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Research Article

Chemometrical examination of active parameters and interactions in flow injection-capillary electrophoresis

The first detailed examination of flow injection-capillary electrophoresis (FI-CE) active parameters and their interactions *via* response surface methodology (RSM) is presented. Specifically, RSM in the form of a Box–Behnken design was implemented to effectively predict the significance of capillary length, voltage and injection volume on the optimization of an in-house built FI-CE analyzer. Initial studies were performed assessing peak height and peak shape of the model compound *N,N*-dimethylformamide. Optimum model conditions were then derived and used in the model separation of two small molecules, nicotinamide adenine dinucleotide, reduced form (NADH) and benzene-sulfonamide. By implementing the RSM approach, detailed examination of active FI-CE parameters was possible, including the ability to reveal a significant interactive effect. This work is not only highly significant for advancing FI-CE developments, but instructive for investigators actively exploring other coupled analytical techniques and associated experimental parameters.

Keywords:

Capillary electrophoresis / Experimental design / Flow injection / Optimization / Response surface methodology
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1 Introduction

The depth of knowledge that can be gained from coupled analytical techniques is profound and becoming increasingly evident in routine analyses. Pioneering studies by Kubáň *et al.* [1] Fang *et al.* [2] and Arce *et al.* [3], for example, introduced the concept of coupling flow injection and capillary electrophoresis (FI-CE). These initial studies showed that many of the limitations of CE including discontinuous and biased sample introduction, fouling of the capillary walls due to matrix complications and limited amount of sample introduced in the capillary resulting in poor concentration limits of detection (LOD) can be reduced or completely eliminated upon coupling CE to an FI front end. Recent studies have built upon initial concepts, presenting state-of-the-art FI-CE technologies with a wide range of applications including biological, environmental, food, medical and pharmaceutical analyses [4–21].

A critical step in developing these methods is to simultaneously optimize the active parameters of both

techniques into a highly robust and sensitive analytical tool. Traditionally, investigators have relied exclusively on univariate approaches to optimize FI-CE techniques. Independent studies on the effects of gap width between the FI interface and the capillary inlet, carrier flow and sample volume were extensively performed [2, 3, 5]. Although successful in their optimization, univariate methods are time consuming in that the response is investigated for each factor while all other factors are held at a constant level. This approach is relatively simple and suitable for factors that are independent. However, univariate methods do not take interactive effects between factors into account, thus limiting the possibility of fully optimized separation and resolution. Recent work by our group utilized a 2³ full factorial design to investigate the main factors (flow rate, injection time and voltage) that affect the desired response (absorbance, mean of three determinations) of an in-house built FI-CE prototype [17]. While providing information identifying the key input parameters affecting the output performance (absorbance), it did not allow full characterization of such parameters, an examination of their interactions and steps needed to fully optimize such a system.

Response surface methodology (RSM) can offer significant progress in understanding the active parameters in FI-CE, their interactions and provide valuable insight into the nature of optimizing coupled techniques. RSMs are

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Abbreviations: FI-CE, flow injection-capillary electrophoresis; RSM, response surface methodology

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multivariate techniques that mathematically fit the experimental domain studied in the theoretical design through a response function [22]. They are valuable for modeling a curved quadratic surface to continuous factors and used when simple linear and interaction models are not adequate, *e.g.* experimentation far from the region of optimum conditions [23–25]. Such a methodology has proved useful in optimizing modern separation methods [26–30] including recent work in our laboratories [31–34].

This study presents the first known examination of active FI-CE parameters using RSM. It focuses on the use of a Box–Behnken design to predict the significance of capillary length, voltage and injection volume on the optimization of an in-house built FI-CE analyzer. The optimum predicted model conditions were then utilized to effectively separate two small molecules, nicotinamide adenine dinucleotide, reduced form (NADH) and benzenesulfonamide. This work is not only highly significant for advancing FI-CE developments, but instructive for investigators actively exploring other coupled analytical techniques and associated experimental parameters.

2 Materials and methods

2.1 Materials and reagents

All chemicals were used as received, unless otherwise noted. Throughout this work, the use of purified water (18.0 M Ω /cm) was obtained from a Nanopure DiamondTM water purification system (Barnstead International, Dubuque, IA, USA). Tris(hydroxymethyl) aminomethane (Tris), glycine (Gly) and DMF were purchased from the Fisher Scientific Company (Fair Lawn, NJ, USA) and were used to prepare the Tris-Gly electrophoresis buffer. The pH of the buffer solution was adjusted with 0.1 M HCl. β -Nicotinamide adenine dinucleotide, dipotassium, reduced form (NADH) and benzenesulfonamide were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Stock solutions of each analyte were prepared by dissolving samples in 192 mM Tris-25 mM Gly buffer solution at pH 8.34. Rinsing of the capillary was done by injecting 0.1 M HCl, purified water (18.0 M Ω /cm), 0.1 M NaOH, and Tris-Gly buffer (pH 8.35) manually by using a 500 μ L syringe. This procedure was repeated twice with a total rinsing time of 12 min.

2.2 Instrumentation

The FI-CE analyzer utilized as a basis for this study was previously described in detail [17]. Unique to the current study is the incorporation of miniature solenoid activated pumps and switching valves (Bio-Chem, Inc., Boonton, NJ, USA) to enhance sample delivery and automate injection timing sequences. All sequences were controlled via an in-house LabVIEW written graphical program (National Instruments, Austin, TX, USA). The in-house-designed,

precisely machined plexiglass FI-CE interface was modified to allow better positioning of the electrodes and sample inlet. A high-voltage power supply (Glassman High Voltage, Inc., High Bridge, NJ, USA), miniature CCD spectrometer and DT Mini UV source (Ocean Optics, Dunedin, FL, USA) and 2 \times 200 μ m fiber optic cables were incorporated to complete the functional unit. Narrow-bore polyetheretherketone tubing (1/32 in od and 0.008 in id) was employed for all sample/reagent lines between the solenoid pumping manifold and the FI-CE interface. All connections were performed with the use of suitable flangeless fittings. Filter screens with a pore size of 0.2 μ m were placed at all inlets into the system to reduce the chance of column blockages.

2.3 Optimization study

DMF (0.014285 mg/mL) was diluted with a buffer (192 mM Tris-25 mM glycine, pH 8.34) and used for chemometric analysis for the search of optimum conditions. The running buffer was injected using the automated solenoid pumps at 0.0178 mL/min at 0.5 cycles/s. Conditions used were capillary length (35, 45, 55 cm), separation voltage (5, 7.5, 10 kV) and injection volume (6, 24, 42 μ L).

2.4 Model separation

Benzenesulfonamide (0.01785 mg/mL) and NADH (0.0625 mg/mL) were used for the analysis of unoptimized (capillary length = 45 cm, voltage = 5 kV and injection volume = 23.73 nL) and optimized (capillary length = 45 cm, voltage = 7.5 kV and injection volume = 40.13 nL) separation conditions using 192 mM Tris-25 mM glycine buffer solution (pH 8.34) at a wavelength of 220 nm. For both conditions, a 5 min separation and a 45 cm capillary length with an inner diameter of 75.0 μ m (Polymicro Technologies, Inc., Phoenix, AZ, USA) were utilized.

2.5 Experimental design

A Box–Behnken response surface design was employed to investigate the effects of capillary length, voltage and injection volume on the peak shape and peak height of DMF. This design is considered an efficient option in RSM and an ideal alternative to central composite designs [22]. Overall, it combines a fractional factorial with incomplete block designs to avoid the extreme vertices and to present an approximately rotatable design with three levels per factor. Table 1 lists the three FI-CE factors and levels selected in which experimental optimization, in terms of overall response (absorbance), could be performed. Factors and ranges considered in this study were based on previous univariate studies and factorial screening methods [17]. A fixed carrier (separation buffer) flow rate of 0.18 mL/min was utilized for all experiments.

2.6 Response surface methodology

The generalized model used in this study had the following quadratic form:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

which contains linear terms for all factors, squared terms for all factors and products of all pairs of factors. In this study, for example, X_1 , X_2 and X_3 terms correspond to capillary length, voltage and injection volume as they relate to predicting absorbance. In the above equation β is the coefficient, akin to a regression coefficient and giving a measure of the rate of change in absorbance per unit change in capillary length, voltage or injection volume.

Our data were analyzed using JMP (SAS Institute) statistical software. 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. These components are then used to compute an F -ratio that evaluates the effectiveness of the model. If the probability associated with the F -ratio is small, then the model is considered a better statistical fit for the data than the response mean alone. In our calculations we assumed that

higher-order interaction terms did not contribute significantly to the behavior of our statistical model.

3 Results and discussion

3.1 Design matrix and linear model

The design matrix generated for the Box–Behnken study is given in Table 2, and the system was fully optimized using the 15 experiments described within. Here, three center points, experiment numbers 7–9, were incorporated to compute an estimate of the error term that does not depend on the fitted model. Included in Table 2 are the mean actual (experimental) and model predicted responses, with the quality of fit expressed by the correlation coefficient and shown visually by a whole model leverage plot (Fig. 1). The points on the plot are actual data coordinates with the horizontal line showing the sample mean of the response. Here we have multiple effects representing a partially constrained model instead of a model fully constrained to a single mean value. Overall, an r^2 value of 0.82 was obtained with a mean ($n = 45$) predicted response (absorbance) of 0.1249.

The basic calculations for the linear model are shown in the ANOVA analysis (Table 3). ANOVA for a linear regression partitions the total variation of a sample into components, which are used to compute the F -ratio. $\text{Prob} > F$ 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 often considered evidence that there is at least one significant regression factor in the model. The effect in a model is tested for significance by comparing the sum of squared residuals with the sum of squared residuals of the model with that effect removed. Residual errors that are

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

Factor	Level (–)	Level (0)	Level (+)
Capillary length (cm)	35	45	55
Voltage (kV)	5	7.5	10
Injection volume (nL)	5.93	23.73	41.53

Table 2. Box–Behnken design matrix with mean actual and model predicted responses

Experiment	Capillary length (cm)	Voltage (kV)	Injection volume (nL)	Mean actual response (absorbance) ($n = 3$)	Model predicted response (absorbance) ($n = 3$)
1	35	5	23.73	0.13814	0.13351
2	55	5	23.73	0.12669	0.11094
3	35	10	23.73	0.13668	0.14110
4	55	10	23.73	0.13748	0.12308
5	45	7.5	5.93	0.03942	0.05566
6	45	7.5	41.53	0.16647	0.20011
7	45	7.5	23.73	0.14446	0.12788
8	45	7.5	23.73	0.14917	0.12788
9	45	7.5	23.73	0.15161	0.12788
10	45	5	23.73	0.14253	0.12223
11	45	10	23.73	0.14576	0.13354
12	35	7.5	5.93	0.04524	0.05453
13	55	7.5	5.93	0.03041	0.06084
14	45	5	41.53	0.16845	0.17907
15	55	7.5	41.53	0.15676	0.17318

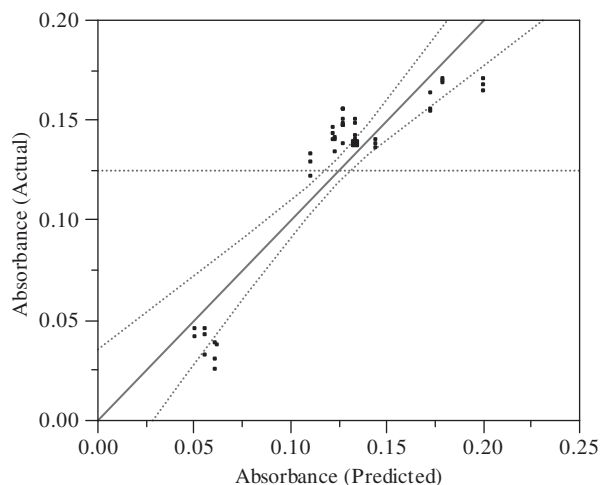


Figure 1. Whole model leverage plot of actual vs. model predicted response values.

Table 3. ANOVA table for the linear model

Source	DF ^{a)}	Sum of squares	Mean square	F-ratio ^{b)}
Model	9	0.09251	0.01027	476.81
Error	35	0.00075	0.00002	Prob > F
C. Total ^{c)}	44	0.09326		<0.0001

a) DF = degrees of freedom.

b) F-ratio = ratio of the mean square for *lack of fit* to the mean square for *pure error*.

c) The total (C. Total) is the sum of squared distances of each response from the sample mean.

much smaller when the effect is included in the model confirm that the effect is a significant contribution to the fit.

An examination of Prob>F from the effect test results (Table 4) revealed that all single factors (capillary length, voltage and injection volume) had significant effects on absorbance with injection volume having the greatest significance. Interestingly, capillary length*voltage showed a strong interactive effect on the response that would not have been detected by traditional univariate methods alone. Graphical displays of the significant single effect (injection volume) and interactive effect (capillary length*voltage) in relation to absorbance leverage residuals are shown in Fig. 2. Such a plot allows 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. Note that the effect in such a model is tested for significance by comparing the sum of squared residuals with 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 Fig. 2A (injection volume) and to a lesser extent (but significant as shown by the effect test results) in Fig. 2B (capillary length*voltage).

Table 4. Effect Test results for active parameters

Source	DF	Sum of squares	F-ratio	Prob > F
Capillary length	1	0.00036	17.0564	0.0002
Voltage	1	0.00012	5.6127	0.0235
Injection volume	1	0.04990	2314.773	<0.0001
Capillary length*voltage	1	0.00014	6.7472	0.0136
Injection volume*voltage	1	0.00005	2.6909	0.1099
Capillary length*injection volume	1	0.00004	1.9241	0.1742

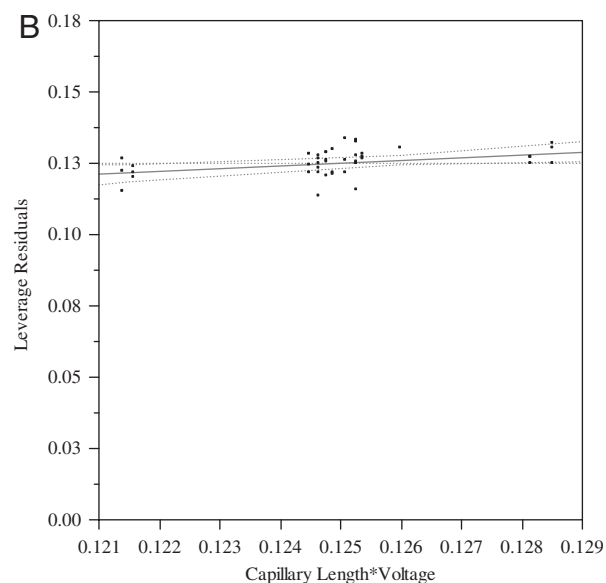
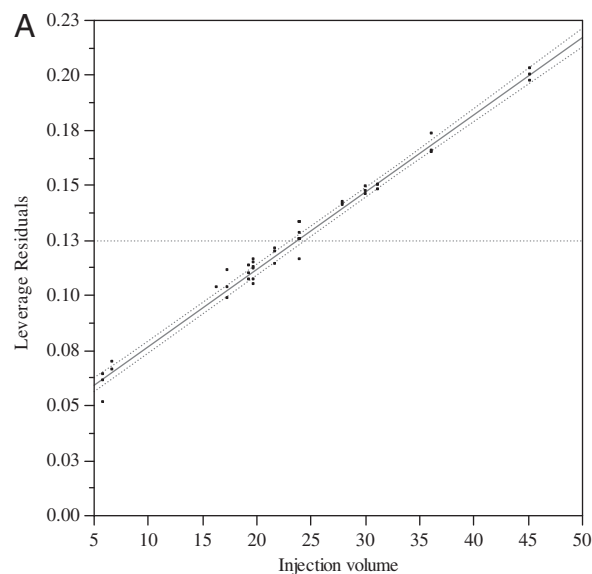


Figure 2. Graphical leverage plot display of effect significance showing for each point what the residual would be both with and without the effect: (A) injection volume and (B) capillary length*voltage.

3.2 Response surface examination and validation studies

The quadratic model (Eq. (1)) allowed the generation of the 3-D response surface image (Fig. 3) for the main interaction between capillary length and voltage. The quadratic terms in this equation model the curvature in the true response function. The shape and orientation of the curvature result 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 *post hoc* review of our model revealed optimum critical values of capillary length = 45 cm, voltage = 7.5 kV and injection volume = 40.13 nL.

The generated optimized model was then validated experimentally by a representative series of replicate ($n = 5$) electropherograms of DMF (in triplicate injections) run at the optimum predicted conditions. Table 5 presents the reproducibility results in terms of peak height (absorbance units) from the series of experiments. As shown, excellent RSD values (<2.0%) were achieved for all experiments. Under these conditions, only a 7.9% discrepancy difference between the experimental values and the values obtained using the model was obtained. Moreover, we found the (LODs) for the injected compounds to be approximately

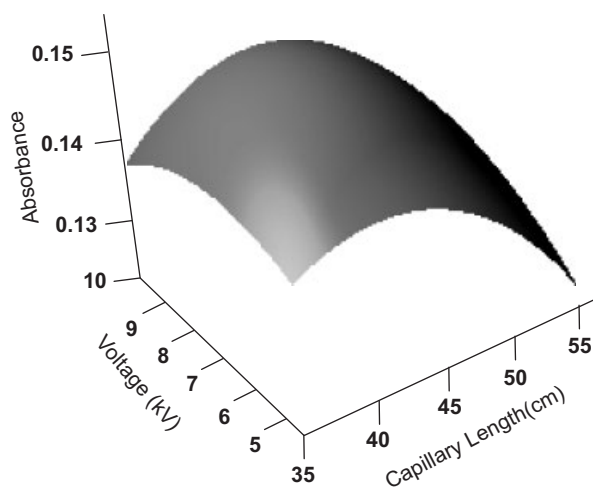


Figure 3. Response surface generated plot showing the interactive effect capillary length*voltage.

Table 5. Reproducibility results from the replicate series of DMF injections

Experiment	Peak height (replicate 1)	Peak height (replicate 2)	Peak height (replicate 3)	Mean ($n = 3$)	RSD (%)
1	0.183407	0.185031	0.184783	0.184407	0.47
2	0.180578	0.178111	0.179863	0.179517	0.70
3	0.181203	0.176190	0.181473	0.179622	1.65
4	0.175247	0.177532	0.179562	0.177447	1.21
5	0.181870	0.187122	0.185879	0.184957	1.48

50 μM . This value is similar to the LODs obtained from our earlier work with like molecules using a commercial instrument.

3.3 Model separation studies

The generated model predicted optimal conditions were further validated experimentally by the model separation of NADH and benzenesulfonamide. Selection of unoptimized conditions (capillary length = 45 cm, voltage = 5 kV and injection volume = 23.73 nL) from the contour profile function of the RSM approach yielded less than the ideal separation conditions (Fig. 4A). As can be seen from the electropherogram, the length of the experiment is long, signal-to-noise is poor, thereby not lending to accurate measurement of migration time for several of the peaks, and the baseline is decreasing. However, noticeable improvements were obtained when utilizing the model optimum conditions (Fig. 4B). In this electropherogram, the peaks for both compounds are symmetrical, thereby making measurement of migration times facile. A greater absorbance is observed as compared with the unoptimized conditions and the length of the experiment is shorter than that in Fig. 4A. Table 6A and B presents the reproducibility results in terms of peak height (absorbance units) from the unoptimized and optimized conditions, respectfully.

4 Concluding remarks

This investigation presented significant progress in understanding active parameters and their interactions in the expanding area of FI-CE. The predicted nature of a validated response surface design allowed successful determination of significant single and interactive effects to aid in overall optimization. Successful separation of NADH and benzenesulfonamide was then shown by the use of the optimized conditions predicted by the model. The limits of detection obtained in this study were comparable to commercial instruments when UV-Vis was the detection system. Given the need for high-throughput techniques that also utilize minute quantities of sample, the present study lays the groundwork for the use of FI-CE in a variety of applications including DNA, protein and other small

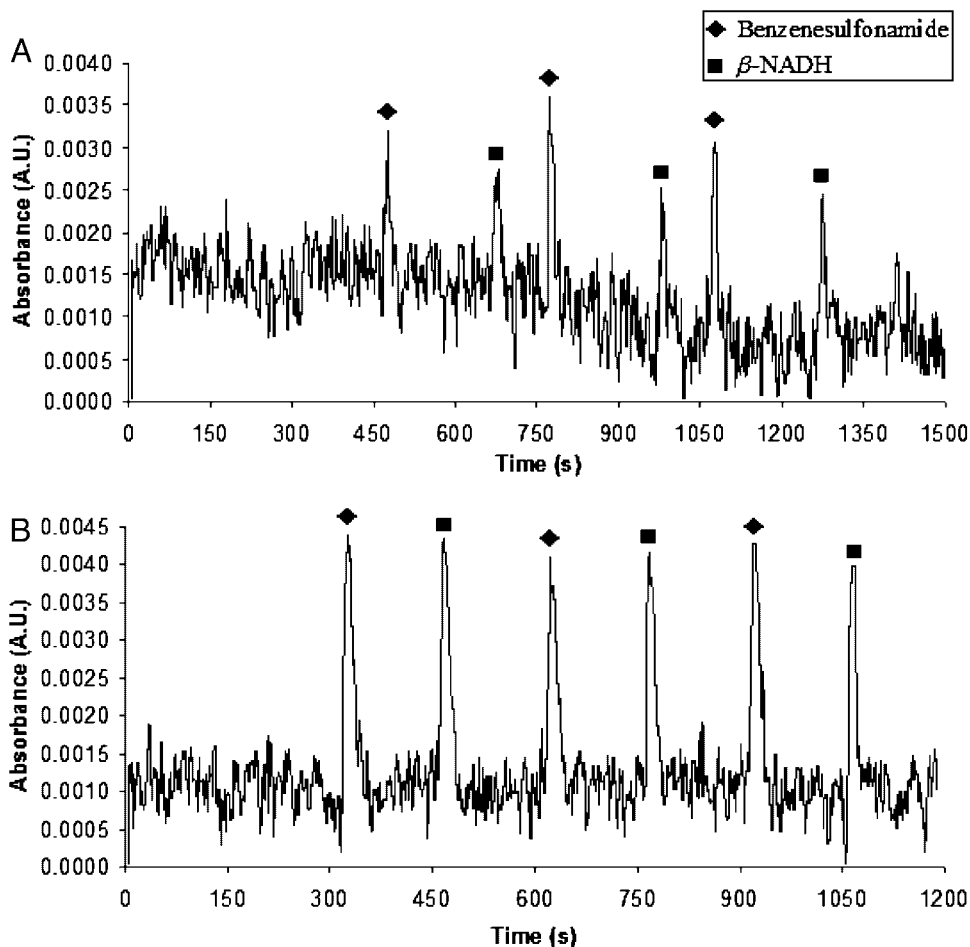


Figure 4. Representative electropherograms showing the separation of benzenesulfonamide (0.01785 mg/mL) and NADH (0.0625 mg/mL) in 192 mM Tris-25 mM Gly buffer solution (pH 8.34) using (A) unoptimized (capillary length = 45 cm, voltage = 5 kV and injection volume = 23.73 nL) and (B) optimized conditions (capillary length = 45 cm, voltage = 7.5 kV and injection volume = 40.13 nL). Injection times for the three replicates were 0 min (initial injection) and 5.00 min and 10.00 min intervals thereafter.

Table 6. Reproducibility results from the model separation study: (A) unoptimized conditions; (B) model optimized conditions

Compound	Peak height (replicate 1)	Peak height (replicate 2)	Peak height (replicate 3)	Mean ($n = 3$)	RSD (%)
(A)					
Benzenesulfonamide	0.002215	0.002566	0.002368	0.002383	7.38
β -NADH	0.001710	0.001951	0.002236	0.001966	13.40
(B)					
Benzenesulfonamide	0.004197	0.003516	0.003709	0.003807	9.21
β -NADH	0.003474	0.003770	0.003758	0.003667	4.56

molecule analyses. The current work is focused on exploring other CE methods that require expeditious and reproducible analysis.

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