Mechanistic Modeling of Reversed-Phase Chromatography of Insulins within the Temperature Range 10–40 °C

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ABSTRACT: In the many published theories on the retention in reversed-phase chromatography (RPC), the focus is generally on the effect of the concentration of the mobile phase modulator(s), although temperature is known to have a significant influence both on the retention and on the selectivity between the adsorbates. The aim of this study was to investigate and model the combined effects of the temperature and the modulator concentrations on RPC of three insulin variants. KCl and ethanol were used as mobile phase modulators, and the experiments were performed on two different adsorbents, with C18 and C2 ligands. The temperature dependence was investigated for the interval 10–40 °C and at two different concentrations of each modulator. The model is derived from the expression for the adsorption equilibrium, which assumes that ethanol is adsorbed to the ligands and displaced by the insulin molecules, similar to the displacement of counterions in the steric mass-action model for ion-exchange chromatography. A good model fit to the new linear-range retention data was achieved by only adding and calibrating three parameters for the temperature dependence of the equilibrium. We found that a lower temperature results in a longer retention time for all adsorbates, adsorbents, and modulator concentrations used in this study, indicating that the adsorption process is enthalpy-driven. A comparison of the different contributions to the temperature dependence revealed that the large contribution from the equilibrium constant is dampened by the significant contributions of the opposite sign from the changes in activity coefficients of insulins and ethanol. Neglect of these effects when comparing different adsorbents and modulators might yield incorrect conclusions because the equilibrium constant varies with both, whereas the activity coefficients should be independent of the adsorbent. As expected, the conditions that promote higher retention also give a higher selectivity between the adsorbates. Nonetheless, in relation to its effect on the retention, the influence of the KCl concentration on the selectivity was significantly stronger than that of the temperature or that of the ethanol concentration.

1. INTRODUCTION

As concluded for hydrophobic interaction chromatography (HIC) by Vailaya and Horváth 20 years ago and for reversed-phase chromatography (RPC) by Pappa-Louisi et al. 8 years ago, studies on the effects on retention have generally been focused on the mobile phase composition, that is, the concentration of salt and/or organic modulator. This is still true, with relatively few investigations of the effect of temperature on retention in these hydrophobicity-based chromatographic systems to be found in the literature, although it has been recognized as having an important influence on the resolution. Vailaya and Horváth present a thermodynamic analysis of the effect of temperature on retention in HIC and suggest how nonlinear van’t Hoff plots for a number of amino acids can be described by the changes in heat capacity, enthalpy, and entropy. By applying the solvophobic theory, they also relate the temperature effects on the retention of some hydrocarbons to physical properties such as surface tension and nonpolar surface area. Dias-Cabral et al. have performed a similar study regarding the effects of temperature and salt concentration on HIC of bovine serum albumin, in which nonlinear van’t Hoff plots were also observed. However, most studies of the effect of temperature on retention and selectivity in RPC seem to focus on small molecules. Hearn and co-workers have published a number of comprehensive studies of the thermodynamics behind the temperature dependence of the retention of polypeptides on RPC adsorbents using methanol or acetonitrile as a mobile phase modulator, but similar studies of proteins with ethanol as a modulator have not been found in the literature. The few studies of the influence of temperature on retention and selectivity in RPC that were found were performed with the abovementioned modulators. In all of the studies mentioned above, the effect of temperature on retention has been attributed to the changes in the equilibrium constant, which have been translated to variations in the changes in Gibbs free energy, enthalpy, and entropy. The values of these might have been over- or
underestimated because possible effects of temperature on the activity coefficients of the modulators and adsorbates have not been investigated. If the aim is to only investigate the temperature dependence of the retention, this is more or less irrelevant. However, if the combined effect of the temperature and modulator concentrations is to be modeled, discrimination between the influence on the equilibrium constant and that on the activity coefficients is important. The equilibrium constant is, per definition, unaffected by changes in concentrations, but it varies with the temperature, whereas the activity coefficients are both concentration- and temperature-dependent. Possible synergy effects on the activity coefficients will be missed if the influence of the temperature is neglected. Additionally, correct comparison of different systems requires separation of the effects on the equilibrium constant and those on the activity coefficients because the former is affected by changes of both adsorbents and modulators, whereas the latter depends only on the modulators. Consequently, we have chosen another approach.

This paper presents a continuation of our previous studies,17,18 in which the effects of dual modulators, ethanol and KCl, on RPC of three insulin variants were investigated and modeled. Here, we have studied the different temperature dependencies of the same process and expanded our model to account for these, within the temperature interval 10–40 °C. The study includes both linear-range and high-load data, and the model can be used for both equilibrium calculations and dynamic simulations. As in the previous study, independent data, for example, vapor–liquid equilibrium data for the water–ethanol system, have been used to separate different effects on the retention. The main aim was to investigate the combined effects of modulator concentrations and temperature on the retention of and selectivity between the three insulin variants as well as to develop a model that describes these effects well.

1.1. Theoretical Basis. The model used in this study is a new version of one that was presented in a previous study by the same authors.17 It has now been further developed to include the effect of temperature. The derivation is based on the assumption that the chromatographic retention is caused by adsorption, and not partitioning, of the substances to be separated.

1.1.1. Adsorption Equilibrium. The organic modulator is assumed to have a dual effect on the adsorption equilibrium: (1) The stationary phase is initially saturated with the organic modulator, which is displaced when the protein is adsorbed and, in turn, causes desorption of the protein by displacing it. Changes in the concentration of the modulator affect its activity coefficient and thereby the adsorption equilibrium. (2) Similarly, the activity coefficient of the protein changes with the modulator concentration. The adsorption mechanism is given by eq 1

\[ P + \nu M \leftrightarrow PL + \nu \xi M \]  

where P, M, and L denote the protein, the organic modulator, and the ligand, respectively. \( \nu \) is the stoichiometric coefficient between the ligand and the protein, that is, the number of ligands that bind to one protein molecule, and \( \xi \) is the number of organic-modulator molecules that bind to one ligand. The equilibrium constant \( K \) for the adsorption process described by eq 1 is given by the first part of eq 2. The second part defines the thermodynamic retention factor \( A \), which is a measure of retention and equal to the initial slope of the adsorption isotherm.

\[
K = \frac{q_P y_{PL}}{q_P y_P} \left( \frac{x_M^{\xi} y_M^{\xi}}{x_M y_M} \right)^\nu_c \Rightarrow A = \frac{q_P}{q_P} = K \left( \frac{x_M^{\xi} y_M^{\xi}}{x_M y_M} \right)^\nu_c
\]  

\[(2)\]

cp and \( q_P \) are the equilibrium concentrations of protein in the mobile and stationary phases, respectively. \( x \) is the molar fraction and \( y \) is the activity coefficient, both of the species indicated by the index. With the assumptions made in the previous study17—a constant ratio between the activity coefficients of the ligand complexes and constant total molarity \( (c_{\text{tot}}) \) of the mobile phase—the thermodynamic retention factor of adsorbate \( i \) on adsorbent \( j \) \( (A_{ij}) \) is given by eq 3.

\[
\ln(A_{ij}) = \ln(A_{ij}^0) + \ln(y(c_{\text{salt}})) + \ln(y(x_M)) - \nu_i \xi_j
\]  

\[(3)\]

The contribution of each term to the variation of \( \ln(A_{ij}) \) with temperature, for the calibrated model, is shown in Figure 2. Two of the terms describe the effects of the organic modulator—the last one, which accounts for the displacement of modulator molecules when the protein adsorbs, and the third one, which describes the effect of the concentration of the organic modulator in the mobile phase on the activity coefficient of the protein (eq 4).

\[
\ln(y(x_M)) = -\frac{\alpha x_M}{\xi x_M^2 + \theta x_M + E_{W,M}}
\]  

\[(4)\]

Equation 4 is a simplified version of Wilson’s equation19 for the water–ethanol—protein system, assuming infinite dilution of the protein. \( E_{W,M} \) is one of the binary interaction parameters for the water–ethanol system, whereas \( \alpha \), \( \xi \), and \( \theta \) are parameters derived from the corresponding parameters for the water–ethanol—desB30 insulin system. The temperature dependence of the binary interaction parameters is given by eq S, where \( v_i \) is the molar volume of species \( i \) and \( \Delta U_{ij} \) is a parameter that describes the effect of temperature on the binary parameter \( E_{ij} \).

\[
E_{ij} = \frac{v_j}{v_i} \exp\left(-\frac{-\Delta U_{ij}}{RT}\right)
\]  

\[(5)\]

The second term in eq 3 is a salting-in potential, which describes the effect of the modulator salt on the activity coefficient of adsorbate \( i \) and is given by eq 6.

\[
\ln(y(c_{\text{salt}})) = -\frac{3N_s}{64\pi\eta\epsilon_\nu RT} \left( \kappa z_i^2 \eta_i^2 + \frac{(z_i F)^2}{\epsilon_\nu RT} \sum_{j=1}^N c_j \psi_j \right)
\]  

\[(6)\]

\( N_s \) is Avogadro’s number, \( \epsilon_\nu \) is the permittivity of the mobile phase, \( R \) is the ideal gas constant, \( T \) is the absolute temperature, and \( F \) is Faraday’s number. \( z_i \) is the charge of adsorbate \( i \), and \( N \) is the number of adsorbates. \( \kappa \) is the inverse of the Debye length and is proportional to the ionic strength. At a high protein load, the effects of the charges on \( \kappa \) and of the dipole moments on the last term in eq 6 of the macroions are included, but these are neglected at a low load (as in the study by Mollerup et al.20). \( \psi_j \) denote \( (\eta_i^2) \), in our previous paper,17 is a parameter linked to the dipole moment and size of adsorbate \( i \). \( \eta_i \) varies with the mobile phase permittivity and thus with both ethanol content and temperature.

The first term in eq 3 is a lumped parameter that contains a number of parameters that are or are assumed to be constants, 

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for example, the ligand density, the ratio between the activity coefficients of the adsorbed species, and the equilibrium constant for the adsorption of adsorbate $i$ on adsorbent $j$ ($K_{ij}$). The temperature dependence of the equilibrium constant (eq 7) can be derived from that of the change in Gibbs free energy upon adsorption ($\Delta G_{ij}$).

$$
K_{ij} = \exp \left( \frac{-\Delta G_{ij}}{RT} \right) \Rightarrow \ln(K_{ij}) = \frac{-\Delta G_{ij}}{RT}
$$

If the changes in enthalpy ($\Delta H_{ij}$) and entropy ($\Delta S_{ij}$) upon adsorption vary insignificantly with temperature, $\ln(K_{ij})$ is linearly dependent on the inverse of the temperature. Possible effects of variations in the column pressure drop and effects of temperature on $\Delta H_{ij}$ and $\Delta S_{ij}$ can be included by expansion of $\Delta H_{ij}$ and insertion of the heat capacity correlations, respectively.

1.1.2. Dynamic Chromatography Model. For the dynamic simulations, the reaction-dispersive model was applied (eq 8).

$$
\frac{\partial q_i}{\partial t} = k_{\text{kin},ij} \left( \frac{A'_{i,j} \rho_i}{\rho_{\text{ads}} \gamma(S_M)} \right) \left( 1 - \sum_{k=1}^{N} \left( b_{i,j} + \sigma_{i,j} q_{k,j} \right) \right) c_{i,j} - \frac{q_i}{\lambda_j} (x_M M)^{\nu_{ij}} c_{ij}
$$

2. EXPERIMENTAL SECTION

2.1. Materials and Methods. The experiments in this study were performed in accordance with our two previous studies, but at other concentrations of KCl and ethanol and at varying temperatures. Thus, the method is only described shortly here, and the reader is referred to the two previous publications for details.

All experiments were performed on a ÄKTA pure 25 chromatography system from GE Healthcare (Uppsala, Sweden) with a U9-M UV monitor. Sample injection was performed with a 50 mL superloop from the same manufacturer and an ALIAS autosampler from Spark Holland BV (Emmen, The Netherlands) for the high-load and linear-range experiments, respectively. The columns were placed inside a ThermoSphere model TS-430 column oven (Phenomenex Inc., Torrance, CA, USA) and equipped with a 2 mL precolumn metal tubing, to ensure isothermal conditions. Two different RPC adsorbents (silica backbone) from Novo Nordisk Pharmatech A/S (Koge, Denmark) were used: one with C18 ligands and one with C4 ligands. Prepacked steel columns (inner diameter 10 mm and length 100 mm) with these adsorbents were purchased from Dr. Maisch HPLC GmbH (Ammerbuch-Entringen, Germany).

Three different human insulin variants (insulin aspart, desB30 insulin, and an insulin ester) were kindly provided by Novo Nordisk A/S (Bagsværd, Denmark). These were chosen because they are very similar in size, shape, and chemical composition—each has one modification compared to human insulin but still differ in pI and hydrophobicity (Table 1), and because they are industrially relevant.

| Adsorbate       | pI | Hydrophobicity (GRAVY) |
|-----------------|----|------------------------|
| Insulin aspart  | 4.8| 0.177                  |
| DesB30 insulin  | 5.3| 0.213                  |
| Insulin ester   | 5.3| 0.231                  |

All experiments were performed at isocratic and isothermal conditions, and the mobile phase flow rates were 3.0 and 1.0 mL/min for the linear-range and high-load experiments, respectively. The lower flow rate at a high load was due to the pressure restrictions for the superloop. A set point with respect to modulator concentrations was chosen at 0.4 mol KCl/kg and 28.4 and 25.6 wt % ethanol for the experiments on the C18 and C4 columns, respectively. Two other mobile phase compositions were used: (1) one with less KCl (0.1 mol/kg) and (2) one with less ethanol (27.5 and 24.7 wt % for the C18 and C4 columns, respectively). All other experimental
conditions remained unchanged. The pH of the elution buffers was 7.5.

All three insulin variants were used for the linear-range experiments, whereas only desB30 insulin was used for the high-load experiments. For the linear-range experiments, the total protein load was kept below 0.03 g/L column, whereas two different load levels were applied for each temperature in the high-load experiments: 12 and 1.2 g of desB30 insulin/L column. The linear-range experiments at the set point cover the temperature range 10–40 °C, with a step of 6 °C, whereas the additional linear-range and high-load experiments were performed at 16, 25, and 34 °C.

2.2. Modeling. 2.2.1. Assumptions, Correlations, and Literature Data. The interstitial, particle, and total porosities for the two columns used in this study were determined previously.17 The mobile phase density was estimated using the correlation by Galleguillos et al.22 for mixtures of water, ethanol, and KCl. This correlation does not include the effect of temperature, but parameters are given for 25 and 40 °C. On the basis of the slight difference in data for these two temperatures observed in that study,22 we used the two parameter sets and applied linear interpolation and extrapolation for the temperature intervals 25–40 and 10–25 °C, respectively.

Any effects of KCl on the permittivity of the mobile phase were assumed to be negligible, and the correlations for water–ethanol mixtures at different temperatures by Akerlof23 were applied. Linear interpolation was used for ethanol concentrations between the levels for which parameter values are given in the paper by Akerlof.23

Using the molar volumes of water (18.069 cm³/mol) and ethanol (58.620 cm³/mol) at 25 °C, ΔU_{W,MM} and ΔU_{W,WM} were estimated to 3670.3 and 487.2 J/(mol-K), respectively, from the vapor–liquid equilibrium data14 used in our previous paper.17 As in that paper, vapor pressures of pure components from the DIPPR 801 Database25 and the modified Raoult’s law, including activity coefficients, were used for the estimation.

2.2.2. Temperature Dependence of Model Terms. Apart from the temperature dependence of eq 6 and that of the Debye length, density, and permittivity of the mobile phase, temperature should not affect the salting-in tendency. A change in the conformation of the adsorbates due to the temperature variations could alter the dipole moment and size of the insulin molecules, but because no maximum in retention was observed, this is not likely to occur.13,16 Consequently, we have chosen to assume that these adsorbate properties are constant enough not to cause any significant variations in the value of the parameter ψ, with temperature.

α is a lumped parameter, including binary interaction parameters for the water–ethanol–insulin system, and should thus have a temperature dependence similar to that of those parameters. Consequently, a modified version of eq 5, using the value at 22 °C (T_{ref}) as a reference (α_{ref}), was applied for this parameter (eq 11).

$$\alpha = \alpha_{ref} \exp \left( \frac{\Delta U_{0,i}}{R} \left( \frac{1}{T_{ref}} - \frac{1}{T} \right) \right) \quad (11)$$

In analogy with the assumption of α having the same value for all three insulin variants in our previous study,17 the same assumption was made for the temperature-dependence parameter ΔU_{0,i}. The main temperature dependence of A_{0,i} (eq 7) should be due to that of K_p. Estimations using the change in molar volume when insulin is adsorbed to an RPC adsorbent14 revealed that the relatively low column pressure drops observed in this study (below 20 bar) would only affect ln(A) by 1% or less. Although the van’t Hoff plots (ln(A) vs 1/T) showed signs of slight curvature, the changes in enthalpy and entropy upon adsorption were assumed to be temperature-independent, and A_{0,i} was assumed to vary with temperature, as described by eq 12.

$$\ln(A_{0,i}) = \frac{-\Delta H'_{i,j}}{RT} + \frac{\Delta S'_{i,j}}{R} \quad (12)$$

ΔH'_{i,j} and ΔS'_{i,j} are lumped parameters because A_{0,i} might contain other effects of temperature than that on the adsorption equilibrium constant.

2.2.3. Calibration of a Linear-Range Equilibrium Model. The parameters of unknown value for the linear-range equilibrium model are ΔU_{0,i}, ΔH'_{i,j} and ΔS'_{i,j}. These three properties are the only parameters in the equilibrium model that were calibrated against the chromatographic data obtained in this study. As mentioned above, the density and permittivity of the mobile phase and the parameters for the temperature dependence of Wilson’s equation (ΔU_{W,MM} and ΔU_{W,WM}) were determined from literature data or correlations. All other parameters have the values that were determined in our previous study.17 ΔU_{0,i} describes the mobile phase properties and is thus adsorbent-independent but has also been assumed to be adsorbate-independent. The other two parameters are both adsorbent- and adsorbate-specific because they describe the interaction between adsorbate and adsorbent. With ΔU_{0,i} being a global parameter for the two adsorption systems studied, simultaneous calibration of the model for all adsorbates and adsorbents was required. The retention volumes were determined from the first moment of each peak, after chromatogram decomposition using the MATLAB function Peak Fitter from MATLAB Central. More details about the method can be found in one of our previous papers.18 The thermodynamic retention factor was calculated from the experimental results using eq 13, and calibration was performed with the MATLAB function lsqcurvefit. lsqcurvefit is a least-squares Gauss–Newton method for nonlinear curve fitting.

$$A_i = \frac{V_{R,i} - V_{NR,i}}{V_{col} \left( 1 - \varepsilon_i \right) } \quad (13)$$

V_{R,i} and V_{NR,i} are the retention and nonretained volumes for adsorbate i, respectively, and V_{col} is the total column volume.

2.2.4. Calibration of a High-Load Dynamic Model. The partial differential equation (eq 8) was converted to a set of ordinary differential equations (ODEs) by discretization, using the finite volume method, and the resulting ODEs were solved by the MATLAB function ode15s. For this study, a two-point backward approximation was used for the first-order derivative in the convection term and a three-point centered approximation was used for the second-order derivative in the dispersion term. The column axis was divided into 100 grid points.

The capacity parameters Λ_i and v_{ij} should not be affected by temperature unless the conformation of the insulin variants is drastically altered, which is assumed not to be. Because the viscosity of the mobile phase increases considerably with decreasing temperature, the mass transfer should be significantly slower at lower temperatures. For the reaction-dispersive model, the effects of mass transfer to and inside the adsorbent particles are lumped with the dispersion in D_{app} and with the
adsorption kinetics in $k_{\text{lim}}$. A change in the value of either of these two parameters affects the peak shape in the same way. As the dispersion is unaffected by the temperature, whereas $k_{\text{lim}}$ should increase exponentially with temperature, the influence of temperature on the peak shape was attributed to the latter. The same type of correlation as applied to $\alpha$ (eq 11) was used to model this effect, with the value calibrated for 22 °C as a reference. Manual calibration was applied; that is, the parameter values were iteratively adjusted after comparison between the simulated chromatograms and the experimental ones.

3. RESULTS AND DISCUSSION

3.1. Linear-Range Equilibrium. As shown in Figure 1, the retention of all three insulin variants increases with decreasing temperature, for all mobile phase compositions applied in this study. This temperature dependence is more or less the opposite of what was reported for another set of insulin variants on a C8 adsorbent, using acetonitrile as a mobile phase modulator, by Szabelski et al.14 Additionally, the van’t Hoff plots in that study were concave, whereas ours (not included) were slightly convex. This demonstrates the possibility to totally alter the adsorption properties by changing modulators. Similar effects on other adsorbates when changing from acetonitrile to methanol as an organic modulator have been reported from other studies.11,27 Unfortunately, no study of the temperature effect on the retention of insulin variants using ethanol as a mobile phase modulator was found in the literature. Although the van’t Hoff plots for our data are slightly convex, there are not any extrema, which supports the assumption that the conformation of the insulin variants does not change with temperature.11 This assumption is further supported by the lack of signs of peak splitting16,28 and the relatively smooth trends in selectivity16 shown in Figure 3.

Another interesting observation, which is not related to the temperature dependence but is clearly shown in Figure 1, is that the relative effects of the modulators change gradually with increasing hydrophobicity of the adsorbates. For the least retained adsorbate, insulin aspart (Figure 1a), the reductions of the concentration of KCl and ethanol, respectively, have approximately the same effect. For the most retained adsorbate, the insulin ester (Figure 1c), the reduction of the concentration of KCl has a considerably larger effect, and the immediately retained adsorbate, desB30 insulin (Figure 1b), shows an intermediary behavior.

There is a good agreement between model and reality (Figure 1), and the model describes the trends with temperature, modulator concentrations, adsorbates, and adsorbents very well. Some discrepancies were anticipated because the parameters for the effects of KCl and ethanol were calibrated against another set of experimental data, produced with other columns packed with the same adsorbents. The assumption of no conformational changes reduced the complexity of the salting-in potential (eq 6). As shown in Figure 1, the effect of the KCl concentration is very well-described by the simplified model, indicating that neither the size nor the dipole moment of the insulin variants is likely to change due to the conformational changes.

The benefit of using the values of $A$, and not $\ln(A)$, for the calibration of the model parameters is that the emphasis is on the longer retention times, where the precision is higher. This approach was chosen to make the calibration more robust with respect to experimental errors. The calibrated parameter values are found in Table 2.

As seen in both Figure 1 and Table 2, temperature has a larger effect on the retention on the C4 adsorbent, and the effect also increases concomitantly with the hydrophobicity of the adsorbates. This difference is, however, rather small, which is reflected by the similarities in parameter values for the two adsorbents. The confidence intervals confirm that all parameters are statistically significant, and the uncertainties for $\Delta H^\circ_{\text{U}}$ and $\Delta S^\circ_{\text{U}}$ (13–20%) are reasonable, especially considering the limited number of data points for each mobile phase composition. The uncertainty for $\Delta U^\circ_{\text{U}}$ (77%) is very high, probably because the temperature dependence of the activity coefficients of ethanol and of the insulin variants affect $\ln(A)$ in a similar way (Figure 2, yellow and purple markers). This could probably be partially redeemed by the use of

![Figure 1](image-url)
Table 2. Parameter Values and Corresponding 95% Confidence Intervals from the Simultaneous Calibration of the Adsorption Model (eq 3) for All Three Insulin Variants

| system       | $\Delta H_i$ [kJ/mol] | $\Delta S_i$ [J/(mol·K)] | $\Delta U_i$ [kJ/mol] |
|--------------|------------------------|--------------------------|-----------------------|
| insulin aspart |                        |                          |                       |
| C18          | $-120 \pm 20$          | $-463 \pm 72$            |                       |
| C4           | $-123 \pm 18$          | $-488 \pm 65$            |                       |
| desB30 insulin |                        |                          |                       |
| C18          | $-124 \pm 22$          | $-483 \pm 79$            | $-3.93 \pm 3.03$      |
| C4           | $-127 \pm 20$          | $-510 \pm 72$            |                       |
| insulin ester |                        |                          |                       |
| C18          | $-128 \pm 25$          | $-507 \pm 91$            |                       |
| C4           | $-135 \pm 23$          | $-557 \pm 84$            |                       |

Figure 2. Contribution to the variation in $\ln(A)$ with temperature from the temperature-dependent terms in eq 3 according to the calibrated model. The model responses for the C18 (filled markers) and C4 (open markers) adsorbents, respectively, at the set-point conditions are shown.

supplementary solubility data for insulin as a function of temperature, similar to the approach used in our previous study. However, attempts to attain such data were futile because no dissolved insulin could be detected at 25 °C, despite several reruns of experiments and analyses, and inconsistent observations were made at higher temperatures.

Because $\Delta H_i$ and $\Delta S_i$ are lumped parameters, it is not possible to draw any quantitative conclusions regarding the temperature dependence of $\Delta G_i$. It is, however, unlikely that the influence of temperature on any of the other lumped parameters is larger than that on the equilibrium constant. Consequently, the qualitative conclusion that the changes in both enthalpy and entropy are negative seems reasonable. This means that the last term on the right-hand side of eq 14 is positive for all (feasible) temperatures and increases concomitantly with the temperature. The lower the value of $\Delta G$, the stronger the adsorption; that is, the negative value of $\Delta H$ provides the driving force for the adsorption. The adsorption is thus enthalpy-driven, and the retention decreases with increasing temperature because the change in Gibbs free energy increases (eq 14).

$\Delta G = \Delta H - T \Delta S$  \hspace{1cm} (14)

The temperature dependence of the linear-range retention in chromatography is generally solely attributed to that of the adsorption equilibrium constant, the natural logarithm of which is proportional to the changes in Gibbs free energy and in turn to the changes in the enthalpy and entropy. This might, however, result in an over- or underestimation of the influence of temperature on the adsorption equilibrium because the values of the activity coefficients of the involved species (in this case, the insulin variants and ethanol) can vary significantly with temperature. It is important to separate the temperature effects on the equilibrium constant from those on the activity coefficients when the modulator concentrations are varied because the former is concentration-independent, whereas the latter are not. Additionally, comparison and simultaneous modeling of two or more systems require discrimination between the behavior related to the modulators and that related to the adsorbents. For these reasons, the variation with temperature of the different terms in eq 3, according to the model, was investigated (Figure 2).

Only the model responses for the set-point conditions, that is, 28.4 and 25.6 wt % ethanol for the C18 and C4 adsorbents, respectively, and 0.4 mol KCl/kg, are shown in Figure 3, but the results for the other mobile phase compositions applied in this study were very similar. The calculations reveal that the effect of temperature on $\ln(A)$, and thereby on $\Delta G$, is almost three times as large as that on $\ln(A)$. The salting-in term gives a positive and concave, but negligible, contribution to the temperature dependence of $\ln(A)$, whereas the contributions from the two terms describing the effects of the ethanol concentration (yellow and purple markers) are significant, approximately linear and negative.

3.2. Selectivity. Because of the interesting synergy effects of temperature, modulator concentrations, and type of adsorbent on the retention indicated in Figure 1, the selectivity between insulin aspart and desB30 insulin (Figure 3a) and that between desB30 insulin and the insulin ester (Figure 3b) were calculated.
Independently of the experimental conditions, the selectivity between both sets of adsorbates decreases with increasing temperature (Figure 3), with a possible exception for that between insulin aspart and desB30 insulin at 0.1 mol KCl/kg on the C4 adsorbent (Figure 3a). It seems, however, more plausible that the data point at 16 °C is an outlier. The C18 adsorbent has a considerably higher selectivity than the C4 adsorbent, for the same temperature and KCl concentration, and ethanol concentrations that give comparable retention times. A higher selectivity is achieved for lower concentrations of KCl and ethanol, which is consistent with our previous findings.18 Compared to the corresponding influence on the retention, the KCl concentration has a considerable effect on the selectivity, whereas that of ethanol only has a minor effect. It is obvious that the selectivity between insulin aspart and desB30 insulin is more sensitive to both the KCl concentration and the temperature, whereas that between desB30 insulin and the insulin ester is almost constant within the studied temperature interval.

3.3. High-Load Dynamics. Our hypothesis for the influence of temperature on the high-load experiments was that apart from the effect on the equilibrium, only the kinetics would be affected. However, the values of the ligand densities had to be reduced to 75 and 50% of the previously calibrated values17 for the C18 and C4 adsorbents, respectively, to achieve a reasonable fit. As shown in Figures 4−6, there is no trend in capacity effects with temperature, and the reason for the adjustment of Λ has nothing to do with the temperature. The likely explanations are a loss of ligands or reduced accessible surface area, caused by wear of the adsorbents. This might also explain the observed decrease in $A_0'$ at 22 °C, compared to the results from our previous study,17 which is the reason why the calibrated value from that study was not used as a reference.

Regarding the neglect of an effect of possible conformational changes on the stoichiometric coefficient $ν$, the occurrence of conformational changes could be the cause of the lack of fit at a high load, but this would also have a very large effect on the influence of the ethanol content on the retention of the insulins (the last term in eq 3). Because the equilibrium model describes the effect of ethanol very well (Figure 1), it is not likely that $ν$ changes with temperature, as a consequence of conformational changes.

The attempts to make $k_{\text{kin}}$ temperature-dependent were futile because it affects the height of the peaks to a much larger extent than it affects their roundness. A more advanced dynamic model, that is, a general-rate model, could give rounder peaks but would likely not give sharp enough fronts at higher temperatures. There is apparently some additional phenomenon that the model cannot capture, and further investigation of this is beyond the scope of this study.

Comparison of Figures 4−6 shows that the best fit for both adsorbents has been achieved at the set-point conditions. At the other two mobile phase compositions, the fit at the higher load (12 g/L column) is better for the C4 adsorbent than for the C18 one, whereas the opposite is true for the lower load (1.2 g/L column).

4. CONCLUSIONS
The combined effects of temperature and the concentrations of KCl and ethanol on the separation of three insulin variants on
C₁₈ and C₄ adsorbents were studied. Our equilibrium and dynamic models for dual modulators from a previous publication were successfully expanded to include the influence of temperature.

A decrease in the retention with increasing temperature for all tested combinations of adsorbates, adsorbents, and mobile phase compositions was observed. This suggests an enthalpy-driven adsorption process. The effect of temperature was stronger for the experimental series performed at lower ethanol concentration (0.4 mol KCl/kg and 27.5 or 24.7 wt % ethanol), using the adjusted values of $A$.

We also found that the increase in $\ln(A_0)$ with the inverse of the temperature is almost three times as fast as that of $\ln(A)$, which is reduced by the simultaneous decrease in the terms describing the ethanol effect. These results demonstrate the importance of accounting for the temperature dependence of the activity coefficients, and not only that of the equilibrium constant, to enable correct comparison of different adsorbents and modulators. Only the equilibrium constant changes with the adsorbent, but a change of modulators also affects the activity coefficients.

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**Notes**

The authors declare no competing financial interest.

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