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Prediction of protein retention at gradient elution conditions in ion-exchange chromatography

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Abstract

This work presents a prediction procedure for protein retention in ion-exchange chromatography, where two linear gradient experiments of different length give the protein retention time at other linear gradients. The procedure predicts the retention time of early and late eluting proteins with similar precision and predictions by extrapolation deviate ≈3% or less from the experimental retention times. By using the ionic strength, this procedure predicts protein retention times obtained with divalent ions in the eluent more accurately than a well-established procedure that uses the protein co-ion concentration.

Keywords: Retention prediction; Gradient elution; Proteins

1. Introduction

Ion-exchange chromatography (IEC) is an important technique for separation and purification of proteins. The major advantage of the technique is that it provides mild separation conditions that enable the proteins to maintain their conformation. The interaction between the protein and the ion-exchange column has a strong salt dependence, i.e., a small change in the ionic strength of the eluent will have a large effect on the retention of the protein. This demands high precision from the analytical equipment and makes the interaction demanding to model. The importance and the intriguing nature of this system has inspired many researchers to investigate the mechanism for protein retention in IEC.

In the published procedures for prediction of protein retention at gradient elution conditions in IEC [1–8], isocratic experiments are used to determine the salt dependence of the analytes. Thereafter, the gradient retention times are calculated from the established salt dependence using mathematical functions describing the gradient profile. Parente and Wetlaufer [5] published a procedure with the best fit between experimental and calculated retention times. The accuracy of their retention predictions was enabled by precise determination of the salt concentration of the eluents, the dwell volume and the void volume of the system. The work of Snyder and co-workers shows that the system is of the non-linear solvent strength (LSS) type [6,7]. Consequently, solute-specific correction factors are required to use the LSS model for predictions, thereby limiting the applicability of the LSS model.

To separate a protein mixture under isocratic conditions in IEC is not feasible since different proteins are likely to demand different eluent ionic strength to attain satisfactory capacity factors. Establishing the isocratic parameters of a protein is time-consuming, especially since the strong salt depen-

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dence of the capacity factor requires thorough system equilibration. In addition, optimal system performance requires regular column regeneration, and the sample amount available for purification is often limited. It would therefore be desirable to predict optimal gradient elution conditions directly from gradient experiments using as few experiments as possible as described for other types of solutes by Yamamoto et al. [9].

The advantage in using isocratic experiments as the basis of retention prediction lies in the constant eluent ionic strength throughout the system. In the gradient system, the ionic strength depends on both time and the position within the column. Keeping in mind the strong salt dependence of protein retention, care must be taken to analyze any source of error in the system. Otherwise, the ionic strength at the point where the proteins are eluted will not be determined accurately.

Snyder et al. [10,11] investigated fundamental sources of error in predicting retention times under gradient conditions. Schoenmakers et al. [12] categorized these as, variations in the gradient program and errors in the relation between the solute capacity factor and the eluent composition. In the first case, differences between the desired gradient program and the actual variation of the gradient with time could be minimized with a well-performing chromatographic system. Snyder et al. found that steeper gradients, lower flow-rates and higher pressures might increase this type of error. Accordingly, longer gradient programs should improve the linearity of the gradient. With the strong salt dependence of protein retention in this system, accurate prediction requires minimal deviation between the programmed and the actual gradient profile.

The second source of error, the accuracy in the relationship between the solute capacity factor and the eluent composition, originates from the accuracy of the isocratic model that the prediction procedure is based on. According to Schoenmakers et al. [13], prediction of retention times at gradient elution conditions only requires a reasonable description of the dependence between the solute capacity factor and the eluent composition. It should, however, be possible to gain precision if the prediction is based on a model with good fit between the theoretical and experimental salt dependence of the protein capacity factor. The procedures mentioned earlier [1–8] are all based on the stoichiometric displacement model [14]. The slab model [15,16] has shown to give better linear fit to isocratic retention data for proteins in IEC than the stoichiometric model. It is derived from fundamental physical theory and is based on the linearized Poisson–Boltzmann equation for two planar, charged surfaces with evenly distributed surface charges of opposite sign, divided by an electrolyte solution [17]. It relates the protein capacity factor at isocratic elution in IEC to the reciprocal square root of the ionic strength of the eluent whereas the stoichiometric displacement model only considers the concentration of the protein co-ions in the eluent.

Combining the fundamental gradient integral [18–20] with the slab model for isocratic elution conditions, this work presents a procedure to describe the salt dependence of protein retention for gradient elution in IEC. It can be used to predict protein retention times at linear gradient elution conditions in IEC based on two linear gradient experiments. The procedure is demonstrated through predictions of the retention times of carbonic anhydrase, conalbumin, ovalbumin and human albumin for a range of linear gradients using both interpolation and extrapolation. The predictions, in terms of accuracy and precision, of the presented procedure are compared to those based on the procedure of Parente and Wetlaufer [5].

2. Theory

For isocratic elution conditions in ion-exchange chromatography, the slab model [15,16] describes the ionic strength dependence of the protein capacity factor, k', as;

$$k' = \Phi e^{-\left(\frac{s}{\sqrt{I}}\right)} \tag{1}$$

where Φ is the phase ratio of the column, assumed to be constant for normal eluting conditions ($I = 50$–500 mM), I is the ionic strength of the eluent and s equals;

$$s = \frac{\sigma_p^2 A_p}{F(2\epsilon_0 \epsilon_r RT)^{\frac{1}{2}}} \tag{2}$$

where σ_p is the charge density of the protein, A_p is half the total protein surface area, F is Faraday's number, ϵ_0 is the vacuum permittivity, ϵ_r is the relative permittivity of water and R is the universal gas constant. Under isocratic conditions, the logarithmic form of Eq. (1) predicts a linear dependency between the protein capacity factor and the reciprocal square root of the eluent ionic strength. The validity of this relation has been verified by several plots of isocratic protein experiments in IEC [15,16].

For the case of a linear gradient, the fundamental gradient elution integral [18–20], becomes [21];

$$\frac{1}{b}\cdot\int_{a}^{a+b(t_R'-\tau)}\frac{d\varphi}{k'(\varphi)}=t_m-\frac{\tau}{k'(a)} \quad (3)$$

where $b=1/t_G$ is the gradient steepness; i.e., the inverse of the total gradient time, a is the initial eluent composition, φ is the volume fraction of the stronger eluent, τ is the instrumental dwell time of the eluent and $k'(\varphi)$ denotes the dependence of the capacity factor on the volume fraction of the stronger eluent. The upper boundary of the integral defines the eluent composition at the column outlet in the moment of solute elution. The constant $\tau/k'(a)$ accounts for solute migration prior to the moment when the solvent overtakes the solute. Through inserting Eq. (1) into Eq. (3) and substituting φ with I, the gradient integral can be written as;

$$\frac{t_G}{\Phi(I_B-I_0)}\cdot\int_{I_0}^{I_0+b(t_R'-\tau)}e^{-\left(\frac{s}{\sqrt{I}}\right)}dI=t_m-\frac{\tau}{k(I_0)} \quad (4)$$

where I_0 is the initial ionic strength and I_B is the ionic strength of the stronger eluent. The upper boundary of the integral defines the ionic strength at the column outlet at the moment of solute elution, which, henceforth will be referred to as I_m. Integration of Eq. (4), through substitution of variables; i.e., $x=s/\sqrt{I}$, gives;

$$\frac{t_G}{\Phi(I_B-I_0)}\left\{\left[e^{-\left(\frac{s}{\sqrt{I}}\right)}(I-s\sqrt{I})\right]_{I_0}^{I_m}-s^2\cdot\int_{s/\sqrt{I_0}}^{s/\sqrt{I_m}}\frac{e^{-x}}{x}dx\right\}=t_m-\frac{\tau}{k'(I_0)} \quad (5)$$

The exponential integral has the rational approximation [22]

$$\int_{s/\sqrt{I_0}}^{s/\sqrt{I_m}}\frac{e^{-x}}{x}dx=\left[\frac{e^{-x}}{x}\cdot\left(\frac{x^2+2.334733x+0.250621}{x^2+3.330657x+1.681534}+\in(x)\right)\right]_{s/\sqrt{I_0}}^{s/\sqrt{I_m}} \quad (6)$$

where the remainder

$$|\in(x)|<5\cdot10^{-5} \quad (7)$$

is negligible in comparison to the rational expression that has a numerical value close to unity. In IEC, the ionic strength interval where solute migration occurs is narrow. Therefore, solute migration at the initial eluent composition, I_0, is usually negligible unless the protein has a low net charge and elutes at very low eluent ionic strength. Consequently, the influence of the initial eluent ionic strength on the final value of the integral is very small; i.e., it can be neglected in all sections of Eq. (5) and the final expression for Eq. (4) becomes;

$$s^2\cdot\frac{t_G}{\Phi(I_B-I_0)}\cdot e^{-\left(\frac{s}{\sqrt{I}}\right)}\cdot\left[\frac{I_m}{s^2}-\frac{\sqrt{I_m}}{s}\left(1-\frac{(s^2/I_m)+2.334733(s/\sqrt{I_m})+0.250621}{(s^2/I_m)+3.330657(s/\sqrt{I_m})+1.681534}\right)\right]=t_m-\frac{\tau}{k(I_0)} \quad (8)$$

2.1. Prediction strategy

Apart from the primary experimental parameters; i.e., the total gradient time and the ionic strengths at the column outlet at solute elution, Eq. (8) contains two unknowns; s and Φ. In principle, these variables can be determined from isocratic experiments, see Eq. (1). Substitution removes the phase ratio by assuming that the phase ratio for each protein is constant in all the experiments performed. Hence, the combination of two linear gradient experiments with different total gradient time and the same initial eluent composition results in an equality with the s value as the only unknown;

$$\frac{t_{G1}}{(I_{B1}-I_{01})}\cdot\int_{I_{01}}^{I_{m1}} e^{-\left(\frac{s}{\sqrt{I_{m1}}}\right)}\mathrm{d}I = \frac{t_{G2}}{(I_{B2}-I_{02})}\cdot\int_{I_{02}}^{I_{m2}} e^{-\left(\frac{s}{\sqrt{I_{m2}}}\right)}\mathrm{d}I \quad (9)$$

The approximate solution of the integrals in the equality of Eq. (9) has the form defined in Eq. (8). The ionic strength difference between the two eluents; $I_B - I_0$, is eliminated when both the experiments in Eq. (9) are conducted with eluents with identical ionic strength. Iteration of the equality in Eq. (9), using the approximation of Eq. (8), gives a point of intersection that corresponds to an apparent *s* value valid for the two combined experiments. By using the obtained *s* value, the upper boundary of one of the integrals in Eq. (9) gives the retention time at any linear gradient program. The unknown retention time is found by inserting the desired gradient time and the obtained *s* value in Eq. (9) and setting it equal to the integral of any of the previously used experiments. Thereafter, iteration gives the elution ionic strength, and hence the protein retention time, that gives equality in Eq. (9).

3. Experimental

3.1. Materials

Salt-free carbonic anhydrase (bovine erythrocyte, No. C-7500, lot 88C-8025, type I conalbumin 98% (C-0755, lot 39C-8000), grade VI ovalbumin ~99% (A-2512, lot 39C-8055) and human albumin (A-8763, lot 55F-9326) from Sigma (St. Louis, MO, USA) were used without further purification. Water, prepared from a Milli-Q water cleaning system (Millipore, Bedford, MA, USA), ethanol amine >98% (Fluka, Buchs, Switzerland) and analytical-reagent grade grade sodium chloride (Merck, Darmstadt, Germany) were used in the preparation of the eluents.

3.2. Equipment

The high-performance liquid chromatography (HPLC) system employed consisted of an ÄKTA Purifier (Amersham Pharmacia Biotech, Uppsala, Sweden) equipped with an A900 autosampler, a 200-μl injection loop, UV and conductivity detection system and a Unicorn control system for system operation, data collection and analysis. Detection was performed with UV absorbance at 280 nm. The chromatographic column was a strong anion-exchange column, MonoQ, HR 5/5, 50×4.6 mm (Amersham Pharmacia Biotech). The packing support was 10 μm in diameter and the protein capacity was specified as in the range of 20–50 mg/column. The experiments were performed at an ambient temperature of 26°C. All calculations were performed by using MathCad 4.0 (MathSoft, Cambridge, MA, USA).

3.3. Buffers

The initial eluent was 20.1 m*M* ethanolamine where the pH value was adjusted with 6.04 *M* HCl to 9.23 using a glass electrode calibrated with reference buffers 4.00, 7.00 and 10.00 (Merck). The resulting initial ionic strength was 13.8 m*M*, as determined from the hydrochloric acid consumption. The final eluent (ionic strength of 514.0 m*M*) was prepared by weighing 1.072 mol sodium chloride into a 2-l volumetric flask followed by dilution with the initial eluent.

3.4. Sample preparation

The protein solutions were prepared to contain approximately 1.5 mg/ml dissolved in the initial eluent. All buffers were filtered through 0.22-μm Millipore filters, type G5, after preparation.

3.5. Gradient linearity test

The integrity of the solvent delivery system was tested by monitoring the conductivity for three different linear gradient programs with total gradient time of 10, 30 and 60 min from 100% initial to 100% final buffer. The procedure was performed with two different instrumental set-ups; the column was first replaced with a 39.6 μl polyether ether ketone (PEEK) tubing, thereafter, the column was installed. With correction for the dwell time, the

conductivity (measured immediately after UV detection) followed the programmed eluent composition when the column was excluded from the system. After the column was installed, only the steepest gradient program with a total gradient time of 10 min gave minor deviations from linearity at the beginning of the chromatogram. The gradient experiments were carried out within this tested interval; i.e., duplicate experiments were performed with the total gradient times of 10, 20, 30, 40, 50 and 60 min, respectively. All the retention times used in the calculations were an average of duplicate experiments.

3.6. Important instrumental parameters

The instrumental dwell volume was determined both from calculation of dead volume based on specifications in the system manual and by extrapolating the conductivity signal of the three gradient experiments to the baseline level. The measured dwell volumes fluctuated around the calculated dwell volume; 1.028 ml. The column void volume, 0.92 ml, was determined through the inflection point of the solvent perturbation signal at the solvent front. The flow was kept at 1 ml/min and was checked using a volumetric flask and a stopwatch.

4. Results and discussion

Table 1 shows the results for an initial evaluation of the accuracy of this prediction procedure. The results in Table 1 include the maximum expected variation of the gradient linearity since the predictions are based on the experiments with the largest difference in total gradient time (10 min and 60 min); the steepest gradient ($t_G = 10$ min) had the largest linearity error.

The retention times calculated by using the prediction procedure outlined in Section 2, agree well with the experimental retention times for the proteins carbonic anhydrase, conalbumin, ovalbumin and human albumin (Table 1). This excellent agreement is shown by the values of the percentage deviation between the predicted retention times and the corresponding experimental retention time. With one exception, the predicted retention times fall within about one percent of the experimental retention times. The over 3% deviation in the prediction of the retention time of carbonic anhydrase at $t_G = 20$ min is due to an unexpected difference between the duplicate experimental retention times; 3.82 and 4.00 min, respectively.

As the gradient time increases, the percentage deviation between the experimental and predicted retention time decreases in a similar manner for all the evaluated proteins. Table 1 includes the percentage deviations in the predicted retention time to facilitate comparisons with previous prediction work. It also includes the deviation in % B at elution in accordance with the recommendations of Snyder and Dolan [23]. They have determined that the intrinsic gradient variability of a well behaved gradient system in the absence of non-ideality is about 0.2% B. As can be seen in Table 1, the obtained deviations in % B at elution are in this region. The presented deviations correspond to a time interval of about 1 to 7 s for CA, 3 to 5 s for CO, 4 to 7 s for OV and 6 to 10 s for HA, respectively. These deviations are small compared to the width of each peak, which is demonstrated in Fig. 1 that shows the precision of the least accurate of the predictions; i.e., the $t_G = 20$ min experiment.

The validity of this prediction procedure is further illustrated by the fact that retention times can be predicted by extrapolation without an unacceptable loss in precision (Table 2). Table 2 shows that extrapolation from the two shortest experiments ($t_G = 10$ and 20 min) can predict the retention times in the longest experiment ($t_G = 60$ min) and vice versa. This extrapolation poses a critical test of the limits for this procedure since the combined experiments are close, i.e., very similar and susceptible to any errors in the input data. Furthermore, the extrapolation is performed over the widest possible range in gradient times and will therefore amplify any changes in the retention of the proteins due to linearity errors that vary with the total gradient time.

The deviations between the predicted and the experimental retention time are about 1 to 3%, except for carbonic anhydrase in the $t_G = 20$ min experiment where the two experimental retention times deviated. A comparison between the Tables 1 and 2 shows that extrapolation results in predictions with higher deviations than those based on interpola-

Table 1
Predicted (t_{calc}) versus the duplicate experimental retention times (t_{exp}) for carbonic anhydrase (CA), conalbumin (CO), ovalbumin (Ov) and human albumin (HA)[a]

Protein	t_G (min)	t_{calc} (min)	t_{exp} (min)	% dev (t)	dev (% B)
Carbonic anhydrase	20	4.03	3.82 4.00	3.06	0.60
	30	4.63	4.60 4.60	0.60	0.09
	40	5.15	5.12 5.13	0.56	0.07
	50	5.63	5.62 5.61	0.27	0.03
Conalbumin	20	8.25	8.17 8.18	0.94	0.39
	30	10.79	10.70 10.71	0.79	0.28
	40	13.19	13.10 13.12	0.64	0.21
	50	15.50	15.47 15.45	0.29	0.09
Ovalbumin	20	10.66	10.57 10.57	0.86	0.45
	30	14.31	14.21 14.19	0.78	0.37
	40	17.80	17.67 17.71	0.63	0.28
	50	21.18	21.13 21.09	0.33	0.14
Human albumin	20	13.24	13.09 13.11	1.08	0.71
	30	18.14	17.98 17.96	0.94	0.56
	40	22.86	22.68 22.73	0.67	0.38
	50	27.45	27.37 27.33	0.37	0.20

[a] The predictions are based on the s-value obtained from the average protein retention times in the experiments with total gradient time (t_G) of 10 and 60 min. The percentage deviation between the predicted and experimental retention times, % dev (t), and the absolute deviation in % B at elution, dev (% B), are calculated from the average of the experimental retention time.

tion. This might be due to the increase in the gradient linearity error at shorter gradient times and/or the possibility that the elution pattern of the proteins depends on the gradient steepness. Overall, the precision of both the interpolative ($\leq$1%) and the extrapolative (1–3%) predictions demonstrates the accuracy of the presented procedure. A comparison of the deviation in the predictions for early

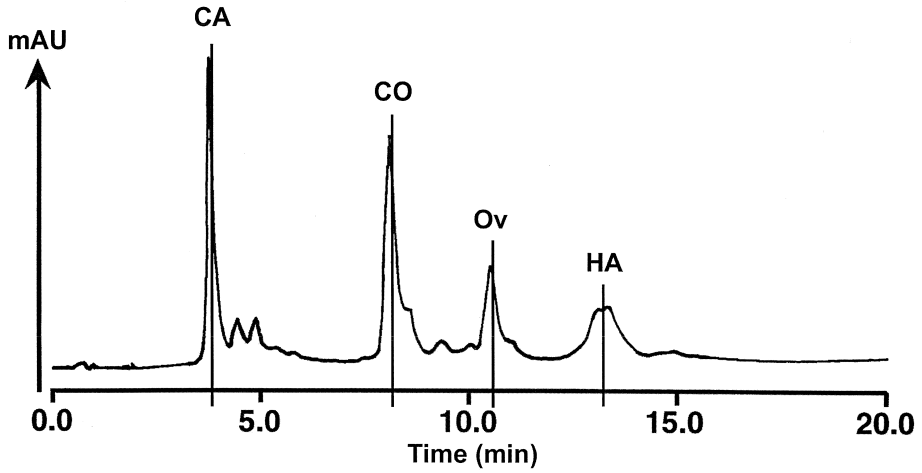

Fig. 1. The precision of the predicted retention time in the experiment with a total gradient time of 20 min from 100% initial (I=13.8 mM) to 100% final buffer (I=514.0 mM). The predicted retention times are indicated by a line. The predicted and experimental retention times as well as the abbreviations are shown in Table 1.

and late eluting proteins shows that the model predicts with similar precision for all the proteins regardless of the prediction method. For this data set, neglecting migration at the initial eluent composition gives no loss of precision in the predictions. Migration at the initial eluent ionic strength would increase

Table 2
Retention times predicted by extrapolation versus experimental retention times[a]

Protein	t_G (min)	t_{calc} (min)	t_{exp} (min)	% dev (t)	dev (% B)
Carbonic anhydrase	10	3.25	3.27 3.33	1.6	−1.0
	60	5.48	6.07 6.07	9.7	−0.5
Conalbumin	10	5.37	5.48 5.50	2.2	−0.8
	60	17.27	17.75 17.74	2.7	−1.2
Ovalbumin	10	6.57	6.72 6.77	2.6	−1.0
	60	23.89	24.49 24.47	2.4	−1.8
Human albumin	10	7.80	8.06 8.05	3.1	−1.5
	60	31.03	31.97 31.93	2.9	−2.5

[a] The calculations and the abbreviations are the same as in Table 1. The retention times in the 10-min experiment are predicted by using the retention times in the 50- and 60-min experiment. The 10- and 20-min experiments are used to predict the retention times in the 60-min experiment.

the deviation in the predicted retention times for the early eluting proteins. On the contrary, there is a slight trend towards higher deviations for later eluting proteins. This possible model error is under current investigation.

To demonstrate the potential advantages of this prediction procedure, it is useful to compare the method to the well-established procedure of Parente and Wetlaufer [5]. Both procedures use the fundamental gradient integral [18–20] to represent the gradient, but the procedures use different models for the salt dependence of protein retention in IEC. Parente and Wetlaufer base their prediction of protein retention times on the slope, m, and intercept, log K, of the stoichiometric log k' versus log $(1/c)$ plot obtained from isocratic experiments. Thus, apart from the difference in the fundamentals of the models, the main difference lies in the description of the eluent composition. Parente and Wetlaufer use the concentration of the protein co-ion, c, to describe the eluent composition whereas this presented procedure uses the ionic strength of the eluent.

By using the equations presented in the publication of Parente and Wetlaufer [5], it is possible to make retention time predictions for gradient experiments in a similar manner as with this presented procedure. The comparison of the two procedures is based on two different data sets; a selection of the data used in this presented study and a selection of the data used in the publication of Parente and Wetlaufer [5]. In the publication of Parente and Wetlaufer, the same experiments were performed at three different flow-rates, and the data obtained at 0.98 ml/min was selected for the comparison. The selection of data is made in order to demonstrate differences in the predictions made by the procedures and the omitted data gives prediction results similar to those presented. In the study of Parente and Wetlaufer, the proteins were eluted with calcium acetate. Since the eluent contains one divalent species, the ionic strength of the eluent is three times higher than the salt concentration. On the other hand, the monovalent salt used in this study gives an ionic strength equal to the salt concentration.

Table 3 summarizes the experimental and predicted retention times of eight proteins. The predictions are based on the presented procedure (slab) and the procedure of Parente and Wetlaufer (PW). The column labeled (dev_{PW}/dev_{slab}) shows the deviation of the retention time predicted by using the procedure of Parente and Wetlaufer divided by the corresponding value for the presented procedure. The data in Table 3 shows that both methods give reasonable prediction. However, as can be seen in the table, the deviation ratio increases considerably when a salt containing divalent ions is used as the eluting salt compared to when elution is performed with a monovalent salt. Thus, it is clearly seen that predictions based on the ionic strength are more accurate than predictions based on the eluent salt concentration. For the case of elution with a monovalent salt, the models predict with similar precision. However, the precision of the presented procedure, relative to that of Parente and Wetlaufer, tends to increase for the early eluting proteins; the deviation ratio is 2.6 for carbonic anhydrase compared to 1.2 for human albumin, respectively. It seems like the better linearity of the slab model, compared to that of the stoichiometric model, gives somewhat better precision in the predictions.

Table 3
A comparison between the predictions made by presented procedure (slab) and the procedure of Parente and Wetlaufer (PW)[a]

	Retention time (min)			dev_{PW}/dev_{slab}
	t_{exp}	t_{slab}	t_{PW}	
Divalent ion in eluent				
α-Chymotrypsinogen	16.34	16.33	16.17	12.8
Trypsin inhibitor	19.09	19.07	18.84	11.6
Cytochrome *c*	21.84	21.84	21.56	93.1
Lysozyme	25.81	25.81	25.46	117.4
Monovalent eluent salt				
Carbonic anhydrase	5.62	5.63	5.65	2.6
Conalbumin	15.46	15.50	15.53	1.5
Ovalbumin	21.11	21.18	21.20	1.3
Human albumin	27.35	27.45	27.47	1.2

[a] In the experimental data of Parente and Wetlaufer, the proteins were eluted on a cation-exchange column from 0.0196 *M* to 0.196 *M* salt where one ionic species is divalent. For these data, the experiments with total gradient time of 20 and 60 min were used to predict the protein retention time in the 40-min gradient experiment. The prediction of data obtained with monovalent salt is based on the protein retention times in the 50-min gradient experiment presented in this work, which are predicted using the experiments with a total gradient time of 40 and 60 min, respectively. The (dev_{PW}/dev_{slab}) ratio is obtained through dividing the deviation from the experimental retention time of each model.

5. Conclusion

The presented prediction procedure gives accurate predictions of protein retention times in a wide range of linear gradient experiments in IEC. The strength of the procedure is seen by the ability to predict retention times with only about 1 to 3% deviation between predicted and experimental retention times even by wide range extrapolations. With this working range, two initial experiments are sufficient to optimize the gradient steepness of a separation, since the model can predict retention times accurately both within, and outside the interval confined by the two initial experiments. Compared to the procedures based on stoichiometric models, the presented procedure gives slightly better predictions of protein retention times, especially in cases when multivalent ions are present in the eluent. Even though both models give quite accurate predictions in the presented case, the better precision of the presented procedure might be necessary in less forgiving calculations; such as the estimation of isocratic elution conditions based on gradient experiments.

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