligand density for the actual adsorbents used in this study is not available. Instead, typical ligand densities for the silica-based RPC adsorbents Kromasil49,50 manufactured by AkzoNobel N.V. (Amsterdam, The Netherlands), were used. A simple estimation, based on the molar weight of insulin, yields a molecular radius of approximately 1 nm. Assuming that the insulin molecules are spherical and can be reached by the ligands below their circular projection (3.14 nm2 = 3.14 × 10−18 m2), maximally seven ligands could interact with each insulin molecule. By assuming that σ = 7 − ν, the number of capacity parameters to calibrate was reduced by one.

The results from the calibration of the capacity and kinetic parameters for desB30 insulin on the C18 and C4 adsorbents are found in Figures 4 and 5, respectively. The protein load levels, 12 and 1.2 g/L column, are included, and all injection volumes have been subtracted. All but one of the experiments performed at 0.7 ml KCl/kg resulted in chromatograms with two peaks. These eluted 1–2 CV after the start of the elution step, suggesting that the retention was low enough to cause partial flowthrough. Consequently, these results were discarded.

The agreement between experiments and simulations is very good for the C4 adsorbent and rather good for the C18 adsorbent. Differences in the retention time occur for both adsorbents at low KCl concentrations, and the simulated peaks are generally sharper than the experimental ones for the C18 adsorbent. The differences in the peak shape might be due to the simplification of the mass transfer effects made using the reaction-dispersive model, in which the rate limitations in the stationary phase—pore diffusion, stagnant films, and adsorption kinetics—are lumped together in kkin. Such effects are not the focus of this study, and therefore, only a simple description of them has been applied.

The slight trend in discrepancy in retention is, however, the same for the experiments and simulations at lower loads, implying that this is not a capacity effect. As shown in Figure 3, the agreement is better for the C4 adsorbent.

Table 1. Parameter Values and the Corresponding 95% Confidence Intervals from the Simultaneous Calibration of the Adsorption Model Including Modulator Displacement (eq 26) and the Solubility Model (eq 27)

| system | ln(A0) (--) | ν (--) | (γff)'kkin × 10−6 (C/m) | σ (--) | α′ × 103 (--) |
|--------|-------------|-------|-------------------------|--------|--------------|
| C18    | −7.41 ± 0.82 | 19.1 ± 0.8 | 1.65 ± 0.08 |
| C4     | −9.32 ± 1.40 | 18.9 ± 1.3 |
| C18    | −7.86 ± 1.02 | 20.2 ± 0.7 | 2.19 ± 0.04 | 18.5 ± 1.0 | 1.96 ± 0.80 |
| C4     | −10.33 ± 1.24 | 20.2 ± 0.9 |
| C18    | −9.03 ± 0.55 | 21.9 ± 0.5 | 2.68 ± 0.06 |
| C4     | −12.72 ± 1.14 | 22.9 ± 1.0 |

“The binary interaction parameters of Wilson’s equation are E_{MN} = 0.7380 and E_{NM} = 0.2532.”

Figure 3. Results from the calibration of the adsorption model (eq 26) against linear-range retention data for (a) insulin aspart and (b) the insulin ester.
2a, the deviation between the experiments and the model response is more noticeable at lower retention volumes—a consequence of the higher impact of data points with high values on the calibration. The deviations in retention at higher loads are thus only a reflection of those observed for the linear range. The estimated values of the kinetic constant for the adsorption, the ligand density, and the stoichiometric coefficient between proteins and ligands are found in Table 2.

On the basis of previously estimated sizes of ligands and ethanol molecules, there is room for $18 - 101$ and $5 - 22$ ethanol molecules on each $C_{18}$ and $C_4$ ligand, respectively, depending on the orientation of the ethanol molecules. With such large ranges and the calibrated values being well inside of its limits, the estimated values of the stoichiometric coefficients and seem reasonable. It might seem strange with a higher ligand density for the $C_{18}$ adsorbent than for the $C_4$ one, but this is probably due to the former having a smaller pore diameter—and thus a larger surface area per volume. The data sheets from Kromasil also give an idea of the common size and range of ligand densities for RPC adsorbents, with $\sim 200$ mol/m$^3$ for a $300$ Å adsorbent$^{40}$ and $\sim 700$ mol/m$^3$ for a $100$ Å adsorbent.$^{39}$

The calibrated values of $\Lambda$ are slightly lower but still seem plausible.

It is, however, known that the strong correlation between $\Lambda$ and $\nu$ makes it difficult to calibrate them simultaneously. A higher value of one of them can, to a large extent, be compensated by a higher value of the other, and effects on the peak shape only become significant for very high values of the two parameters. Consequently, a reasonable integer value was chosen for the stoichiometric coefficient and the ligand density was subsequently tuned to maximize the similarity between the experimental and simulated chromatograms. The important conclusion that neither the salt nor the ethanol affects the adsorption capacity is nevertheless still valid.

**Table 2. Calibrated Values of the Ligand Density, the Stoichiometric Coefficient Between Ligands and Insulin, and the Kinetic Constant for Adsorption**

| adsorbent | $\Lambda$ (mol/m$^3$) | $\nu$ (—) | $k_{\text{kin}} \times 10^{-17}$ (s$^{-1}$) |
|-----------|----------------------|-----------|----------------------------------------|
| C$_{18}$  | 440                  | 3         | 2.9                                    |
| C$_4$     | 190                  | 3         | 3.9                                    |

$^a$The ligand density is based on the total column volume and refers to the number of moles of complete $C_{18}$ and $C_4$ ligands.

Figure 4. Results from the calibration of the dynamic model for desB30 insulin on the $C_{18}$ column at (a) 0.1 mol KCl/kg and (b) 0.4 mol KCl/kg. Two protein load levels, 12 and 1.2 g/L column, are included for each ethanol concentration. Solid and dashed lines represent experimental and simulated chromatograms, respectively.

Figure 5. Results from the calibration of the dynamic model for desB30 insulin on the $C_4$ column at (a) 0.1 mol KCl/kg and (b) 0.4 mol KCl/kg. Two protein load levels, 12 and 1.2 g/L column, are included for each ethanol concentration. Solid and dashed lines represent experimental and simulated chromatograms, respectively.

ACS Omega 2017, 2, 136–146

DOI: 10.1021/acsomega.6b00248
4. CONCLUSIONS

Two RPC models have been evaluated against the retention data for three insulin variants on two RPC adsorbents, as well as the solubility data for one of the insulin. The difference between the two models is the adsorption mechanism: one assumes a pure association between a protein molecule and a number of ligands, whereas the other assumes that the ligands initially are saturated with organic modulator and that a number of adsorbed modulator molecules are displaced upon the adsorption of an adsorbate molecule. The experiments were performed with dual modulators, KCl and ethanol, to evaluate the performance of models that combine the theories for the effect of salt and that of an organic modulator on protein retention.

Our main findings were that the effect of ethanol on the retention of desB30 insulin is stronger than that on the solubility of the same protein, and that there is no significant effect of the modulator concentrations on the adsorption capacity. The first observation means that the change in the activity coefficient of desB30 insulin, which results in a variation in solubility, cannot alone explain the effect of ethanol on retention. Consequently, one or more additional phenomena must be involved in the adsorption process. We believe that this additional phenomenon is the displacement of ethanol upon adsorption of the insulin, and we have shown that a model including ethanol displacement is in good agreement with the experimental data, both within the linear range and at high-protein loads.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: karolina.arkell@chemeng.lth.se. Phone: +46 46 222 82 90. Fax: +46 46 222 45 26 (K.A.).

ORCID
Karolina Arkell: 0000-0003-4069-1988

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Novo Nordisk A/S, the Swedish Foundation for Strategic Research (SSF), and the Process Industry Centre at Lund University (PIC-LU) are gratefully acknowledged for their financial support. The authors would also like to thank Anna-Margrethe Flarup, Novo Nordisk (Bagsværd, Denmark) for performing the experiments for the solubility study; as well as Jørgen Mollerup, PrepChrom (Klampenborg, Denmark), Ernst Broberg Hansen, Lars Sejergaard, Thomas Buddde Hansen, and Karol Lacki, Novo Nordisk (Bagsværd, Denmark) for useful comments and fruitful discussions.

■ REFERENCES

(1) Chandler, D. Interfaces and the driving force of hydrophobic assembly. Nature 2005, 437, 640–647.
(2) Snyder, P. W.; Lockett, M. R.; Moustakas, D. T.; Whitesides, G. M. Is it the shape of the cavity, or the shape of the water in the cavity? Eur. Phys. J. Spec. Top. 2014, 223, 853–891.
(3) Tabor, R. E.; Griesser, F.; Dugasini, R. R.; Chan, D. Y. C. The hydrophobic force: Measurements and methods. Phys. Chem. Chem. Phys. 2014, 16, 18065–18075.
(4) Kauzmann, W. Some factors in the interpretation of protein denaturation. Adv. Protein Chem. 1959, 14, 1–63.
(5) Stillinger, F. H. Structure in Aqueous Solutions of Nonpolar Solutes from the Standpoint of Scaled-Particle Theory. J. Solution Chem. 1973, 2, 141–158.
(6) Freitag, R. Chromatographic Techniques in the Downstream Processing of Proteins in Biotechnology. In Animal Cell Biotechnology—Methods in Molecular Biology, 3rd ed.; Pörtner, R., Ed.; Humana Press: New York, USA, 2014; Vol. 1104, pp 419–458.
(7) Junghauer, A. Chromatographic media for bioseparation. J. Chromatogr. A 2005, 1065, 3–12.
(8) Melander, W.; Horváth, C. Salt Effects on Hydrophobic Interactions in Precipitation and Chromatography of Proteins: An Interpretation of the Lyotropic Series. Arch. Biochem. Biophys. 1977, 183, 200–215.
(9) Arakawa, T. Thermodynamic analysis of the effect of concentrated salts on protein interaction with hydrophobic and polysaccharide columns. Arch. Biochem. Biophys. 1986, 248, 101–105.
(10) Perkins, T. W.; Mak, D. S.; Root, T. W.; Lightfoot, E. N. Protein retention in hydrophobic interaction chromatography: Modeling variation with buffer ionic strength and column hydrophobicity. J. Chromatogr. A 1997, 766, 1–14.
(11) Staby, A.; Mollerup, J. Solute retention of lysozyme in hydrophobic interaction perfusion chromatography. J. Chromatogr. A 1996, 734, 205–212.
(12) Mollerup, J. M.; Breil, M. P.; Vogelpolh, C.; Sadowski, G. Simultaneous correlation of hydrophobic interactions in HIC and protein solubility in aqueous salt solutions and mixed solvents. Fluid Phase Equilib. 2011, 301, 163–170.
(13) Horváth, C.; Melander, W.; Molnár, I. Solvophobic Interactions in Liquid Chromatography with Nonpolar Stationary Phases. J. Chromatogr. A 1976, 125, 129–156.
(14) Dill, K. A. The Mechanism of Solute Retention in Reversed-Phase Liquid Chromatography. J. Phys. Chem. 1987, 91, 1980–1988.
(15) Marquese, J. A.; Dill, K. A. Solute partitioning into chain molecule interphases: Monolayers, bilayer membranes, and micelles. J. Chem. Phys. 1986, 85, 434–444.
(16) Ying, P. T.; Dorsey, J. G.; Dill, K. A. Retention Mechanisms of Reversed-Phase Chromatography: Determination of Solute-Solvent Interaction Free Energies. Anal. Chem. 1989, 61, 2540–2546.
(17) Snyder, L. R.; Poppe, H. Mechanism of solute retention in liquid—solid chromatography and the role of the mobile phase in affecting separation. J. Chromatogr. A 1980, 184, 363–413.
(18) Scott, R. P. W.; Kucera, P. Solute interactions with the mobile and stationary phases in liquid—solid chromatography. J. Chromatogr. A 1975, 112, 425–442.
(19) Scott, R. P. W.; Kucera, P. Solute-solvent interactions on the surface of silica gel. J. Chromatogr. A 1978, 149, 93–110.
(20) Nikitas, P.; Pappa-Louis, A. Retention models for isotropic and gradient elution in reversed-phase liquid chromatography. J. Chromatogr. A 2009, 1216, 1737–1755.
(21) Johansson, K.; Frederiksen, S. S.; Degerman, M.; Breil, M. P.; Mollerup, J. M.; Nilsson, B. Combined effects of potassium chloride and ethanol as mobile phase modulators on hydrophobic interaction and reversed-phase chromatography of three insulin variants. J. Chromatogr. A 2015, 1381, 64–73.
(22) Wilson, G. M. Vapor-liquid equilibrium. XI. A new expression for the excess free energy of mixing. J. Am. Chem. Soc. 1964, 86, 127–130.
(23) Yamamoto, H.; Terano, T.; Nishi, Y.; Tokunaga, J. Vapour-Liquid Equilibria for Methanol + Ethanol + Calcium Chloride + Ammonium Iodide, and + Sodium Iodide at 298.15 K. J. Chem. Eng. Data 1995, 40, 472–477.
(24) Nikitas, P.; Pappa-Louis, A.; Agafiotou, P. Effect of the organic modifier concentration on the retention in reversed-phase liquid chromatography I. General semi-thermodynamic treatment for adsorption and partition mechanisms. J. Chromatogr. A 2002, 946, 9–32.
(25) Vailaya, A.; Horváth, C. Retention in reversed-phase chromatography: Partition or adsorption? J. Chromatogr. A 1998, 829, 1–27.
(26) Jaroniec, M.; Martire, D. E. A general model of liquid—solid chromatography with mixed mobile phases involving concurrent adsorption and partition effects. J. Chromatogr. A 1986, 351, 1–16.
(27) Brooks, C. A.; Cramer, S. M. Steric Mass-Action Ion Exchange: Displacement Profiles and Induced Salt Gradients. AIChE J. 1992, 38, 1969–1978.
(28) Scott, R. P. W.; Simpson, C. F. Solute—Solvent Interactions on the Surface of Reverse Phases. Interactive Characteristics of Some Short-Chain Aliphatic Moderators Having Different Functional Groups. Faraday Symp. Chem. Soc. 1980, 15, 69–82.
(29) Staby, A. Novo Nordisk, Bagsværd, Denmark. Personal communication, Aug 26, 2016.
(30) Schmidt-Traub, H. Preparative Chromatography of Fine Chemicals and Pharmaceutical Agents; Wiley-VCH: Weinheim, Germany, 2005.
(31) Galleguillos, H. R.; Taboada, M. E.; Graber, T. A.; Bolado, S. Compositions, Densities, and Refractive Indices of Potassium Chloride + Water + Sodium Chloride + Water Solutions at (298.15 and 313.15) K. J. Chem. Eng. Data 2003, 48, 405–410.
(32) Akerlof, G. Dielectric constants of some organic solvent—water mixtures at various temperatures. J. Am. Chem. Soc. 1932, 54, 4125–4139.
(33) Edsall, J. T.; Wyman, J. Biophysical Chemistry; Academic Press, Inc.: New York, USA, 1958; Vol. I.
(34) DIPPR Project 801 Design Institute for Physical Property Research; American Institute of Chemical Engineers, 2007.
(35) MATLAB Release 2015a; The MathWorks, Inc.: Natick, Massachusetts, USA, 2015.
(36) Jakobsson, N.; Karlsson, D.; Axelsson, J. P.; Zacchi, G.; Nilsson, B. Using computer simulation to assist in the robustness analysis of an ion-exchange chromatography step. J. Chromatogr. A 2005, 1063, 99–109.
(37) Takashima, S.; Asami, K. Calculation and Measurement of the Dipole Moment of Small Proteins: Use of Protein Data Base. Biopolymers 1993, 33, 59–68.
(38) Laogun, A. A.; Sheppard, R. J.; Grant, E. H. Dielectric properties of insulin in solution. Phys. Med. Biol. 1984, 29, 519–524.
(39) AkzoNobel. Kromasil 100 Å Data Sheet. 2014 (accessed Sept 17, 2014).
(40) AkzoNobel. Kromasil 300 Å Data Sheet. 2014 (accessed Sept 17, 2014).