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Research Article

Use of chemometric methodology in optimizing conditions for competitive binding partial filling affinity capillary electrophoresis

This work expands the knowledge of the use of chemometric response surface methodology (RSM) in optimizing conditions for competitive binding partial filling ACE (PFACE). Specifically, RSM in the form of a Box–Behnken design was implemented in flow-through PFACE (FTPFACE) to effectively predict the significance of injection time, voltage, and neutral ligand (neutral arylsulfonamide) concentration, $[L_0]$, on protein–neutral ligand binding. Statistical analysis results were used to create a model for response surface prediction *via* contour and surface plots at a given maximum response ($\Delta RMTR$) to reach a targeted $K_b = 2.50 \times 10^6 \text{ M}^{-1}$. The adequacy of the model was then validated by experimental runs at the optimal predicted solution (injection time = 2.3 min, voltage = 11.6 kV, $[L_0] = 1.4 \mu\text{M}$). The achieved results greatly extend the usefulness of chemometrics in ACE and provide a valuable statistical tool for the study of other receptor–ligand combinations.

Keywords:

Affinity capillary electrophoresis / Capillary electrophoresis / Chemometrics / Response surface methodology
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1 Introduction

Molecular recognition refers to the recognition and interaction between two or more molecules *via* noncovalent bonding and is at the root of essentially all biological processes and, hence, much of medicine. All cellular functions in the human body depend on protein–protein, protein–small molecule, protein–nucleic acid, or other intermolecular interactions. These biological interactions are essential to life and are involved in all of the enzyme-based reactions involved in cell division, cell death, and cell transformation. Hence, biological interactions are important in the initiation, progression, and effects of all human disease including Parkinson's, Alzheimer's, AIDS–HIV, and cancer [1, 2].

Currently, there are a variety of techniques available to measure affinity parameters between biological species including equilibrium dialysis, RIA, fluorescence quench-

ing, ultracentrifugation, NMR, and slab gel electrophoresis. If the amount of bound and free ligand in solution can be distinguished, these techniques can provide reasonable estimates of binding constants (K_b).

ACE has been shown to be a versatile microanalytical technique to estimate affinity constants, and has emerged as a useful and sensitive method for studying bimolecular noncovalent interactions and for determining binding and dissociation constants of formed complexes. The first reports detailing the use of ACE to measure affinity parameters between biological species were published in the early 1990s [3–7]. Since these informative studies, a multitude of other interactions including protein–ligand, peptide–peptide, protein–peptide, protein–antibody, polymer–peptide, and antibody–antigen have been examined successfully using ACE [8–38].

ACE differentiates between bound and unbound receptor (R) as a function of free ligand (L) concentration only when the R–L complexation yields a sizable difference in mass or charge-to-mass ratio. In a typical ACE experiment, a sample of receptor and noninteracting markers are reacted with an increasing concentration of ligand in a running buffer, thereby, causing a shift in the migration of the receptor peak. Subsequent analysis of these changes in migration time yields a value for K_b [20].

To minimize the amount of sample needed in an ACE assay, partial filling techniques in ACE were developed. In PFACE, the capillary is partially filled with ligand (or receptor)

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Abbreviations: CAB, carbonic anhydrase B; FTPFACE, flow-through partial filling ACE; HHM, horse heart myoglobin; MO, mesityl oxide; RMTR, relative migration time ratio; RSM, response surface methodology

and a sample plug of receptor (or ligand) is introduced into the capillary and electrophoresed. During electrophoresis the zones of samples overlap within the capillary and an equilibrium is established prior to the point of detection. An extension of PFACE is flow-through PFACE (FTPFAFCE) [9, 11, 19]. In this technique, the capillary column is partially filled with an even smaller plug of ligand than that used in PFACE (Fig. 1). A sample containing receptor and markers are subsequently injected and electrophoresed. Upon application of a voltage gradient, the ligand, and sample zones first overlap then and an equilibrium is established between ligand and receptor. As long as the time of contact between ligand and receptor is sufficient to establish equilibrium prior to the point of detection, a value for K_b can be elucidated.

Previously a univariate approach to optimizing conditions for FTPFAFCE was described in the binding of 4-carboxybenzenesulfonamide (CBSA) and carbonic anhydrase B (CAB) [11]. Here, the minimal injection time of ligand required in FTPFAFCE to ensure an equilibrium between ligand and receptor was determined. Univariate techniques are time-consuming (especially in regards to new method development) and do not take interactive effects between factors into account. Fortunately, various chemometric experimental design and response surface methodology (RSM) techniques have been utilized in CE to aid in designing and optimizing analyses [39–46]. In the latter [46], we

successfully implemented RSM in the form of a Box–Behnken design to effectively predict the significance of capillary length, voltage, and injection time on protein–ligand binding. This was the first known application of RSM in ACE and showed the predicted nature of the Box–Behnken design in determining which factors had the greatest influence of reaching a targeted response.

Herein, we demonstrate the successful expansion of the use of RSM in a competitive binding FTPFAFCE study to effectively predict the significance of injection time, voltage, and $[L_0]$ on protein–neutral ligand binding. It greatly extends the knowledge of chemometrics in ACE by showing that uncharged ligands for receptors can also be modeled effectively using response surface methodology and obtain viable values for affinity parameters. Results were used to create a model for response surface prediction *via* contour and surface plots at a given maximum response. The adequacy of the model was then validated by experimental runs at the predicted solutions.

2 Materials and methods

2.1 Chemicals and reagents

All chemicals were of analytical grade. CAB (EC 4.2.1.1 containing CAA and CAB isozymes from bovine erythrocytes),

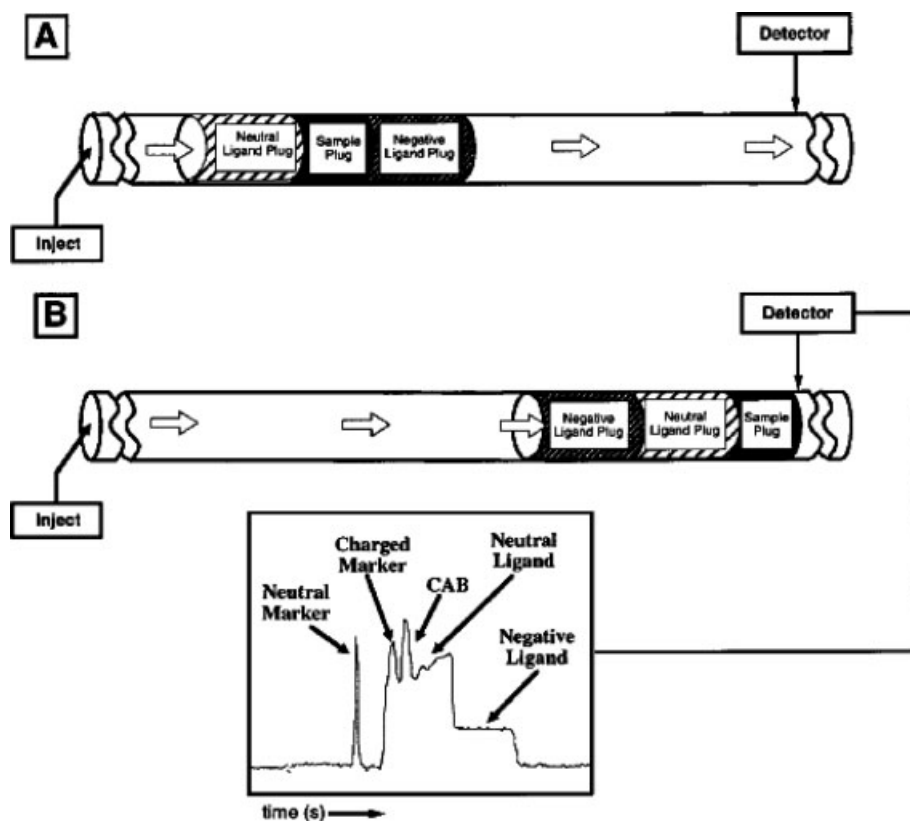


Figure 1. Schematic of a competitive binding FTPFAFCE experiment. The sample plug is enlarged to best pictorially represent the technique.

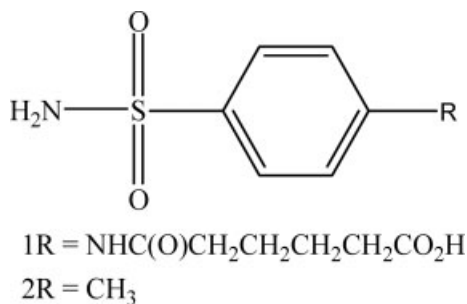


Figure 2. Structures of [[4-(aminosulfonyl)phenyl]methyl]-amino]-6-oxohexanoic acid (**1**) and *p*-toluenesulfonamide (**2**) used in this study.

and horse heart myoglobin (HHM) were purchased from Sigma Chemical Company (St. Louis, MO, USA) and used without further purification. Mesityl oxide (MO) was purchased from Calbiochem (San Diego, CA, USA). *p*-Toluenesulfonamide, **2**, (Fig. 2) was purchased from Aldrich (Milwaukee, WI, USA). [[4-(Aminosulfonyl)phenyl]methyl]-amino]-6-oxohexanoic acid (**1**) was synthesized using methods from known literature procedures [16]. For FTPFACE: Stock solutions of bovine CAB (1 mg/mL), MO (100 μ L/1000 μ L buffer), and HHM (1 mg/mL) were each prepared by dissolving in buffer (192 mM glycine-25 mM Tris; pH 8.4). A stock solution of ligand **1** (1 mg/mL) was prepared by dissolving the molecule in buffer and diluting to 20 μ M. A stock solution of ligand **2** (1 mg/mL) was prepared by dissolving the molecule in buffer and diluting to the following concentrations: 0, 2.5, 5.0, 7.5, 9.0, 15, 20, 30, 40, and 50 μ M.

2.2 Instrumentation

All analyses were carried out using a P/ACE 5500 CE instrument (Beckman Instruments, Fullerton, CA, USA). The capillary tubing (Polymicro Technologies, Phoenix, AZ, USA) was of uncoated fused-silica with an id of 50 μ m, length from inlet to detector of 30.5 cm, with a length from detector to outlet of 6.5 cm. Data were collected and analyzed with Beckman System Gold software.

2.3 Procedure

For CAB, a sample of a negative arylsulfonamide (20 μ M), L₋, was vacuum-injected into the capillary for 0.1 min, for example, at high pressure (20 psi) followed by a sample (3.6 nL) of solution for 3 s containing 0.26 mg/mL of CAB and HHM, respectively and 1.0 μ L/mL of MO in buffer. The electrophoresis was carried out using Tris-glycine buffer and repeated at increasing concentrations of a neutral arylsulfonamide, L_o (0–50 μ M) for 4.0 min. L₋ and L_o are molecules **1** and **2**, respectively.

2.4 Form of analysis

In our earlier work [46], we used a single marker form of analysis based on changes in electrophoretic mobility ($\Delta\mu$) to estimate the value for K_b . While this form of analysis is generally accepted, a single marker does not always compensate for changes in EOF that can occur due to changes in ionicity of the buffer and/or Joule heating. In the present work, a dual marker form of analysis, called the relative migration time ratio (RMTR), was used to obtain a value for K_b between a neutral arylsulfonamide and CAB relative to two non-interacting standards (Eq. 1).

$$\text{RMTR} = \frac{t_t - t_{s'}}{t_{s'} - t_s} \quad (1)$$

Here, $t_{s'}$ and t_s are the measured migration times for the noninteracting markers (MO and HHM) and t_t is the migration time for CAB. Equation (2) is used to obtain the value for K_b of L_o from the change in RMTR as a function of the [L_o] ($\Delta\text{RMTR}/[\text{L}_{o}]$) for the interaction of L₋ and CAB on a relative time scale with noninteracting markers [10]. Equation (2) represents the general equation used to obtain a linear plot and hence the K_b of the competitive binding system we are investigating:

$$\frac{\Delta\text{RMTR}_{\text{R,L}}}{[\text{L}_{o}]} = \frac{1 - \Delta\text{RMTR}_{\text{R,L}}}{(K_{b_o}^{-1})(1 + ([\text{L}_{-}])_i)(K_b)} \quad (2)$$

Here, K_b and L are the known values for the binding constant and concentration of ligand **1** in the running buffer, respectively. In this experiment, K_{b_o} and L₋ are $4.16 \times 10^6 \text{ M}^{-1}$ and 20 μ M, respectively. The values of $\Delta\text{RMTR}_{\text{R,L}}/[\text{L}_{o}]$ over a range of concentrations of neutral ligand (0, 2.5, 5.0, 7.5, 9.0, 15, 20, 30, 40, and 50 μ M) were then used for analysis. A maximum $\Delta\text{RMTR}_{\text{R,L}}/[\text{L}_{o}]$ response value is expected to yield a target K_{b_o} of $2.50 \times 10^6 \text{ M}^{-1}$ which is based on previous work on FTPFACE and with the charged arylsulfonamide system.

2.5 Experimental design and optimization procedures

For this study three factors (injection time, voltage, and [L_o]) were chosen and tested at three levels in a Box–Behnken response surface design (Table 1). Response surface methodologies are multivariate techniques that mathematically fit the experimental domain studied in the theoretical design through a response function. The Box–Behnken design is an efficient option in RSM and an ideal alternative to central composite designs [47]. It combines a fractional factorial with incomplete block designs to avoid the extreme vertices and presents an approximately rotatable design with only three levels *per* factor. It uses a smaller number of data points to estimate the coefficients for all linear, quadratic, and first-order interaction terms in a polynomial model relating response variables to input factors. In this manner, a complete

Table 1. Experimental factors and levels used in the Box–Behnken design

Factor	Level (-)	Level (0)	Level (+)
Injection time (min)	1	2.5	4
Voltage (kV)	5.0	12.5	20
[L ₀] (μM)	1	5	9

second-order model approximating the desired response in *N*-dimensional space is obtained. Ultimately, this model can be used for predictions within the simulated space and for sensitivity analysis.

Factor significance was calculated in ANOVA models that were estimated and run up to their first order interaction terms. ANOVA for a linear regression partitions the total variation of a sample into components. In our calculations we assumed that higher order interaction terms did not contribute significantly to the behavior of our statistical model. In each of our designs experiments were replicated three times so as to reduce the type I error rate and increase the power of our analyses.

The generalized model used in this study had the quadratic form (Eq. 3):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (3)$$

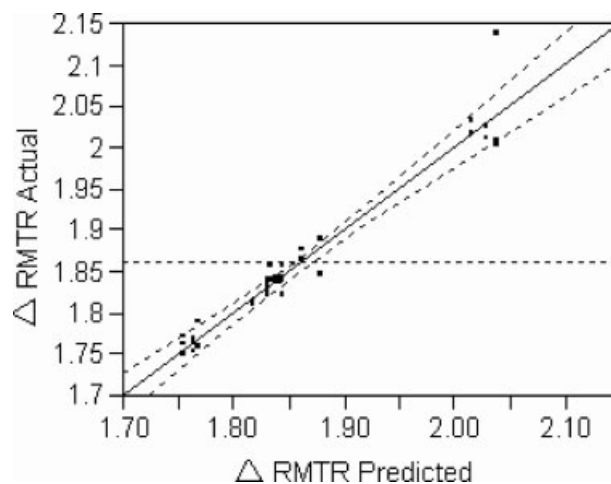
which contains linear terms for all factors, squared terms for all factors and products of all pairs of factors. In this study, X_1 , X_2 , and X_3 terms correspond to injection time, voltage, and [L₀] as they relate to predicted the response (Δ RMTR). In the above equation β is the coefficient, akin to the regression coefficient and giving a measure of the rate of change in Δ RMTR *per* unit change in injection time, voltage, and [L₀].

Factors, levels, and a target K_{bo} value of $2.50 \times 10^6 \text{ M}^{-1}$ were selected based on our previous univariate work on FTPFACE and with the CAB-charged arylsulfonamide system [9]. This K_{bo} value is in agreement with previous ACE studies and in studies using other assay techniques [8, 33]. Although variable values for K_b have been reported for the detection of 2 with CAB, values for K_b are pH and buffer dependent. To minimize variances in K_b , the current study was conducted by the same operator and utilized the same CE instrument.

3 Results and discussion

3.1 Design matrix and linear model

The design matrix (including actual (experimental) and model predicted responses) generated for the Box–Behnken study is shown in Table 2. Here, three center-point experi-

**Figure 3.** Whole model leverage plot of actual *versus* predicted Δ RMTR.

ments were incorporated to compute an estimate of the error term that does not depend on the fitted model. A whole model leverage plot (Fig. 3) was generated to show actual values of the response plotted against the model predicted values with the quality of fit expressed by the coefficient of determination (r^2). This coefficient is the variation in the response around the mean that can be attributed to terms in the model rather than to random error. Typically, the points on the leverage plot are actual data coordinates and the horizontal line the sample mean of the response.

Shown in the leverage plot (Fig. 3) are confidence curves. These indicate whether the test is significant at the 5% level by showing a confidence region for the line of fit. If the confidence region between the curves contains the horizontal line, then the effect is not significant. If the curves cross the line (as in this study), the effect is significant. Overall, an r^2 value of 0.86 was obtained with a mean response of 1.861. The basic calculations for the linear model are shown in the ANOVA (Table 3). $\text{Prob}>F$ is the significance probability for the *F*-ratio, which states that if the null hypothesis is true, a larger *F*-statistic would only occur due to random error. Significance probabilities of 0.05 or less are considered evidence that there is at least one significant regression factor in the model.

A close examination of $\text{Prob}>F$ from the Effect test results (Table 4) revealed that voltage and [L₀] had significant single effects on the response (Δ RMTR) with $\text{Prob}>F$ values of 0.0135 and <0.0001 , respectively. Interestingly, injection time did not have a significant single effect ($\text{prob}>F = 0.5505$) on Δ RMTR but was significant ($\text{prob}>F = 0.0186$) as an interactive effect when combined with voltage. The shift in the migration time of the peak for CAB is predicated on both the amount of time the zone of sample exists within the plug of neutral ligand and the concentration of that neutral ligand the former being greatly dependent on the voltage. The values of injection time

Table 2. Box–Behnken design matrix including mean actual (experimental) and model predicted responses

Experiment	Injection time (min)	Voltage (kV)	[L ₀] (μM)	Mean actual Response r(ΔRMTR) (n = 3)	Model predicted response (ΔRMTR) (n = 3)
1	1	5	5	1.83842	1.84415
2	4	5	5	1.80813	1.81802
3	1	20	5	1.83929	1.83974
4	4	20	5	1.87217	1.87679
5	2.5	12.5	1	2.01261	2.02874
6	2.5	12.5	9	1.75861	1.73236
7	2.5	12.5	5	1.83419	1.82945
8	2.5	12.5	5	1.82916	1.82945
9	2.5	12.5	5	1.82501	1.82945
10	2.5	5	5	1.84946	1.83383
11	2.5	20	5	1.86609	1.86101
12	1	12.5	1	2.02052	2.01454
13	4	12.5	1	2.0476	2.03746
14	1	12.5	9	1.76667	1.76646
15	4	12.5	9	1.75883	1.75445

Table 3. ANOVA table for the linear model

Source	Degrees of freedom	Sum of squares	Mean square	F-ratio
Model	8	0.32471623	0.064943	49.9090
Error	36	0.05074805	0.001301	Prob>F
Corrected total	44	0.37546428	–	<0.0001

Table 4. Effect test results for the Box–Behnken design

Source	Degrees of freedom	Sum of squares	F-ratio	Prob>F
Injection time (min)	1	0.00017874	0.3632	0.5505
Voltage (kV)	1	0.00332354	6.7533	0.0135
[L ₀] (μM)	1	0.31730580	644.7529	<.0001
Injection time*voltage	1	0.00299321	6.0821	0.0186
Voltage*[L ₀]	0	0.00000000	1.3478	0.8176
Injection time*[L ₀]	1	0.00091495	1.8591	0.1812

chosen for this study are sufficient to cause equilibrium to be achieved between ligand and receptor unbeknownst to voltage. This is not to say that any voltage will be sufficient to create an equilibrium but that typical voltage values (most ACE studies are run in excess of 20 kV) will yield accurate values for the binding constant. In the present experiment, the extreme values for the voltage outweighed the injection time and had an effect on the experimental values for ΔRMTR. Such an interaction would not have been detectable by use of classical univariate optimization methods.

A graphical plot display of all single effects and the significant interactive effect in relation to ΔRMTR leverage

residuals is shown in Fig. 4. Such a plot allows closer examination and maximum insight into how the fit carries the data and shows for each point what the residual would be both with and without that effect in the model. This type of examination was absent in the first chemometric-based ACE study [46]. Note that the effect in the current plot is tested for significance by comparing the sum of squared residuals to the sum of squared residuals of the model with that effect removed. As with the whole model leverage plot above, when the confidence curves cross the line, the effect is considered significant. This is evident in Figs. 4B–D which depict the leverage plots for voltage, neutral ligand and the interactive effect of injection time*voltage. The former two proved to be significant single effects in the Effects test and the latter being the only significant interactive effect.

3.2 Response surface model and validation studies

The quadratic model (Eq. 3) allowed the generation of the 3-D response surface image (Fig. 5) for the main interaction between injection time and voltage. The quadratic terms in this equation models the curvature in the true response function. The shape and orientation of the curvature results from the eigenvalue decomposition of the matrix of second-order parameter estimates. After the parameters are estimated, critical values for the factors in the estimated surface can be found. For this study, a posthoc review of our model revealed optimum critical values of injection time = 2.3 min, voltage = 11.6 kV, and [L₀] = 1.4 μM.

The generated optimized model was then validated experimentally by a representative series of replicate (n = 6) electropherograms (Fig. 6) of CAB and markers (HHM and MO) in capillaries partially filled with increase in [L₀] = 1.4 μM run at the conditions of injection time =

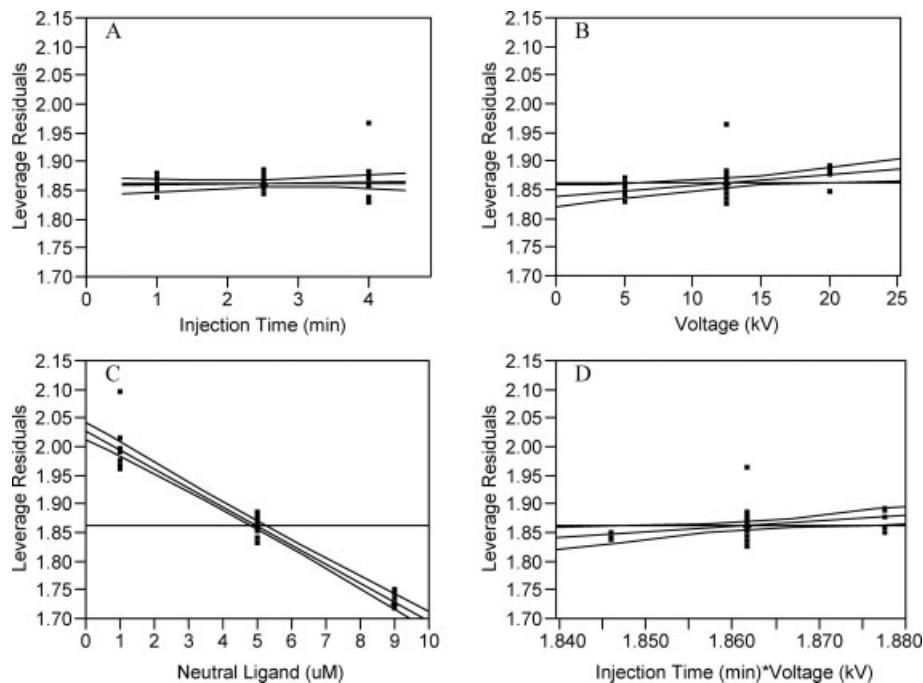


Figure 4. Graphical leverage plot display of effect significance showing for each point what the residual would be both with and without the effect: (A) injection time, (B) voltage, (C) neutral ligand, (D) the interactive effect injection time*voltage.

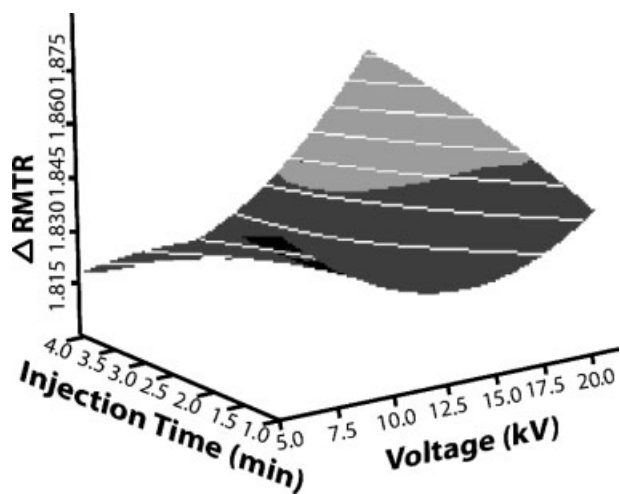


Figure 5. Response surface generated plot showing the main interactive effect injection time*voltage.

2.3 min and voltage = 11.6 kV. Under these conditions, the experimental and model predicted had a percent discrepancy difference of only 10.1%. The (+) is an isozyme (CAA) of CAB and exists in the sample purchased from the vendor. CAB is a zinc protein of the lyase class that catalyzes the equilibration of dissolved carbon dioxide and carbonic acid. It is strongly inhibited by sulfonamide-containing molecules [48]. We chose the CAB system for several reasons: (i) it does not absorb to the walls of the uncoated capillaries; (ii) it is commercially available and inexpensive; (iii) we have data describing its electrophoretic behavior in other circum-

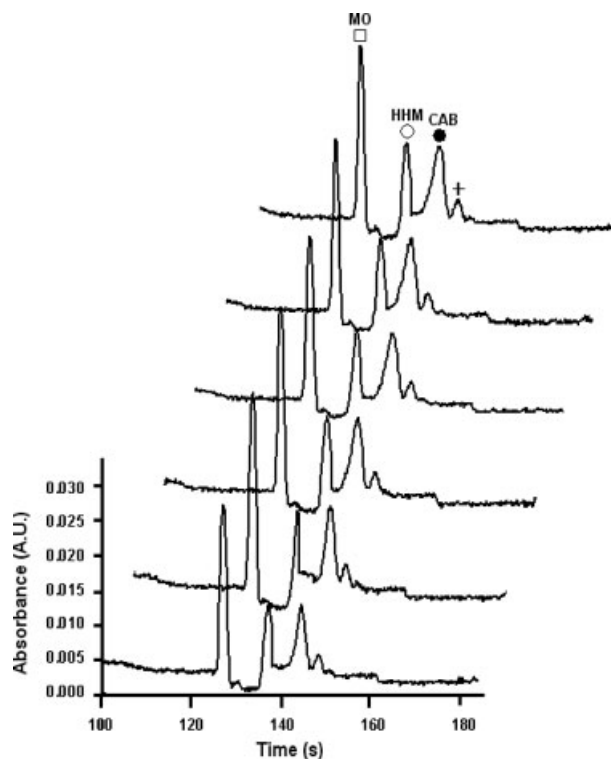


Figure 6. A representative set of stacked electropherograms of CAB in 0.192 M glycine-0.025 M Tris buffer (pH 8.3) containing 2 (1.4 μM) using the competitive binding FTPFACE technique. The total analysis time in each experiment was 3.0 min at 11.6 kV (current, 6.0–13.6 μA) using a 30.5 cm (inlet to detector), 50 μm id open, uncoated quartz capillary. MO (open square) and HHM (open circle) were used as internal standards.

stances; (iv) ligands for it can be easily synthesized; and (v) many ligands bind to it with values of K_b between 10^5 and 10^9 M^{-1} .

The generated model predicted optimal conditions were further validated experimentally by an ACE experiment. Figure 7 is a representative series of electropherograms of CAB in capillaries partially filled with increasing concentrations of L_o at the conditions of injection time = 2.3 min, voltage = 11.6 kV. At the point of detection, separate peaks for CAB, HHM, and MO are observed. The complex that forms between CAB and L_o is more negatively charged than

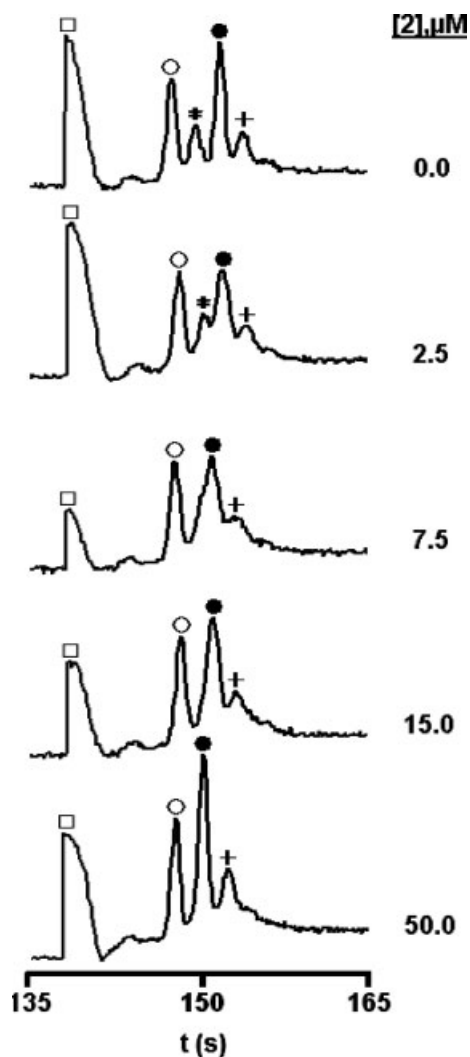


Figure 7. A representative series of electropherograms of CAB (darkened circle) in 192 mM glycine–25 mM Tris buffer (pH 8.4) containing various concentrations of $[L_o]$ using the FTPFACE technique. The total analysis time in each experiment was 4.0 min at, 11.6 kV (current 1.7–2.7 μA) using a 47-cm (inlet to detector), 50 μm id open, uncoated quartz capillary. MO (open square) and HHM (open circle) were used as internal standards. The asterisk (*) and cross (+) are discussed in the text. CAA (+) is an isozyme of CAB and gives values of K_b indistinguishable from CAB using the FTPFACE technique.

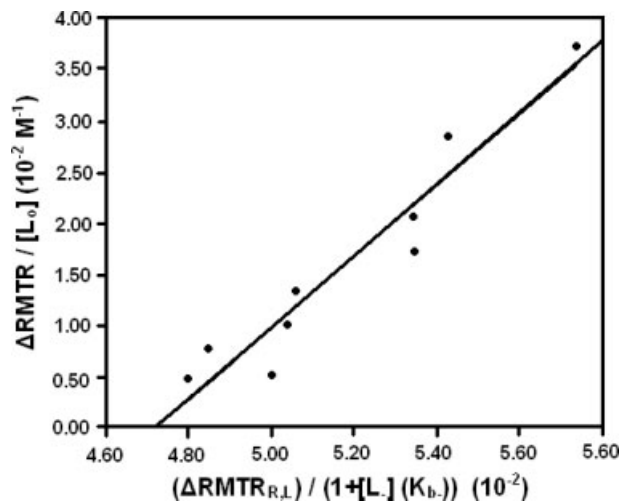


Figure 8. Scatchard plot of the data for CAB according to Eq. (2).

CAB complexed to L_o and, hence, the peak for the CAB– L_o complex shifts to shorter migration time on increasing the concentration of $[L_o]$ partially filled in the capillary column. A fourth peak (designated with an asterisk (*)) appears under the original CAB peak and is designated as inactive CAB. This inactive CAB does not effect the measurement of a K_b . The difference in peak height of MO and HHM with increase in $[L_o]$ is the result of the ramping up of voltage from the three levels, –, 0, +, to the final voltage of 25 kV of which electrophoresis continues. The (+) is an isozyme (CAA) of CAB and gives values of K_b indistinguishable from CAB using the FTPFACE technique. Figure 8 is the linear plot of the data for CAB and L_o using Eq. (2).

4 Concluding remarks

This paper demonstrates the successful estimation of binding constants for neutral ligands to CAB using competitive FTPFACE in a multivariate chemometric response surface design approach. Here, we used a two-marker form of analysis (RMTR) that we have found can be more universally applied in ACE analysis. Whereas the original ACE investigation used K_d ($K_d = 1/K_b$) as the response for the chemometric study, the present work used RMTR instead which was a major goal of the study. The change in RMTR, upon varying the concentration of neutral ligand, was modeled *via* a Box–Behnken design and compared to values obtained from both individual neutral ligand concentrations and, furthermore, in a subsequent ACE competitive assay to validate the chemometric work. This model effectively predicted the significance of injection time, voltage and $[L_o]$ on protein–neutral ligand binding and was validated by a series of experimental runs. The predicted results were in good agreement with experimental data. This work uniquely showed that RSM can model ACE studies using different

forms of analysis and K_d and K_b interchangeably, thereby demonstrating the universal power of chemometric methodologies. In the future, the detailed chemometric tools will be applied to other receptor–ligand combinations.

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