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Research Article

Response surface examination of the relationship between experimental conditions and product distribution in electrophoretically mediated microanalysis

This work presents the first known use of response surface methodology (RSM) in electrophoretically mediated microanalysis. This concept is demonstrated by examining the optimization of reaction conditions for the conversion of nicotinamide adenine dinucleotide to nicotinamide adenine dinucleotide, reduced form by glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) in the conversion of glucose-6-phosphate to 6-phosphogluconate. Experimental factors including voltage, enzyme concentration, and mixing time of reaction at the applied voltage were selected at three levels and tested in a Box–Behnken response surface design. Upon migration in a capillary under CE conditions, plugs of substrate and enzyme are injected separately in buffer and allowed to react at variable conditions. Extent of reaction and product ratios were subsequently determined by CE. The model predicted results are shown to be in good agreement (7.1% discrepancy difference) with experimental data. The use of chemometric RSM provides a direct relationship between electrophoretic conditions and product distribution of microscale reactions using CE, thereby offering a new and versatile approach to optimizing enzymatic experimental conditions.

Keywords:
CE / Electrophoretically mediated microanalysis / Optimization / Response surface methodology

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1 Introduction

In the past two decades, CE has emerged as a powerful and versatile separation tool due to its high sensitivity, resolution, and ability to detect minute quantities of samples [1–11]. CE differentiates charged species on the basis of mobility differences under the influence of an applied electric field. This remarkable versatility coupled with a mechanism of analyte resolution that is complimentary to more traditional techniques, such as HPLC, has made it the technique of choice in many analytical laboratories especially when analysis of small sample volumes is required.

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Abbreviations: EMMA, electrophoretically mediated microanalysis; **G6P,** glucose-6-phosphate; **G6PDH,** glucose-6-phosphate dehydrogenase; **MO,** mesityl oxide; **NAD,** nicotinamide adenine dinucleotide; **NADH,** nicotinamide adenine dinucleotide, reduced form; **RSM,** response surface methodology

The ability of CE to analyze minute quantities of material resulting from a chemical reaction was recognized early on by Regnier *et al.* [12–14] who showed that enzyme reactions could be monitored *via* a technique they termed electrophoretically mediated microanalysis (EMMA). In EMMA, differential electrophoretic mobility is utilized to merge distinct zones of analyte and analytical reagent(s) under the influence of an electric field [15–32]. The reaction is then allowed to proceed within the region of reagent overlap either in the presence or absence of an applied potential, and the resultant product is transported to the detector under the influence of an electric field (Fig. 1). This technology has been used for different biochemical applications such as for assays of enzyme activity [33], determination of Michaelis constants [20], and inhibition constants [21].

Although the use of CE and EMMA in enzyme assays is well-documented little work has focused on optimizing conditions for an enzyme assay. In open-tubular CE, other experimental parameters (mobility differences, voltage, length of plug–plug overlap, Joule heating) unique to CE must be considered when developing and optimizing conditions for assay. In earlier work, we determined a mathematical relation whereby the electrophoresis parameters, voltage

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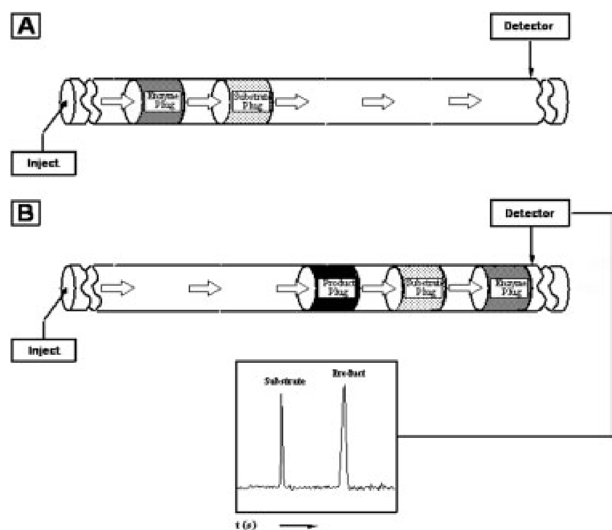

Figure 1. Schematic representation of an in-capillary enzyme-catalyzed microreactor (A) before reaction and (B) after reaction.

(*V*), enzyme concentration (*E*), and mixing time of reaction (*M*) at the applied voltage, were correlated to product ratios [17]. In this work, we used as a model system the conversion of nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dinucleotide, reduced form (NADH) in the oxidation of glucose-6-phosphate (G6P) to 6-phosphogluconate by glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49). G6PDH is considered the key regulatory enzyme of the pentose phosphate pathway with its product, NADH, playing a critical role, for example, in the defense against free radicals. More detailed information on the enzyme kinetics can be found in ref. [16]. The simulated results were shown to be in good qualitative agreement with experimental data. This work, though, used a univariate approach to optimization and did not examine interactive effects between electrophoresis parameters.

Fortunately, various chemometric multivariate techniques including experimental design and response surface methodology (RSM) have been devised to aid in optimizing the performance of a system, including studies on the optimization of CE methods [34–42]. In addition, we have recently shown the first incorporation of RSM in ACE to effectively predict the significance of experimental conditions on protein–ligand binding [43]. The current study presents the first known use of RSM in EMMA, ultimately providing investigators a new and significant approach in optimizing enzymatic experimental conditions.

2 Materials and methods

2.1 Chemicals and reagents

All chemicals were of analytical grade. G6P, G6PDH (EC 1.1.1.49), and NAD were purchased from Sigma–Aldrich and used without further purification. Mesityl oxide (MO) was purchased from Calbiochem (San Diego, CA, USA). Stock solutions of G6PDH (0.5, 2, and 3 mg/mL separately), MO (100 μL/1000 μL buffer), and NAD (2 mg/mL) were each prepared by dissolving in buffer (200 mM G6P–30 mM Tris; pH 7.85).

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 internal diameter of 50 μm, length from inlet to detector 40.5 cm, with a length from detector to outlet of 6.5 cm. In a 50 mm (id) capillary, a one second injection at low pressure (0.5 psi) using a Beckman Coulter CE instrument equals 1.2 nL of solution. Data were collected and analyzed with Beckman System Gold software.

2.3 Procedure

A sample solution (3.6 nL) containing G6PDH in buffer was introduced by pressure injection onto the capillary equilibrated with buffer. A solution (3.6 nL) of NAD and MO was next introduced by pressure injection and the electrophoresis run at a given contact voltage (1.0, 13, and 25 kV) for the desired mixing time (0.2, 0.8, and 1.4 min) then the voltage was increased to 25 kV to complete elution of all species. UV detection was performed at 260 nm.

2.4 Experimental design

A Box–Behnken design was employed to locate the optimum conditions for product distribution in EMMA by mapping the electrophoretic response surface. This design is considered an efficient option in RSM and an ideal alternative to central composite designs [44, 45]. 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 shows the three electrophoretic factors and levels selected in which experimental optimization, in terms of overall response (% conversion), could be performed. Experimental factors and levels in this study were selected on the basis of previous univariate studies [17] and electrophoretic intuition.

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

Factor	Level (−)	Level (0)	Level (+)
Mixing time (*M*) (min)	0.2	0.8	1.4
Voltage (*V*) (kV)	1.0	13	25
Enzyme concentration (*E*) (mg/L)	0.5	2.0	3.5

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2.5 RSM

RSM is designed to allow investigators to estimate interactions, thereby, providing them an idea of the shape of the response surface they are studying. This approach is often used when simple linear and interaction models are not adequate (*e.g.*, experimentation far from the region of optimum conditions) [44–49]. Here, the experimenter can expect curvature to be more prevalent requiring a mathematical model to represent the curvature. The model used in this study had the quadratic form:

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

that 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 mixing time, voltage, and enzyme concentration, respectively, as they relate to predicting % conversion. In the above equation, β is the coefficient, akin to a regression coefficient. In other words, it provides a measure of the rate of change in % conversion *per* unit change in mixing time or voltage.

Our data were analyzed in 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 (ratio of mean square for lack of fit to mean square for pure error) 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. 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.

3 Results and discussion

3.1 Experimental design

The design matrix generated for the Box–Behnken study is shown in Table 2. 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 second-order model approximating the desired response in *N*-dimensional space is obtained. Three center point experiments 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), model predicted responses, and discrepancy difference (%) between the experimental and model predicted.

Figure 2 shows the whole model leverage plot of actual *versus* predicted responses (based on all effects) with the quality of fit expressed by the coefficient of determination (r^2). This coefficient is variation in the response around the mean that can be attributed to terms in the model rather than to random error. The black points on the plot are actual data coordinates and the horizontal line (blue dashed) 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. As shown, the

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

Experiment	Mixing time (min)	Voltage (kV)	Enzyme concentration (mg/mL)	Mean experimental response (% conversion) (n = 3)	Mean model predicted response (% conversion) (n = 3)	% Discrepancy difference
1	0.2	1.0	2.0	24.1	21.2	13.6
2	1.4	1.0	2.0	24.3	22.4	8.40
3	0.2	25	2.0	26.3	22.8	10.9
4	1.4	25	2.0	24.9	22.5	10.6
5	0.8	13	0.5	8.10	4.30	88.3
6	0.8	13	3.5	38.8	32.0	21.2
7	0.8	13	2.0	30.6	30.8	0.60
8	0.8	13	2.0	32.4	30.8	5.19
9	0.8	13	2.0	29.3	30.8	4.87
10	0.8	1.0	2.0	15.0	19.7	23.8
11	0.8	25	2.0	14.7	20.5	28.3
12	0.2	13	0.5	3.60	5.90	38.9
13	1.4	13	0.5	5.40	6.80	20.5
14	0.2	13	3.5	30.1	33.9	11.2
15	1.4	13	3.5	31.0	33.9	8.55

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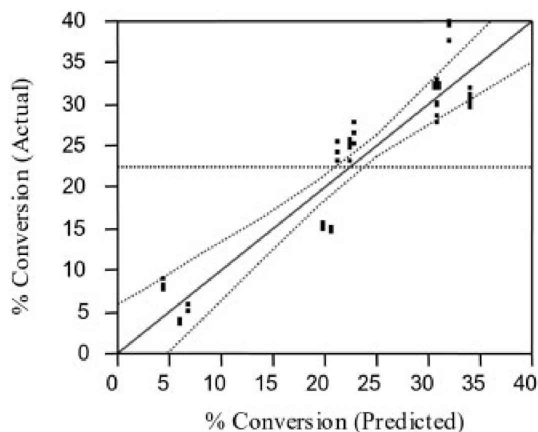

Figure 2. Whole model leverage plot of actual *vs.* predicted responses.

confidence curves (red dashed lines) cross the horizontal line, thus the test is considered significant at the 5% level. The solid red line represents the line of fit. Overall, an r^2 value of 0.87 was obtained with a mean predicted response of 22.6%.

The basic calculations for the linear model are shown in the ANOVA table (Table 3). 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. An examination of Prob>*F* from the effect test results (Table 4) revealed that enzyme concentration had a significant single effect on % conversion. This is to be expected as the extent and rate of conversion are highly dependent on the amount of enzyme used in a given assay. The other two single factors (voltage and mixing time) were not significant in our model. However, when combined, these two factors had a significant interactive effect on % conversion. Here, the extent of contact between substrate and enzyme is dictated by the difference in electrophoretic mobilities, which is in turn dictated by mixing time and voltage. Such an interaction would not have been possible by use of classical univariate optimization methods.

Table 3. ANOVA table for the linear model used in this study

Source	DF[a]	Sum of squares	Mean square	*F*-ratio
Model	9	1.676	0.1862	26.16
Error	35	0.249	0.0071	Prob>*F*
C. Total	44	1.925	–	<0.001

a) Degrees of freedom for each source of variation.

3.2 Response surface modeling

The quadratic model from the Box–Behnken design allowed us to generate a response surface image (Fig. 3) for the main interaction voltage and mixing time. Here, we assessed how the predicted responses change with respect to changing these factors simultaneously, while keeping enzyme concentration constant (2.82 mg/mL). A *post-hoc* review of our model revealed optimum critical values of: mixing time = 0.78 min, voltage = 13.2 kV, enzyme concentration = 2.82 mg/mL, and a predicted conversion of 31.2%. A series of five validation experiments using the optimum critical values were performed. A mean experimental conversion of 29.0% was obtained with a 7.1% discrepancy difference from the model predicted. The generated model was validated experimentally by a representative electropherogram (Fig. 4) showing the separation of NAD and NADH after reaction with G6PDH. Here, the critical values predicted by the model (see section above) were used. These values were utilized in an actual EMMA experiment and yielded a conversion of NAD to NADH similar to that predicted.

4 Concluding remarks

We have successfully demonstrated the use of RSM in EMMA. We used as a model system the conversion of NAD to NADH by G6PDH (EC 1.1.1.49) in the conversion of G6P to 6-phosphogluconate. Three experimental factors were examined and evaluated using a Box–Behnken response surface design. The predicted results are in good agreement with experimental data. This work provides further basis for

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

Source	DF[a]	Sum of squares	*F*-ratio	Prob>*F*
Mixing time	1	1.0837	0.0665	0.7979
Voltage	1	3.0422	0.1867	0.6682
Enzyme concentration	1	3427.9	210.39	<0.001
Mixing time/voltage	1	0.0532	7.3804	0.0102
Mixing time/enzyme concentration	1	0.6075	0.0373	0.8480
Voltage/enzyme concentration	1	1.7633	0.1082	0.7441

a) Degrees of freedom for each source of variation.

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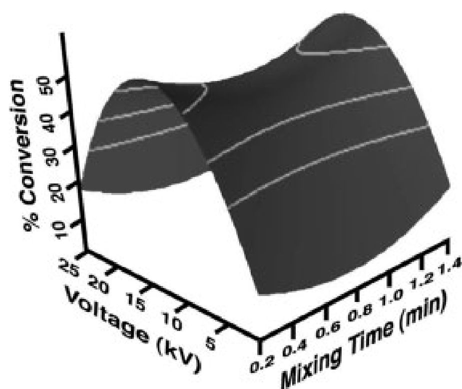

Figure 3. Response surface image for the main interactive effect of voltage/mixing time at predicted critical values with enzyme concentration kept constant.

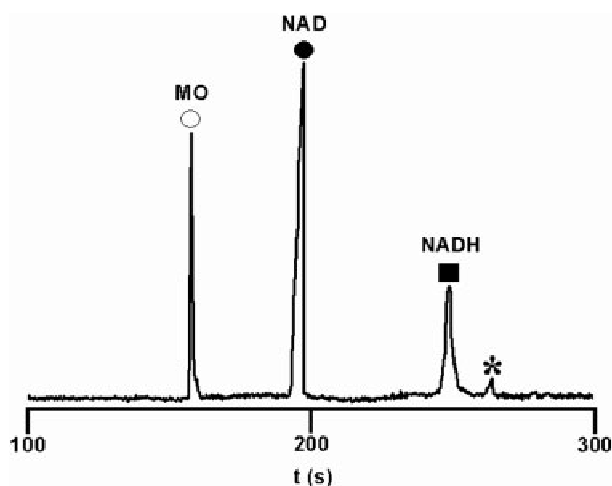

Figure 4. Representative electropherogram showing the separation of NAD and NADH after reaction with G6PDH in 30 mM Tris buffer (pH 7.85). The total analysis time in this experiment was 8.0 min at 13.2 kV (current 22.8 μA) using a 40.5 cm (inlet to detector), uncoated capillary. MO was used as an internal standard. The peak marked * is an impurity.

integrating chemometrics in CE and especially in applications where optimizing experimental conditions are time consuming, require large amounts of expensive reagents and/or where a univariate approach to optimization yields results of marginal confidence and accuracy.

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The authors have declared no conflict of interest.

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