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## Journal of Chromatography A

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# Evaluation of selectivity in multimodal anion exchange systems: *A priori* prediction of protein retention and examination of mobile phase modifier effects

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### ARTICLE INFO

#### Article history:

Received 28 April 2011  
Received in revised form 25 July 2011  
Accepted 26 August 2011  
Available online xxx

#### Keywords:

Multimodal chromatography  
Quantitative structure property relationship  
Mobile phase modifier  
Protein selectivity

### ABSTRACT

Although recent advances in multimodal chromatography have shown significant potential for selective protein purification, there is a need to establish a deeper understanding of the nature of selectivity in these systems. In this work, the adsorption behavior of a library of commercially available proteins with varying physicochemical properties was investigated. Linear gradient experiments were carried out with the multimodal anion exchanger and the results were compared to those obtained with a traditional anion exchange material. Proteins were more strongly retained on the multimodal resin and a different elution pattern was obtained as compared to anion exchange. Quantitative structure–property relationship models using a support vector regression technique were developed and the resulting models were shown to have good predictive abilities in both systems. Molecular descriptors selected during the generation of these models suggested that multiple interaction modes contributed to the stronger retention and different elution patterns observed in the multimodal system. Finally, mobile phase modifiers such as ethylene glycol, urea and arginine were shown to be able to impart unique selectivity trends in multimodal chromatography.

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## 1. Introduction

Recent advances in the design of multimodal chromatographic systems have produced new classes of chromatographic materials which can provide alternative and improved selectivities as compared to traditional single mode chromatographic materials [1–6]. These advances have resulted in an increased utilization of multimodal chromatography for a number of high-resolution separations for complex biological mixtures in the pharmaceutical and biotechnology industries [7–9]. While recent advances in multimodal chromatography have shown significant potential for protein purification, there is a significant need to establish a deeper understanding of the nature of selectivity in these systems. In addition, methods development with these multimodal chromatographic systems can be quite involved since there are many possible parameters such as salt type, mobile phase modifiers and gradient conditions, to optimize during the development of the separation protocol.

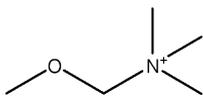
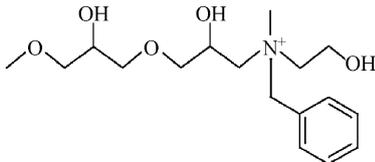
Several investigators have examined the distinctive separation attributes in multimodal chromatographic systems for the separation of small molecules [10], proteins [2,4,5,11], antibodies [12] and nucleic acids [1]. Recently, Oehme and Peters used commercial mixed-mode materials for the capture and purification of several

recombinant therapeutic proteins from various expression systems [13]. Kallberg et al. have explored pH dependent multimodal chromatography to separate glycosylated from non-glycosylated proteins [14].

The unique selectivity in multimodal chromatography has been found to depend on ligand chemistry and protein–ligand interactions. Yang et al. [15] have shown that subtle variations in ligand design can have an impact on selectivity. Further, several studies have suggested that multimodal interactions can be used to enhance protein binding [16,17]. Several mechanical models have also been proposed in the literature. Pitiot et al. [16] have shown promising results when modeling the retention profile with a salt dependent Langmuir isotherm. Li et al. [17] have studied the mechanics of protein adsorption in a multimodal system, and have determined that while the protein obeys a Langmuir isotherm in electrostatic attraction regimes the binding behavior becomes more complex when operated near the isoelectric point. This is believed to be due to a multilayer or rearrangement effect that occurs when electrostatic interactions give way to hydrophobic interactions. Nfor et al. [18] have recently developed an isotherm formalism of protein adsorption on multimodal adsorbents which includes both hydrophobic and electrostatic interactions. In that work, isotherm parameters were determined for five model proteins on four multimodal resins and were shown to be able to predict the adsorption behavior in batch experiments. Despite these advances, to date there have been minimal efforts to elucidate the binding mechanisms in these multimodal chromatographic

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**Table 1**  
Comparison of ligand Q and Capto adhere.

Resin type	Q	Capto Adhere
Functional group		
Matrix	Highly cross-linked agarose, 6%	Highly cross-linked agarose
Total ionic capacity	0.18–0.25 mmol Cl <sup>-</sup> /ml	0.09–0.12 mmol Cl <sup>-</sup> /ml
Mean particle size	90 μm	75 μm

systems at the molecular level. Further, most studies in the literature with multimodal chromatography have been limited to relatively small protein data sets.

We have recently employed a library of cold shock protein B (CspB) mutant variants with varying charge density and distribution to examine differences in protein binding behavior in multimodal cation exchange chromatography (Capto MMC) [19]. In that work, it was found that single site mutations introduced on the protein surface resulted in markedly different retention behavior in the multimodal system. Further, the selectivity trend with this homologous library was found to be quite different in the multimodal as compared to the single mode ion exchange material. We have also recently employed a homologous library based on ubiquitin mutants to examine preferred binding regions on proteins for multimodal interactions [20]. While these studies with homologous protein libraries shed light on some of the interactions in these MM systems, there is a need to examine protein libraries with more diverse properties. In addition, to date there is no systematic examination of the effects of mobile phase modifiers in multimodal chromatography for such protein data sets.

Quantitative structure–property relationship (QSPR) models have been successfully used for the prediction of chromatographic retention [21,22]. We have recently used predictive QSPR modeling with support vector machine (SVM) regression to investigate displacer efficacy and selectivity [23,24], and protein retention in ion-exchange [25–27], hydrophobic interaction [28] and multimodal chromatography using a homologous protein library [19]. In the current work, we develop a QSPR model

for multimodal chromatography which is based on data from a commercial protein library with diverse properties. The SVM method of nonlinear regression [29] has many important properties, including an effective avoidance of overfitting through capacity control and regularization. These features improve its ability to build models using large numbers of molecular property descriptors with relatively few experimental results in the training set [30].

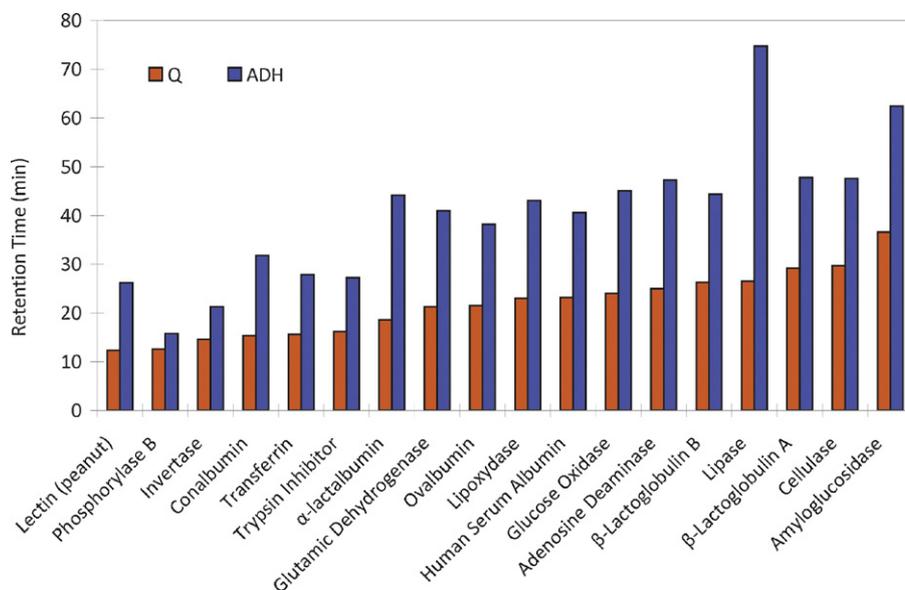
In this work, a set of proteins with a wide range of physicochemical properties (e.g. size, shape, surface area, charge and hydrophobicity) is employed to investigate affinity and selectivity in multimodal anion exchange chromatographic systems. GE Capto Adhere (ADH) is used as a model system to evaluate protein binding under different mobile phase conditions. The selectivities achieved with ADH are then compared to a traditional anion exchanger. QSPR models using a SVM regression algorithm are then developed to predict the binding affinity of proteins to both ADH and the traditional anion exchanger. Finally, the effects of mobile phase modifiers are evaluated in the ADH chromatographic system.

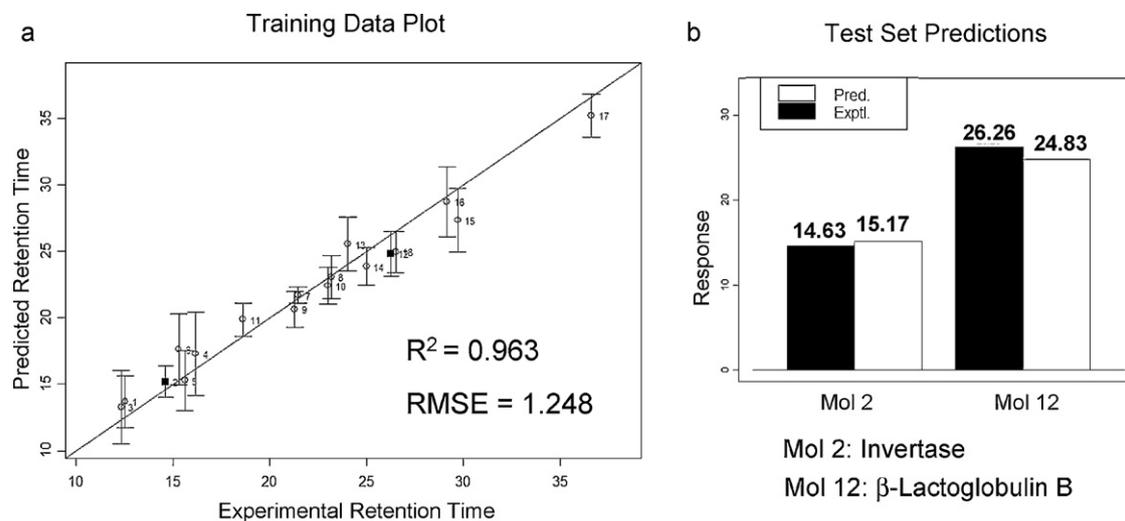
## 2. Materials and methods

### 2.1. Materials and equipment

#### 2.1.1. Materials

Multimodal anion exchanger Capto Adhere and strong anion exchange Q Sepharose FF was purchased from GE Healthcare

**Fig. 1.** Retention times of the protein library in Q and ADH.



**Fig. 2.** QSPR model results for the ion exchanger. (a) QSPR model for prediction of the entire data set, where the open circles indicate the results for the training data set, cross-validated  $R^2 = 0.963$ , the solid squares are the results for the test set data. (b) Comparison of predicted and experimental retention time for the test set.

(Uppsala, Sweden). The following proteins were purchased from Sigma (St. Louis, MO): lectin, phosphorylase B, invertase, conalbumin, transferrin, trypsin inhibitor,  $\alpha$ -lactalbumin, glutamic dehydrogenase, ovalbumin, lipoxylase, human serum albumin, glucose oxidase, adenosine deaminase,  $\beta$ -lactoglobulin B, lipase,  $\beta$ -Lactoglobulin A, cellulase, amyloglucosidase. Sodium phosphate (mono- and dibasic), sodium chloride, urea, ethylene glycol and arginine were also purchased from Sigma.

### 2.1.2. Equipment

Analytical linear gradient chromatographic experiments were carried out using a GE Healthcare Akta explorer 100 controlled by Unicorn 5.0 chromatography software.

## 2.2. Procedures

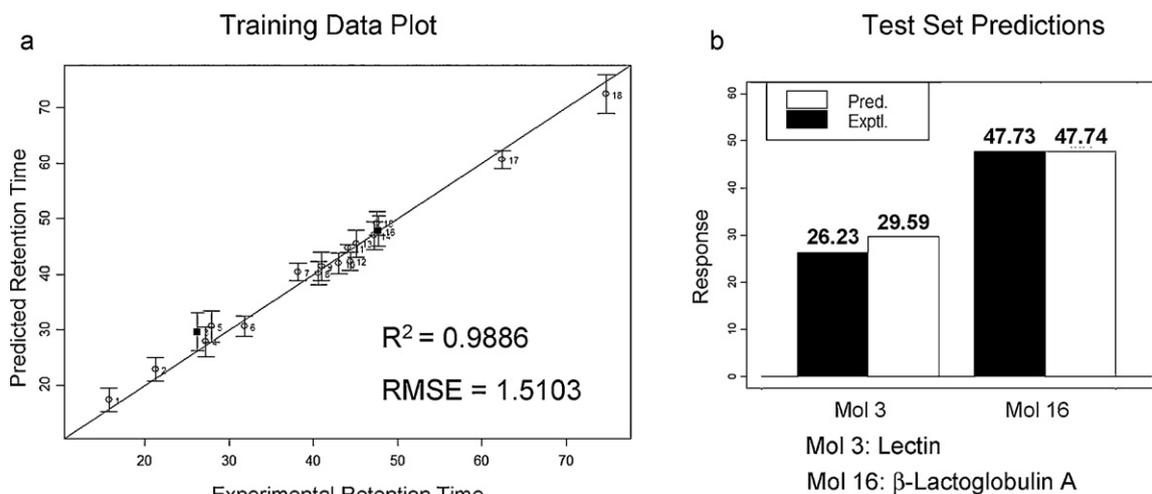
### 2.2.1. Linear gradient chromatographic experiments

The same linear gradient chromatographic experiments were carried out on both the 1 ml Capto Adhere and 1 ml Q Sepharose FF columns (0.5 cm i.d.  $\times$  5 cm) at pH 7. Linear gradient experiments were carried out with a constant slope between buffer A (10 mM Na phosphate, pH 7.0) and buffer B (10 mM Na phosphate, pH 7.0

with 1 M NaCl). The linear gradient slope for these experiments was 25 mM NaCl per column volume. 25  $\mu$ l of protein samples were injected into the column at a concentration of 2 mg/ml in equilibration buffer, and the experiments were carried out in duplicate at a flow of 0.5 ml/min. The column effluent was monitored at 280 nm. For experiments run under ethylene glycol, urea and arginine conditions, the same protocol was used except that 20% (v/v) ethylene glycol, or 2 M urea or 0.1 M arginine was added to the buffer species (buffer A and buffer B) as mobile phase modifiers. All samples were incubated in the buffer for at least 2 h before column experiments.

### 2.2.2. QSPR modeling and SVM regression model

QSPR prediction models were generated using a support vector machine (SVM) regression approach which helps to control the complexity of the model and to minimize the risk of overfitting. In order to construct predictive QSPR models for the retention times of the protein library, the protein crystal structures were downloaded from the RCSB Protein Data Bank (<http://www.rcsb.org/>). Homology modeling of proteins was first conducted to “clean” the protein structures using the Molecular Operating Environment (MOE) software from CCG (Chemical Computing Group,



**Fig. 3.** QSPR model results for ADH. (a) QSPR model for prediction of the entire data set, where the open circles indicate the results for the training data set, cross-validated  $R^2 = 0.9886$ , the solid squares are the results for the test set data. (b) Comparison of predicted and experimental retention time for the test set.

**Table 2**  
PDB ID and pI of the proteins used in this study and retention times for Q and ADH.

Protein Name	PDB	pI	Q (min)	ADH (min)
Lectin (peanut)	2PEL	5.8	12.35	26.23
Phosphorylase B	1GPB	5.5	12.56	15.73
Invertase	2AC1	4.4	14.63	21.27
Conalbumin	1AIV	6.3	15.31	31.79
Transferrin	1A8E	6.2	15.63	27.89
Trypsin Inhibitor	1AVU	4.5	16.19	27.25
$\alpha$ -lactalbumin	1F6R	4.5	18.63	44.13
Glutamic Dehydrogenase	1NR7	6.7	21.29	40.95
Ovalbumin	1OVA	4.7	21.47	38.19
Lipoxydase	1F8N	5.7	23.02	43.02
Human Serum Albumin	1AO6	5.2	23.19	40.61
Glucose Oxidase	1CF3	4.2	24.04	45.07
Adenosine Deaminase	1VFL	4.8	25.00	47.25
$\beta$ -Lactoglobulin B	1BSQ	5.3	26.26	44.37
Lipase	3TGL	4.1	26.51	74.76
$\beta$ -Lactoglobulin A	3BLE	5.1	29.16	47.73
Cellulase	1EG1	4.5	29.71	47.55
Amyloglucosidase	1LF6	3.6	36.61	62.43

Inc.) [31]. A variety of descriptors were then calculated based on the three-dimensional protein structures, including traditional two-dimensional and three-dimensional descriptors computed using the MOE software package [31]. In addition, pH-dependent electrostatic potential (EP) descriptors and molecular lipophilicity potential (MLP) descriptors [27] and surface hydrophobicity descriptors were calculated as described elsewhere [28]. Invariant descriptors were then removed by using a partial least squares for PC (PLS-PC) software package [32]. Subsequently, a sparse linear l1-norm SVM regression algorithm [29] was applied in the feature selection mode to identify a subset of descriptors relevant for each response. Finally, independent non-linear predictive retention SVM-QSPR models [30] were generated using the descriptors from the feature selection step and were examined for quality and consistency (given by the cross-validated  $R^2$  and RMSE for the training set) and predictive ability (for an external test set of molecules not used in the feature selection or generation of the model). In this work, the original training set of proteins was randomly subdivided into a validation set, with the remaining proteins used as a training subset. A large number of models for different combinations of training and internal validation sets of proteins (i.e., bootstrapping) were generated. Models were then created for different sets of training proteins and used to make predictions on the internal validation set of proteins left out of the training set. The SVM algorithm used in both the feature selection and model-building minimizes the possibility of fortuitous correlations and ensures that the resultant models have general predictive ability over the chemical space within which the QSPR models are built [33]. A more detailed background on QSPR theory and regression procedures are described elsewhere [27,29,30,34].

**Table 3**  
Definitions of selected descriptors from ion exchange and multimodal QSPR models.

Categories	Descriptors	Description of descriptors	Ion exchange	Multimodal
Electrostatic potential and charge related	PEOE.VSA.FPNEG (MOE)	Fractional negative polar Van der Waals surface area	10.2%	
	EP2.HF.6.PH7	Electrostatic potential energy	8.3%	
	DIPOLE (MOE)	Dipole moment calculated from the partial charges of the molecule	7.7%	
	Q.VSA.FPOS (MOE)	Fractional positive Van der Waals surface area	-19.7%	-3.3%
	FCHARGE (MOE)	Total charge of the molecule (sum of formal charges)	-8.3%	-9%
Molecular lipophilicity potential (MLP)	MLP1.W17 MLP1.W19	Molecular lipophilicity/hydrophobicity Potential (MLP) on the protein surface		6.1%
	E.STR (MOE)	Bond stretch potential energy		5.5%
Protein potential energy	SHAPE	Protein Shape	6.5%	-5.8%
	GLOB (MOE)	Globularity		5.4%

**Table 4**  
Summary of the main effects of studied mobile phase modifiers.

Name	Structure	Overall Effects		
		Hydrophobic	H-bonding	Ionic
Ethylene Glycol	<chem>OCCO</chem>	-		
Urea	<chem>NC(=O)N</chem>	-	-	
Arginine	<chem>NC(CCNC(=O)O)C(=O)N</chem>	-	-	-

Note: "–", sign in the table indicates a reduction in this effect.

### 3. Results and discussions

#### 3.1. Protein binding in multimodal and anion exchange systems

The retention behavior of a library of eighteen proteins was investigated in both multimodal (Capto Adhere, ADH) and anion exchange systems using gradient conditions. The proteins in this library were selected based upon protein pI's, the availability of protein structures (PDB files), and the diversity of their properties such as charge, hydrophobicity, size, shape and surface area. The structures of the immobilized ligands used in the ADH and Q resins and properties of these resins are shown in Table 1. While both the ADH and Q ligands contain charged amine groups, the ADH ligand has an aromatic ring for hydrophobic and aromatic interactions as well as hydroxyl groups for hydrogen bonding. Linear gradient experiments were carried out with both resins as described in Section 2. Fig. 1 compares the gradient retention times of the protein library on both the ADH and Q systems. As can be seen in the figure, the proteins exhibited stronger retention on the ADH system, even though the total ion capacity is lower in ADH (Table 1). This indicates that the additional interactions afforded by the ADH ligand (e.g. hydrophobic and hydrogen bonding) are also playing an important role in the protein retention. This is in agreement with other results in the literature [19,27]. The retention data in Fig. 1 is presented based on the retention order observed with the Q resin. Clearly, the selectivity trends on these two types of anion exchange resins were quite different. In addition, while all of the protein exhibited stronger retention on the ADH resin, some proteins (e.g. lectin, conalbumin,  $\alpha$ -lactalbumin and lipase) were observed to have significantly elevated binding in ADH as compared to the traditional anion exchanger. These results with both the anion and the multimodal anion exchange resins indicate that both affinity and selectivity can be significantly different in these two systems. While one could propose possible reasons for the selectivity differences observed with this data, the approach taken in this paper is to employ QSPR modeling to get a more

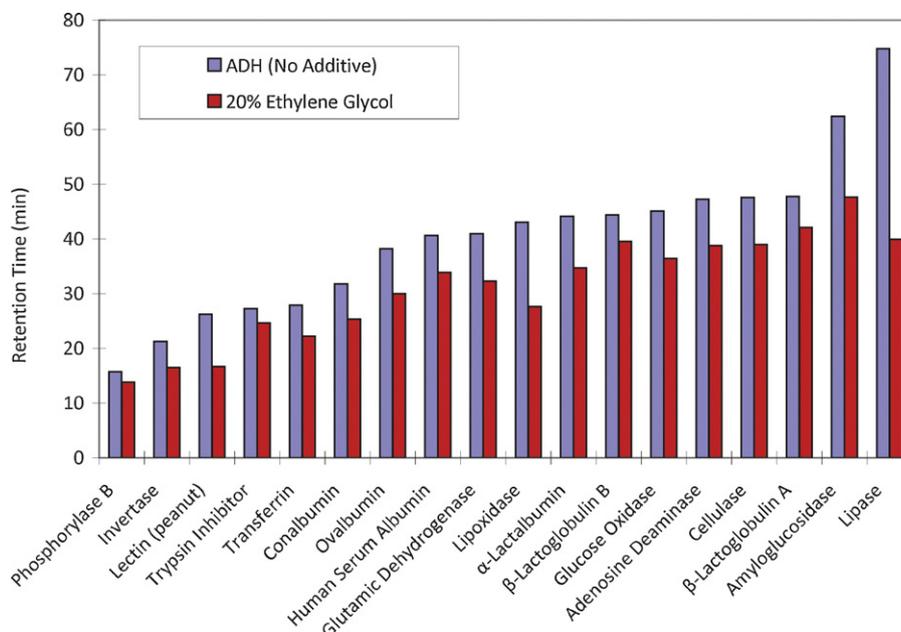


Fig. 4. Protein retentions in ADH with and without 20% (v/v) ethylene glycol.

global understanding of the selectivity as described in the next section.

### 3.2. QSPR modeling of protein retention

In order to examine these multimodal effects in more detail, the set of experimental retention data was also employed to model the retention behavior of proteins in Q and ADH systems. QSPR models were created for the *a priori* prediction of protein retention behavior in these systems. Table 2 shows the names of the proteins, corresponding PDB files, protein pI and retention times for Q and ADH that were used for this work. As described above in the procedures section, a sparse SVM regression algorithm was applied for feature selection and non-linear predictive retention

SVM-QSPR models were then generated using the selected molecular descriptors.

The resulting QSPR models for the Q and ADH resins are shown in Figs. 2 and 3, respectively. Figs. 2a and 3a show the correlation between the experimental and predicted results. In these figures, the open symbols represent the training data, and the filled symbols indicate the test set proteins. The error bars in the figure indicate the standard deviation of the predicted retention values obtained from all bootstraps involved in the generation of the ensemble model. The cross-validated  $R^2$  for these models were 0.9630 and 0.9886, respectively, which indicated that the predicted values for protein retention were in good agreement with the experimental data. Validation of the QSPR models was carried out by comparing the experimental and predicted data for the test molecules which were not included in the training set or in the generation of the

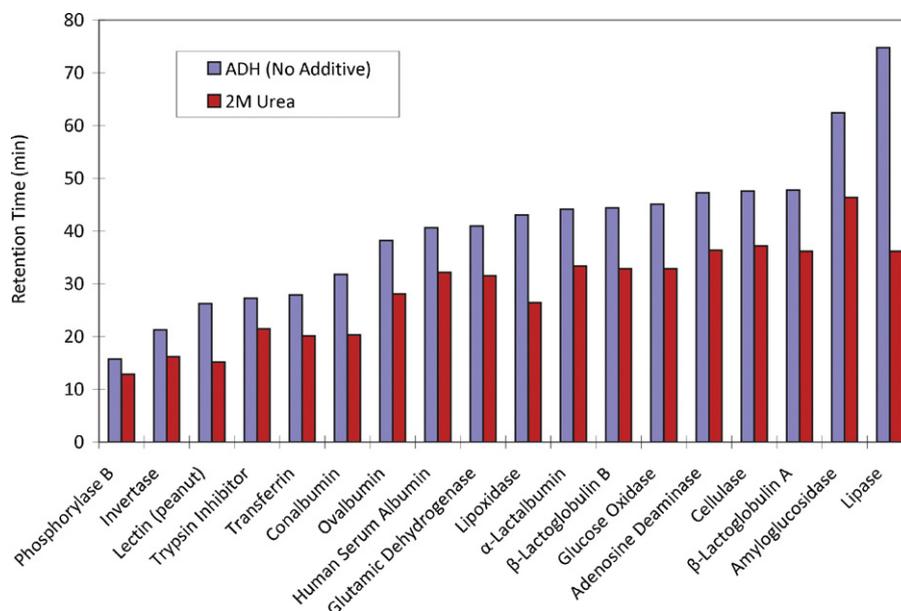


Fig. 5. Protein retentions in ADH with and without 2 M urea.

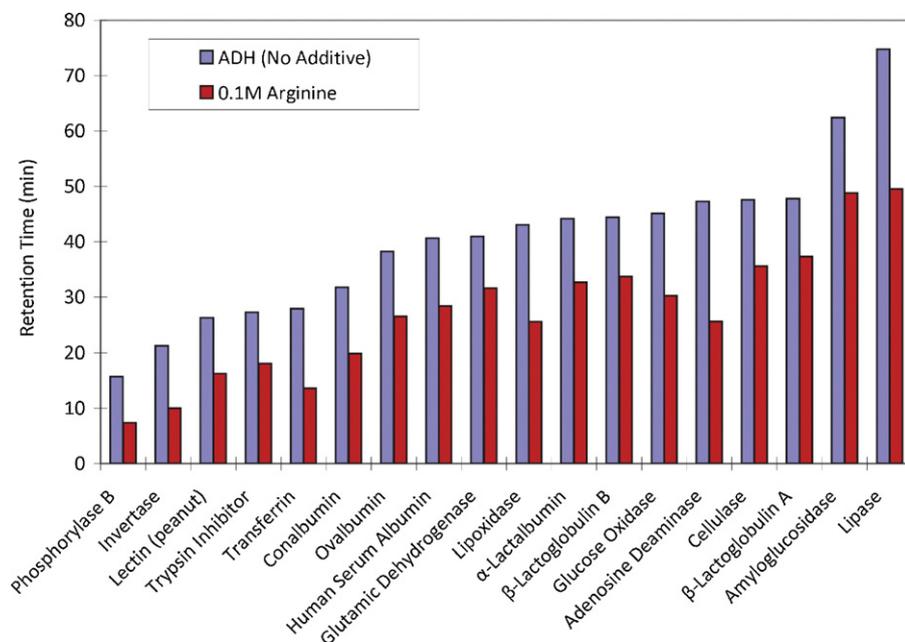


Fig. 6. Protein retention in ADH with and without 0.1 M arginine.

models. The predicted results for the test set proteins are shown in Figs. 2b and 3b. As can be seen in these figures, the predicted values for the test proteins were also in good agreement with the experimental data. These results confirm the predictive power of these QSPR models for a wide range of proteins in both anion exchange and multimodal systems.

The QSPR models can also aid in the interpretation of the main factors that affect protein retention in multimodal anion exchange chromatography. In order to carry out this analysis, the results from the multimodal system (ADH) are compared to a pure ion exchanger (Q). The definitions and contributions of the significant positive or negative descriptors in the QSPR models for Q and ADH are provided in Table 3 and the detailed molecular descriptor values for the protein library are listed in the Supplementary Table S1.

Before evaluating the multimodal system, it is important to establish the behavior of the anion exchanger under the exact same mobile phase conditions. As seen in Table 3, for the Q resin the most important positive contributors were fractional negative polar van der Waals surface area, electrostatic potential energy and dipole moment. In addition, protein shape was also found to be important in the model. The most important negative contributors to the Q model were positive protein partial charge and

protein net charge. These descriptors are similar to those previously identified [35,36].

The results for the multimodal system are also presented in Table 3. As can be seen in the table, the most important contributors to the ADH model were two molecular lipophilicity potential (MLP) descriptors. This is in sharp contrast to the Q system where hydrophobic interactions were not observed to play any role. The protein shape (globularity) was also a positive contributor. Two important negative contributors to the ADH model were also observed in the Q model, namely, protein positive partial charge and net charge. This is not surprising, since electrostatic interactions are expected to also play an important role in the ADH system. Finally, the E-STR descriptor which represents the bond stretch potential energy was also seen to be an important negative contributor to the ADH model. This analysis of the selected molecular descriptors provided further evidence for the importance of hydrophobic interactions in ADH protein chromatography.

### 3.3. Effects of mobile phase modifiers

The retention data presented in Fig. 1 indicated that the selectivity of multimodal systems can be significantly different from traditional ion exchange. Further, the results from the QSPR

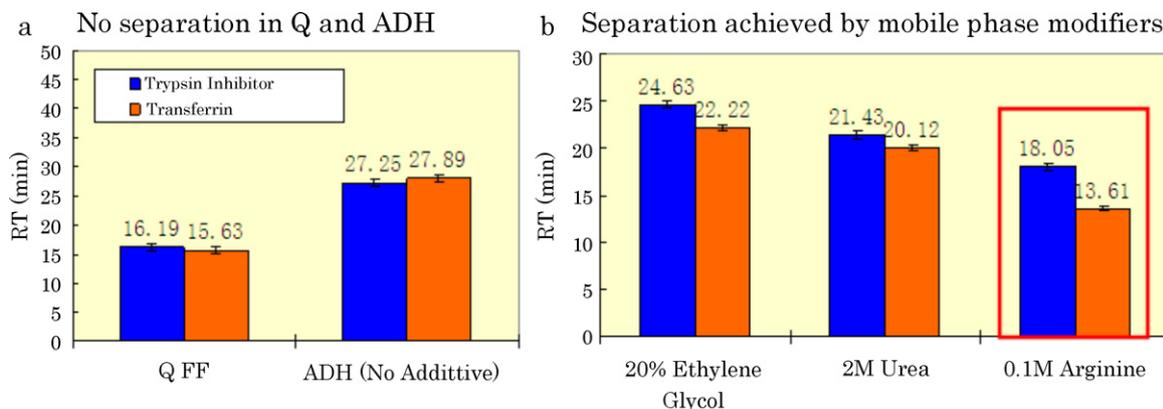


Fig. 7. Achieving selectivity for a protein pair using mobile phase modifiers in ADH.

models confirmed the importance of multiple interaction modes (e.g. hydrophobic and electrostatic interactions) in these systems. Thus, in order to further improve the selectivity of multimodal systems, it was of interest to examine various mobile phase modifiers which have the ability to modulate these intermolecular interactions to varying extents.

Accordingly, the effects of ethylene glycol, urea and arginine on protein retention on the ADH resin were examined. The main effects of these mobile phase modifiers on protein elution properties are summarized in Table 4. Ethylene glycol is known to primarily reduce hydrophobic interactions [37–39]. In addition, it can also result in slight increases in electrostatic interactions. Urea can affect both hydrophobic and hydrogen bonding interactions by interacting directly with protein polar side chains and backbones and/or by altering the solvation of proteins [40,41]. Urea can also result in slight decreases in electrostatic interactions.

Arginine can interact with protein side chains by hydrogen bonding and aromatic moieties via  $\pi$  electron–cation interactions [39,42]. While the behavior of arginine is quite complex, it is generally accepted that this additive can result in the reduction of hydrophobic, hydrogen bonding and electrostatic interactions. The effects of these mobile phase modifiers on protein retention in ADH were examined in a series of chromatographic gradient experiments. The experimental results are provided in Figs. 4–6 for ethylene glycol, urea and arginine, respectively. The protein elution orders in these figures were sorted based on the experimental retentions in ADH when no additives were present. As seen in these figures, the presence of these mobile phase modifiers resulted in reductions of protein retentions albeit to varying degrees.

As can be seen in Figs. 4 and 5, the effects of ethylene glycol and urea on protein retention were qualitatively similar for this protein library. Both modifiers resulted in similar reductions in the retention times and modifications of the selectivity between various protein pairs. The fact that the retention pattern is similar with these two modifiers, indicates that hydrophobic interactions are likely playing a more important role than hydrogen bonding in the ADH system under these conditions. In contrast, the effect of arginine as a mobile phase modifier was markedly different from either ethylene glycol or urea with respect to both the reductions in retention times and the selectivity (Fig. 6). This difference in selectivity may be due to the effect of arginine on not only hydrophobic interactions and hydrogen bonding, but on electrostatic interactions as well.

The results in Figs. 4–6 indicate that significant changes in retention patterns can be produced with the presence of appropriate mobile phase modifiers. Further, under appropriate conditions, these modifiers can be employed to result in improved selectivity. For example, trypsin inhibitor and transferrin were very difficult to separate using either Q or ADH without any modifiers (Fig. 7a). On the other hand, when mobile phase modifiers were employed with ADH (Fig. 7b), a significant improvement in selectivity was achieved with 0.1 M arginine resulting in the best separation. These results indicate that mobile phase modifiers may be helpful when dealing with difficult separation problems which cannot be achieved using traditional single mode or even multimodal chromatography.

#### 4. Conclusions

In this work, the adsorption behavior of a library of commercially available proteins with varying physicochemical properties was investigated. Linear gradient experiments were carried out with the multimodal anion exchanger and the results were compared to those obtained with a traditional anion exchange material. Proteins were more strongly retained on the multimodal resin and a different elution pattern was obtained as compared to anion exchange.

Quantitative structure–property relationship models using a support vector regression technique were developed and the resulting models were shown to have good predictive abilities in both systems. Molecular descriptors selected during the generation of these models suggested that multiple interaction modes contributed to the stronger retention and different elution patterns observed in the multimodal system. The analysis of the molecular descriptors selected in the generation of the QSPR ADH model confirmed the importance of hydrophobic interactions in these systems. Finally, mobile phase modifiers such as ethylene glycol, urea and arginine were employed to modulate the selectivity in multimodal chromatography. While ethylene glycol and urea elicited similar changes in the protein elution pattern, arginine resulted in a distinctly different pattern. This work indicates that there may be significant potential to employ appropriate mobile phase modifiers with multimodal chromatography to address challenging biopharmaceutical separations. Further, by combining multimodal ligand design and mobile phase modifier selection it may be possible to design multimodal separation systems that are uniquely well suited to resolve closely-related protein variants. A more detailed molecular level understanding of the effects of mobile phase modifiers on multimodal chromatography and the effects of non-linear adsorption will be examined in future work.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.08.080.

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