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Optimal operation conditions for protein separation in hydrophobic interaction chromatography☆

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Abstract

Protein retention in hydrophobic interaction chromatography is determined by protein physicochemical properties and by system characteristics. In this paper we present an attempt to determine the optimal operation conditions that would allow the separation of binary protein mixtures. The statistically significant system variables were determined, and then empirical models were obtained which explained more than 92% of variability in dimensionless retention time based on salt properties, ionic strength of the initial eluent and substitution degree of the resin. These variables were optimized in order to achieve the maximum retention time difference between two proteins in a mixture. The optimum operation conditions as predicted by the models were tested experimentally, showing a good agreement with predicted separation. We concluded that it would be possible to determine the system conditions that allow the maximum separation of two proteins based on the main system properties. The methodology proposed here presents potential to be applied to partially characterized systems, however, it could be improved if protein's properties were included explicitly in the models.

Keywords: Hydrophobic interaction chromatography; Operation conditions; Optimization

1. Introduction

Hydrophobic interaction chromatography (HIC) is one of the key techniques used for protein purification and also largely used in industrial operations [1]. In HIC, proteins bind to a weakly hydrophobic stationary phase under moderately high concentration of a kosmotropic salt. Elution is achieved by decreasing the ionic strength in the mobile phase in a linear or a stepwise way [2–4]. HIC technique requires a minimum of sample pre treatment and thus can be used in combination with precipitation and other chromatographic techniques [5].

The factors that affect HIC performance can be divided into system characteristics and protein physicochemical properties. The main protein properties that affect protein retention in HIC are average hydrophobicity [5–8], surface hydrophobicity distribution [9,10], and protein size [2]. The main system characteristics are concentration and type of salt [3,11–18] and matrix properties [3,4,17,19,20]. Additionally, there are other system properties that can affect HIC performance to a lesser extent, such as pH of the mobile phase [15,18,21,22] and temperature [3,21,22,23].

Several efforts have been made towards elucidating the way these factors affect protein retention in HIC. Considering only a protein's average surface hydrophobicity it has been possible to obtain predictive models which have been shown to be valid for use in a purification process design [6,7]. Besides, considering the surface hydrophobicity distribution as the only design variable it has been possible to deduce empirical models that were able to predict a protein's retention time in HIC, under fixed operational conditions [9,10]. However, to use these models in process design it is necessary to know a priori the three-dimensional structure of the protein of interest and that of the main contaminants. Then the application of these models is limited to well characterized systems.

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On the other hand, many attempts have been made in order to understand and describe the way that operational conditions affect protein retention in HIC. Geng et al. [24] developed a stoichiometric displacement retention model which described isocratic retention of proteins in HIC considering the system's properties as independent variables. The model considered protein properties implicitly in the parameters.

In this work we focused on operational conditions because these factors can be manipulated, in contrast to protein properties, which are difficult to know when a purification process of a new system is being developed. Our aim was to propose a methodology to estimate optimal conditions which could be used with a partially characterized system.

2. Theory

2.1. Mobile phase effect

The mobile phase characteristics are defined mainly by ionic strength, type of salt, and pH. The first theoretical framework to describe the effect of salt on electrostatic and hydrophobic interactions was developed by Melader and Horváth [12]and Melader et al. [14], based on the Manning's counter-ion condensation theory for electrostatic interactions [25], and an adaptation of the Sinanoglu's solvophobic theory [26] to the salting-out of proteins and their retention in HIC. They proposed that the hydrophobic interaction is proportional to the surface tension increment of the salt used in the mobile phase and to its molal concentration. In a later study, it was found that specific salt–protein interactions and protein hydration also affect protein retention [15]. Based on the interaction between protein and salt the preferential interaction theory was developed [18,27,28], showing that selectivity reversals exist when different types of salt are used in the mobile phase.

2.2. Stationary phase effect

An increase in the chain length of an alkyl ligand increases the strength and specificity of the hydrophobic interaction [29]. However, resolution decreases when chain length is higher [5], thus the selectivity of an HIC resin can be manipulated by changing the ligand density [19]. Recently, Machold et al. [20] quantified the apparent hydrophobicity characteristic of different protein–resin systems and compared the performance of the sorbents. Base support chemistry, as well as type and density of the hydrophobic ligand, were found to affect selectivity of an HIC system. Based on a quantitative structure property relationship (QSPR) model, it was shown that selectivity of HIC systems is affected by ligand and/or base support chemistry [8]. Based on the preferential interaction theory [27], Xia et al. [18] reported that the total number of released water molecules is proportional to the total hydrophobic area of the resin, which in turn reflects the chemical properties of the resin.

We present an attempt to optimize the main operation conditions in an HIC system, based on an empirical model that explains a high percentage of variability on protein retention time. The models consider the operation conditions explicitly as independent variables and the properties inherent to proteins were considered implicitly in the parameters.

3. Experimental

3.1. Materials

Phenyl Sepharose 6FF (high substitution), Butyl Sepharose 4FF and Octyl Sepharose 4FF were donated by GE Healthcare (Uppsala, Sweden). Sodium chloride and ammonium sulfate were purchased form Sigma (St. Louis, MO), trisodium citrate dihydrate was purchased from Merk. α-amylase, thaumatin and α-chymotrypsinogen A were purchased from Sigma (St. Louis, MO). Water, prepared from a Milli-Q water cleaning system (Millipore, Bedford, MA) and Bis–Tris buffer was used in the preparation of the mobile phase.

3.2. Equipment

The high-performance liquid chromatography system employed, consisted of a FPLC (GE Healthcare, Uppsala, Sweden) equipped with a 100-μL injection loop. A 1-mL column (100 mm × 5 mm ID) was used to pack the chromatographic resins. The experiments were performed at room temperature (22 °C), using a flow rate equal to 0.75 mL/min and a 10 CV decreasing elution gradient. The column effluent was monitored at 280 nm and the retention volume of the proteins was recorded. The conductivity of the eluent was also monitored.

3.3. Sample preparation

Single protein solutions were prepared to contain approximately 2.0 mg/mL dissolved in the initial eluent. Solutions containing a mixture of two proteins were prepared to contain 2.0 mg/mL of each protein. All samples were filtered through 0.22-μm Millipore filters after preparation.

3.4. Buffer

The initial eluent was Bis–Tris 20 mM pH 7.0 plus a maximum salt concentration as shown in Table 1. The final eluent was Bis–Tris 20-mM pH 7.0 (Buffer A). All buffers were filtered through 0.22-μm Millipore filters after preparation and degassed with helium for 10 min.

3.5. Experimental conditions

Experiments were performed using different combinations of type of matrix (Butyl Sepharose, Octyl Sepharose or Phenyl Sepharose), salt type (ammonium sulphate, sodium citrate or sodium chloride) and salt concentration at the beginning of elution gradient (varying as shown in Table 1). The experimental conditions used in each experiment are shown in Table 1. Elution was achieved using a decreasing salt gradient, with a steepness of 7.5% B/min (a 10 CV gradient). All experiments were performed in duplicate.

Table 1
Experimental conditions and quantitative variables that represent each condition

Experiment number	Type of salt	σ_s[a] [$\times 10^3$ dyn g/cm mol]	Ionic strength	Salt concentration [M]	Type of matrix[b]	Ligand surface density[c] [μmol/mL gel]	Sorbent selectivity[d]
1	Sodium chloride	1.64	2.0	2.0	Butyl	50	6.10
2	Ammonium sulfate	2.16	1.5	0.5	Butyl	50	6.10
3	Sodium chloride	1.64	4.5	4.5	Butyl	50	6.10
4	Ammonium sulfate	2.16	4.5	1.5	Butyl	50	6.10
5	Sodium chloride	1.64	2.0	2.0	Phenyl	40	16.85
6	Ammonium sulfate	2.16	1.5	0.5	Phenyl	40	16.85
7	Sodium chloride	1.64	4.5	4.5	Phenyl	40	16.85
8	Ammonium sulfate	2.16	4.5	1.5	Phenyl	40	16.85
9	Sodium citrate	3.12	3.0	0.5	Octyl	50	0.20

[a] Molal surface tension increment of the salt, as reported by Melander and Horváth [12].
[b] Base support in all cases is Sepharose.
[c] Information proportioned by the manufacturer.
[d] As reported by Machold et al. [20], values correspond to the sum of squares for each sorbent.

3.6. Protein retention time

The chromatographic behaviour of proteins was represented by the parameter "dimensionless retention time" (DRT), and was calculated as follows [6,7,9,10,30]:

$$\mathrm{DRT} = \frac{\mathrm{RT} - t_0}{t_f - t_0} \quad (1)$$

where RT is the retention time of a protein (determined as the elution volume divided by the flow rate, in this case, 0.75 mL/min), t_0 is the time corresponding to the start of the elution gradient, and t_f is the time corresponding to the end of the elution gradient. If a protein is not retained by the resin, DRT is equal to zero, and if a protein elutes only after the gradient has been completed, its DRT is equal to one. The values of t_0 and t_f were obtained directly from the conductivity curve in the chromatogram.

3.7. Data analysis

The retention data of the three proteins under the different experimental conditions were related to the quantitative parameters that represent each condition (Table 1) using multiple regression analysis. The independent variables analyzed were molal surface tension increment of the salt (σ_s), ionic strength in the initial buffer, surface ligand density on the resin, and resin selectivity. The statistically significant variables were determined by ANOVA, and the best model for each protein was chosen, based on statistical criteria such as *r*-squared and *p*-value.

3.8. Optimization

The operation conditions that allow the best separation of two proteins were determined based on the statistical models obtained for each protein. Three protein binary mixtures were analyzed (thaumatin–α-amylase; thaumatin–α-chymotrypsinogen A, α-amylase–α-chymotrypsinogen A), and the model corresponding to each protein in the mixture was used. The optimal conditions were determined by maximizing the difference between DRT values predicted by the model corresponding to each protein in the mixture. The values of the independent variables were set within the experimental constraints, and the optimization process was subject to the intervals the model was fit. σ_s varied between 1.64 (which corresponds to sodium chloride) and 3.12 (corresponding to citrate); ionic strength varied between 1.5 and 4.5; ligand surface density on the resin ranged between 40 (Phenyl Sepharose) and 50 (Butyl Sepharose and Octyl Sepharose).

The Newton's method was used in the optimization, using progressive derivatives and linear estimation. 100 iterations were made for each estimation, with 5% tolerance and convergence of 0.0001.

Table 2
Statistical analysis of the independent variables' effect on dimensionless retention time showed by the three proteins studied

Variable	*p*-value		
	α-Chymotrypsinogen A	Thaumatin	α-Amylase
Ionic strength	0.0065	0.0137	0.0129
Resin selectivity	0.6692	0.9692	0.8855
σ_s	0.0501	0.1722	0.2660
Surface ligand density	0.2593	0.4189	0.3197

Table 3
Statistical analysis of the effect of the statistically significant variables on dimensionless retention time observed for the three proteins studied

Variable	*p*-value		
	α-Chymotrypsinogen A	Thaumatin	α-Amylase
Ionic strength	0.0023	0.0053	0.0093
σ_s	0.0055	0.0194	0.5689
Surface ligand density	0.0035	0.0021	0.0010

4. Results and discussion

4.1. Statistical analysis of retention data and model selection

The dimensionless retention time obtained in the different experimental runs was related to the operation conditions variables, for each of the three chosen proteins. A multiple regression analysis was carried out, considering that the independent variables are the ionic strength of initial buffer, molal surface tension increment of the salt (σ_s), surface ligand density on the resin and selectivity of the resin. The effect of each variable on DRT was analyzed statistically, and the results are shown in Table 2. In all cases, the highest *p*-value corresponds to the variable "resin selectivity", being greater than 0.1. This means that this variable is statistically insignificant at a 90% or higher confidence level. The statistical model obtained considering the four variables can then be simplified by removing the not significant variables, in this case, "resin selectivity". The analysis was repeated, but this time considering only the significant variables. The results are shown in Table 3. For the proteins α-chymotrypsinogen A and thaumatin, the variables, ionic strength, surface ligand density and σ_s were significant at a 99% confidence level, while for α-amylase, it still remained a insignificant variable corresponding to σ_s. In this case, σ_s could be removed from the model, but we decided not to remove it because of the importance of analyzing the effect of type of salt on protein retention. Besides, this situation occurred with only one protein, and to consider fewer variables in one case would make it difficult to compare the performance of the three models and to carry out the optimization.

The multiple regression analysis resulted in linear models that depend on three independent variables and four parameters or coefficients, showing the form:

$$\mathrm{DRT} = A + B\mathrm{Is} + C\sigma_s + D\mathrm{Ld} \quad (2)$$

Where *A*, *B*, *C* and *D* are the model coefficients, Is is the ionic strength of the initial buffer, σ_s is the surface tension increment of the salt, and Ld is the surface ligand density on the hydrophobic resin. The protein hydrophobicity is considered implicitly in the parameters; it was not considered explicitly as an additional variable because it is a property inherent of proteins and thus it can not be manipulated for the optimization. The coefficients obtained for each protein are given in Table 4, as well as the statistics of each model.

The parameters in the three models were in the same order of magnitude, however a sign change was observed in the parameter C for α-amylase with respect to the other proteins. This is probably due to the different net charge of α-amylase, which has a p*I* equal to 6.0, while thaumatin and chymotrypsinogen A have a p*I* of 9.7 approximately. Then, we can suppose that salt interacts in a different way with α-amylase, which explains the different sign of parameter *C*, which accompanies σ_s in the models.

In all cases, the *p*-value of the model was less than 0.01, what means that a statistically significant relation exists between the independent variables at a 99% confidence level. Besides, the *r*-squared was greater than 92% in all cases, and so the models are able to at least explain 92% of the variability in DRT, considering the three operational variables Is, σ_s and Ld. These results are showing that the main operation conditions that determine protein retention in HIC are ionic strength of the initial buffer, the salt properties and the surface ligand density on the hydrophobic resin. If these three variables determine maximum protein retention time in HIC, then interest should be focused on the optimization of these main variables, in order to design the optimal separation process. Additionally, we can conclude that ionic strength is a more suitable design variable than salt concentration, because it considers the effect of charge. A multiple regression analysis was carried out considering salt concentration instead of ionic strength, but the results were not satisfactory (data not shown). These models explained only 80% of the vari-

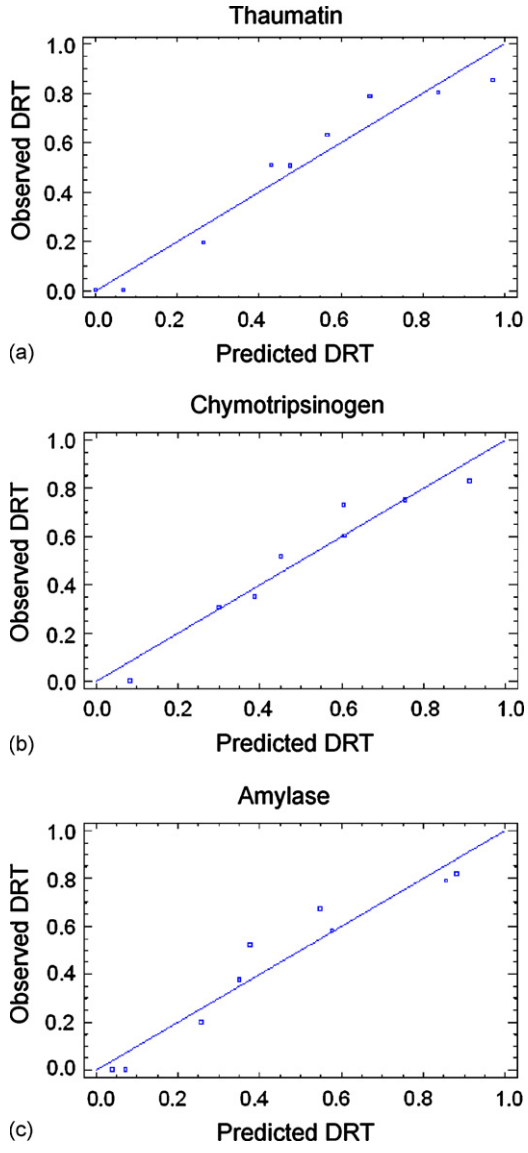

Fig. 1. Comparison of experimental and predicted dimensionless retention time (DRT) of the proteins thaumatin (a), α-chymotrypsinogen A (b) and α-amylase (c), using the statistical models shown in Table 4.

Table 4
Statistical parameters and coefficients of the models obtained for the three proteins

Protein	Model parameters				*p*-value	*r*-squared (%)
	A	*B*	*C*	*D*		
α-Chymotrypsinogen A	2.0673	0.1229	−0.3005	−0.0304	0.0009	95.40
Thaumatin	2.4740	0.1205	−0.2593	−0.0405	0.0014	94.46
α-Amylase	2.2963	0.1119	0.0496	−0.0506	0.0027	92.86

The general form of the models is $DRT = A + BIs + C\sigma_s + DLd$, where *A*, *B*, *C*, *D* are the model coefficients, Is is the ionic strength of the initial buffer, σ_s is the surface tension increment of the salt, and Ld is the surface ligand density on the hydrophobic resin.

ability in DRT, and the *p*-values indicated that the variables were related at a 95% confidence level.

On the other hand, σ_s represented very well the salt properties in a quantitative way. Surface ligand density on the resin appeared also as a good parameter, but it has to be noted that the range we studied was relatively narrow (it fluctuated between 40 [μmol/mL gel] and 50 [μmol/mL gel]).

Resin selectivity resulted as statistically insignificant, which is in contradiction with literature [20]. Probably the parameter used to represent this variable was not adequate; another way to quantify it should be investigated. Besides, the chromatographic resins used have the same base support, and so differences in selectivity may not be of great importance.

Fig. 1 shows the correlation between observed and predicted DRT for thaumatin (a), α-chymotrypsinogen A (b) and α-amylase (c). A very good agreement can be observed; the models describe extremely well, the chromatographic retention of the proteins. Then, these models could be used to predict protein retention in HIC under certain operation conditions. Additionally, the models could be used to determine the conditions that produce the maximum difference in DRT of two proteins.

4.2. Optimization of the operation conditions

The models obtained previously were used to determine the operation conditions (ionic strength, type of salt and resin) that produce the maximum difference between the retention of two proteins. Using the Newton's method, the values of the model variables were determined, in order to give the highest DRT difference between two proteins. The conditions that would theoretically allow the best separation of two proteins in a mixture were determined for the combinations α-chymotrypsinogen A–thaumatin, α-chymotrypsinogen A–α-amylase, and α-amylase–thaumatin. After that, the conditions predicted by the optimization were tested experimentally with the protein mixtures. The results were compared with the separation obtained under nonoptimal conditions, in order to test if the conditions predicted as optimal were really the best ones.

Table 5
DRT difference predicted by the models under the optimum operation conditions, in comparison with experimental values

Mixture components	Predicted separation[a]	Experimental separation[a]	Deviation (%)[b]
Thaumatin α–amylase	0.215	0.255	15.7
α-Chymotrypsinogen A–amylase	0.257	0.242	6.2
Thaumatin α–Chymotrypsinogen A	0.041	0.000	–

[a] Separation was quantified as the difference between DTR of proteins.
[b] Deviation $= \frac{|DRT_{experimental} - DRT_{prediced}|}{DRT_{experimental}} \times 100$

The experimental conditions that would result in the best separation as predicted by the optimization were ionic strength equal to 4.5; σs equal to 1.64 (which corresponds to sodium chloride) and ligand density equal to 50 (corresponding to Butyl Sepharose). In Table 5 the experimental separation between two proteins is compared to the optimal separation as predicted by the models. The chromatograms of the protein mixtures are shown in Fig. 2.

Using the optimal experimental conditions, the separation of two mixtures was reached: thaumatin–α-amylase and α-chymotrypsinogen A–α-amylase. The predicted separation, measured as the difference between DRTs, showed a relatively

Table 6
Separation of the binary mixtures under different experimental conditions

Experimental conditions[a]	Thaumatin–α-chymotrypsinogen A			Thaumatin–α-amylase			α-Chymotrypsinogen A–α-amylase		
	DRT 1	DRT 2	Separation[b]	DRT 1	DRT 2	Separation[b]	DRT 1	DRT 2	Separation[b]
2	**0.622**	**0.622**	**0.000**	**0.623**	**0.368**	**0.255**	**0.331**	**0.573**	**0.242**
3	0.727	0.727	0.000	0.787	0.569	0.218	0.619	0.619	0.000
4	0.278	0.278	0.000	***0.213***	***0.000***	***0.213***	***0.000***	***0.271***	***0.271***
5	0.758	0.758	0.000	0.810	0.810	0.000	0.784	0.784	0.000
6	0.524	0.524	0.000	0.527	0.527	0.000	0.499	0.499	0.000
7	0.446	0.446	0.000	0.000	0.000	0.000	0.605	0.431	0.174
8	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

The best operation conditions are presented in bold. Cases where one of the protein was not retained are presented in italics.
[a] As shown in Table 1.
[b] Separation was measured as the difference between both DRTs.

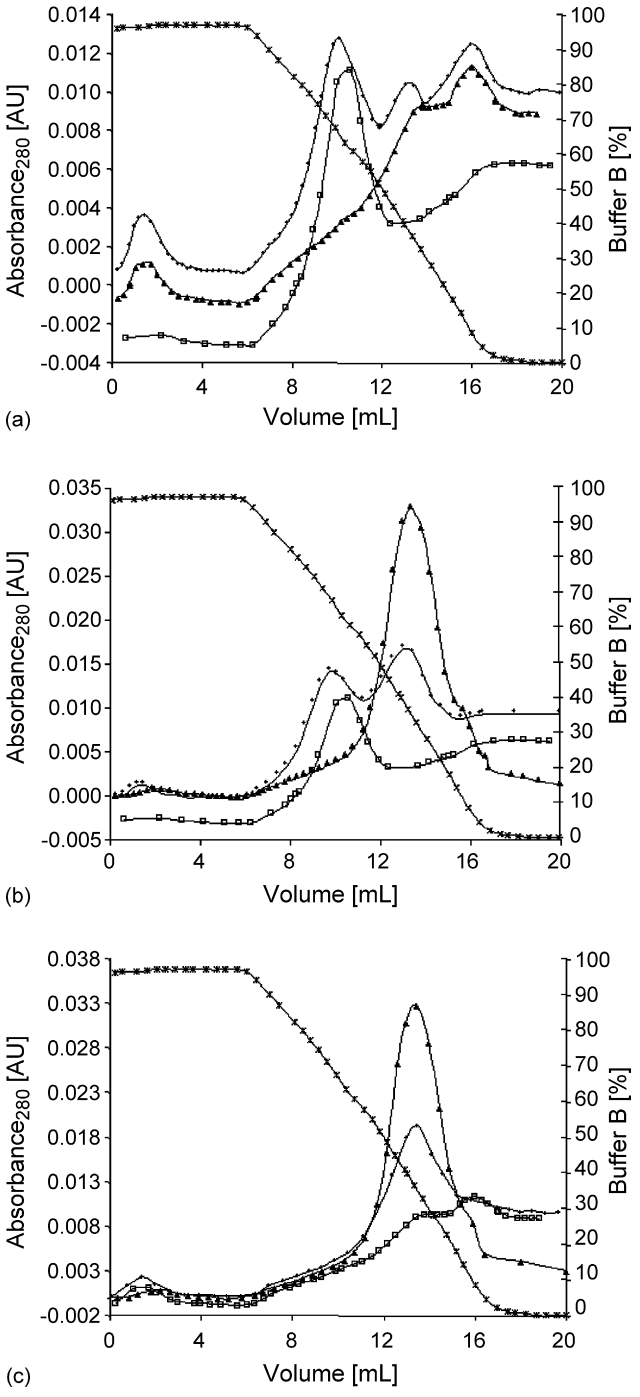

Fig. 2. Chromatograms of protein mixtures compared with the chromatograms of individual proteins. In all graphs: (*) buffer B percentage (%); (●) protein mixture. (a) (▲) Thaumatin; (□) α-amylase. (b) (▲) α-Chymotrypsinogen A; (□) α-amylase. (c) (▲) α-Chymotrypsinogen A; (□) thaumatin.

low deviation from the experimental results: 15.7% for the mixture thaumatin–α-amylase and 6.2% for α-chymotripsynogen A–α-amylase. These results show that it would be possible to estimate a protein's retention time in HIC considering only the main operation variables. Additionally, our results suggest that based on such models it would be possible to determine the most adequate conditions to reach the maximum separation of two proteins.

However, as shown in Table 5 and Fig. 2, it was not possible to reach the separation of α-chymotrypsinogen A from thaumatin, despite the models predicting a DRT difference equal to 0.04. The average surface hydrophobicity of thaumatin is 0.269, and that of chymotrypsinogen is 0.306 [6]; then if separation was determined exclusively by the hydrophobicity difference, these proteins would be expected to separate, but the experimental results do not show this. We can suppose that there are some specific interactions between these proteins and the resin that explain why the technique is not able to separate them.

We think that the prediction of protein retention obtained by this methodology could be improved if the protein's hydrophobicity was included explicitly in the model parameters. This means giving a physical meaning to the parameters, which should be a topic in our future work.

The mixtures were chromatographed under the experimental conditions given in Table 1, and the separation was determined. The results are given in Table 6. It can be observed that the only conditions that resulted in a better separation than that obtained with the optimal ones, corresponded to cases where one of the two proteins did not elute during the salt gradient. Thus, the methodology proposed in this work would be valid only for those cases in which both proteins are delayed by the system.

5. Conclusion

The main system's factors that determine protein retention are salt type (measured as the molal surface tension increment (σ_s)), ionic strength of the initial buffer, and ligand density on the resin surface. If these three variables were able to explain more than 92% of the variability in DRT of model proteins, then the optimization of these variables would be useful in a purification process design. It was possible to determine the optimum operation conditions that would allow the separation of two binary protein mixtures, based on empirical models that relate system characteristics with DRT of the proteins. The predicted separation agreed extremely well with experimental results, thus the methodology presented here shows potential application to partially characterized systems. However, we think that more accurate predictions could be obtained if protein properties such as average surface hydrophobicity were included explicitly as part of the parameters of the models. In order to give more generality to the methodology proposed in this work, a higher number of protein mixtures should be analyzed, and the physical meaning of the parameters should be examined.

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