Orthogonal Array Design for Optimizing the Capillary Zone Electrophoretic Analysis of Heterocyclic Amines

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Abstract

Orthogonal array design (OAD) has been applied to the optimization of the capillary zone electrophoretic (CZE) analysis of heterocyclic amines (HCAs), carcinogenic and mutagenic compounds that are found in cooked foods. The factors affecting separation efficiency, such as the pH of the buffer solution, organic modifier and buffer concentrations, the applied potential, and the operating temperature, are studied in two steps under OAD. In the first experiment, each variable is tested at two levels using an OA_{16} (2^{15}) matrix to determine the most important factors and the interactions between them. Based on the results of the first step, the second experiment is implemented according to a three-level OAD with an OA_9 (3^4) matrix, in which more exact values for the important variables are chosen. Finally, the determined optimum conditions for the CZE analysis of the 12 HCAs are applied and evaluated.

Introduction

Heterocyclic amines (HCAs) are mutagens and carcinogens mainly found in cooked meat products (1–4). They have been shown to have strong mutagenic and carcinogenic activities in several animal species, including monkeys (5,6). HCAs have also been implicated in human carcinogenesis (7–13). Along with other mutagenic and carcinogenic natural toxins such as aflatoxins and polycyclic aromatic hydrocarbons in food, HCAs have received considerable attention in recent years (14). The determination of HCAs in cooked food is not straightforward because of matrix interferences and because of their very low concentrations (parts per billion) in food. Several methods have been developed to determine HCAs in cooked food such as negative ion chemical ionization gas chromatography–mass spectrometry (15,16), liquid chromatography–mass spectrometry (17,18), high-performance liquid chromatography (HPLC) (19–21), and immunoaffinity chromatography (22). However, these techniques are restricted to the determination of a selected group of mutagenic HCAs, and they require sophisticated and costly equipment that is beyond the reach of many laboratories.

In capillary zone electrophoresis (CZE), several essential parameters affecting electroosmotic flow (EOF) and solute interactions with the capillary walls have to be considered if the separation of mixtures containing compounds with very similar mobilities is to be optimized. Otherwise, efficiency and resolution will be compromised. Some of these parameters are, to some extent, synergistic or antagonistic with one another within a range of variation. For example, generally speaking, the higher the voltage, the better the resolution; however, Joule heating becomes a problem at high voltages. In addition, some factors have interactions, that is, the state or value of one factor influences that of another. To develop a systematic optimization protocol, multiple factors should be considered simultaneously, and the significance of each factor and their interactions should be estimated and possibly quantitated.

To date, in most applications, the "best" CZE separation conditions are obtained by the use of simple univariate procedures (i.e., varying one parameter at a time while keeping the others constant). These trial-and-error methods are, however, often ineffective in locating the true optimum, and they tend to be time-consuming and tedious.

There are some methods that use a simultaneous multivariate optimization approach to obtain a global rather than a local optimum. For example, mixture designs and factorial designs have been developed for the optimization of HPLC separations (23–25), and these may be applied to CZE and other capillary electrophoresis (CE) modes. A Plackett–Burman statistical design has been used to optimize the resolution of testosterone esters (26). For the investigation of five factors all related to buffer composition at two levels, the method required only eight experimental trials instead of the 2^5 that would have been required by factorial design. However, the reduction in the number of experiments was achieved by focusing only on the main parameters, and possible effects caused by interaction between parameters were ignored. In addition, no fixed rules existed for the selection of lower and upper limits of the parameter values used in the optimization procedure.

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Mixture designs such as the overlapping resolution mapping (ORM) scheme have been adapted for the optimization of CE separation. A two-dimensional rectangular ORM approach was used to optimize two parameters by implementing seven trials (27,28), and a three-dimensional scheme was used for micellar electrokinetic capillary chromatography (29). Although the ORM scheme is capable of locating the global optimum within the selected range of experimental conditions, the significance of the different factors considered is not clear, and only three variables related to the composition of the electrolytic system can be considered each time. In addition, an appropriate computer program is necessary for the data analysis.

Another approach for optimizing CZE–CE separations is to use the orthogonal array design (OAD) procedure. OAD has been applied to analytical chemistry since 1989 (25,30–32), and studies done by our group include chromatographic separations (33,34), solid-phase extraction (35), microwave dissolution of biological samples (36–38), polarography (39,40), and recently, CZE in which OAD was applied as a preliminary factor screening step to identify critical separation parameters before the use of ORM (41).

As a chemometric approach, OAD has some advantages over other techniques in that orthogonal arrays are used to assign factors (the analytical parameters) to a series of trial combinations whose results can then be analyzed using a common mathematical procedure. OAD is a fractional factorial design method, but the number of the experiments can be reduced considerably because when the effect for one factor is calculated, the influence of the others is taken out of consideration. This is achieved by a well-balanced assignment of all factors. The interactions between the factors can be assigned as independent factors and can easily be evaluated quantitatively by using the associated triangular table. Emphasis is placed on identifying controlling factors and quantitating the magnitude of effects rather than just identifying statistically significant effects. In addition, the technique can deal with both continuous and discrete factors.

In the present study, OAD was used to systematically examine the factors that affect the separation of 12 HCAs by CZE.

Experimental

Apparatus

Analyses were performed using an HP^{3D} capillary electrophoresis system (Waldbronn, Germany) with a UV–vis diode-array detector. The unmodified fused-silica tubing used for separation was 51 cm in length; the effective length was 42.5 cm. A constant potential of 20 kV was applied. UV absorbance was monitored at 190, 220, 240, and 263 nm. Pressurized injection was carried out at 5 mbar·s. The capillary temperature was maintained at 25°C.

Reagents

Chemicals and solvents were of HPLC or analytical grade. Water was obtained from a Milli-Q water purification system (Millipore; Bedford, MA). 2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) were kindly provided by Dr. K. Wakabayashi, National Cancer Center Research Institute, Japan. 2-Amino-4-methylimidazo[4,5-*f*]quinoline (Iso-IQ), 1-methyl-9H-pyrido[3,4-*b*]indole (H), 9H-pyrido[3,4-*b*]indole (NH), 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-1-methyl-6-phenlyimidazo[4,5-*b*]pyridine (PhIP), 2-amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-2), and 2-amino-9H-pyrido[2,3-*b*]indole (AαC) were purchased from Toronto Research Chemicals (Toronto, Canada). They were dissolved in methanol at 250 ng/µL, and a mixture solution containing 10 ng/µL of each compound in methanol prepared from this stock was used as the working solution. Stock solutions of 1M Na_2HPO_4, 1M citric acid, and 1M NaCl were obtained by dissolving them in water; the separation buffer was freshly prepared just before use. The buffer pH was adjusted with H_3PO_4.

Optimization criteria

An electropherogram is more than a simple unique number in one dimension; therefore, proper optimization criteria, which can be the objective goals of an optimization process, are imperative. As in a chromatogram, the quantitation of the amount of separation in an electropherogram can be seen as an expansion of the characterization of the separation achieved for each pair of peaks; at the same time, the number of distinguishable peaks detected and the analysis time should be reflected in the criteria. A specific form of the CRF (chromatographic response function) was developed (42–44) for the automated optimization of reversed-phase HPLC separations:

$$\mathrm{CRF} = \Sigma R_i + n^a - b\,|T_A - t_{RL}| - c\,(t_0 - t_{R1}) \qquad \text{Eq 1}$$

where R_i is the resolution between adjacent peak pairs; n is the number of peaks detected; t_{R1} is the retention time of the first peak; t_0 is a specified minimum desired retention time for the first detected peak; T_A is an acceptable analysis time; t_{RL} is the retention time of the last detected peak; and a, b, and c are weighting factors selected by the operator that are usually set in the range 0–3.

In the present work, Equation 1 was modified for CZE (Equation 2), and values of a = 1.5, b = 1.5, c = 1, T_A = 20 min, and t_0 = 5 min were used:

$$\mathrm{ERF} = \Sigma R_i + n^{1.5} - 1.5\,|\,20 - t_{RL}\,| - (5 - t_{R1}) \qquad \text{Eq 2}$$

where *ERF* is electrophoretic response function. Equation 2 was used as an optimization function to evaluate the quality of the electropherograms. In Equations 1 and 2, ΣR_i is the sum of the resolution between each pair of components.

The experimental values of the migration times can be used to calculate the resolutions (R_i) according to the equation:

$$R_i = 2\,(t_2 - t_1)/w_1 + w_2 \qquad \text{Eq 3}$$

where t_1 and t_2 are the migration times of two adjacent peaks, and w_1 and w_2 are the widths of the peaks.

Optimization strategy

Successful CZE separations are usually obtained when both EOF and solute mobility properties are optimized. Methods to control EOF include the alteration of the applied electric field; the selection of neutral hydrophilic polymer and covalent coating (45); the variation of capillary temperature; and a change in the nature of the electrolytic system such as buffer pH, ionic strength, organic modifier, and the addition of surfactants. The influences of these factors are different, and some of them are known to interact with one another. In this work, three factors related to the buffer (pH value, methanol concentration, and supporting electrolyte concentration) and two factors related to the instrument (capillary temperature and the applied potential) were investigated.

In the first experiment, 16 trials were carried out at two levels. The assignment of trials was based on a relatively large OAD, using an OA_{16} (2^{15}) matrix in which the effects of five factors and the interaction effects between pairs of them were considered.

Based on the results of the first experiment, the second experiment was implemented by using a three-level OAD with an OA_9 (3^4) matrix in which the two most influential factors were considered over a narrower range of levels. Here the interactive effects between factors were neglected.

Table I. Assignment of the Factors and Their Levels for the First Experiment

Factor	Symbol	Level 1	Level 2
pH	A	2.5	3.5
MeOH (%)	B	0	35
NaCl (mM)	C	0	30
Temperature (°C)	D	35	25
Voltage (kV)	E	20	15

Results and Discussion

Table I shows the assignment of factors and their levels for the first experiment. Sixteen experimental trials were implemented according to the matrix shown in Table II. The response of each trial was calculated according to Equations 2 and 3 and is listed in Table II. The sum of responses at each level was computed and listed as K_i in Table II. Taking factor B as an example, K_1 was the sum of responses at level 1 (trials 1, 2, 3, 4, 9, 10, 11, and 12), and K_2 was the sum of responses at level 2 (trials 5, 6, 7, 8, 13, 14, 15, and 16). The effects due to different levels of the relevant factors could be directly evalu-

Table II. Assignment of the Factors and Their Levels for the First Experiment by Using an OA_{16} (2^{15}) Matrix Along with the Responses

Trial	A	B	A×B	C	A×C	E×F with B×C	C×F with B×E	D	A×E	E	A×F	F	B×F with C×E	R*
1	1	1	1	1	1	1	1	1	1	1	1	1	1	5.08
2	1	1	1	1	1	1	2	2	2	2	2	2	2	9.33
3	1	1	1	2	2	2	1	1	1	1	2	2	2	7.78
4	1	1	1	2	2	2	2	2	2	2	1	1	1	8.02
5	1	2	2	1	1	2	1	1	2	2	1	1	2	12.25
6	1	2	2	1	1	2	2	2	1	1	2	2	1	7.66
7	1	2	2	2	2	1	1	1	2	2	2	2	1	11.34
8	1	2	2	2	2	1	2	2	1	1	1	1	2	12.21
9	2	1	2	1	2	1	2	1	1	2	1	2	2	4.04
10	2	1	2	1	2	1	1	2	2	1	2	1	1	4.97
11	2	1	2	2	1	2	2	1	1	2	2	1	1	1.36
12	2	1	2	2	1	2	1	2	2	1	1	2	2	3.37
13	2	2	1	1	2	2	2	1	2	1	1	2	1	3.58
14	2	2	1	1	2	2	1	2	1	2	2	1	2	0.46
15	2	2	1	2	1	1	2	1	2	1	2	1	2	6.90
16	2	2	1	2	1	1	1	2	1	2	1	2	1	2.10
K_1†	73.67	43.95	43.25	47.37	48.05	55.97	47.35	52.33	40.69	51.55	50.65	51.25	44.11	100.45
K_2	26.78	56.50	57.20	53.08	52.40	44.48	53.10	48.12	59.76	48.90	49.80	49.20	56.34	
Δ‡	46.89	12.55	13.95	5.71	4.35	11.49	5.75	4.21	19.07	2.65	0.85	2.05	12.23	

* R is the response, which is equal to the electrophoretic response function minus 47.00.
† K_1 is the sum of responses at level 1; K_2 is the sum of responses at level 2.
‡ $\Delta = |K_1 - K_2|$.

Table III. An Analysis of Variance Table for the First Experiment

Source of variance	Sum of squares	Degrees of Freedom	Mean square	*F* value	Significance*
A (pH)	137.41	1	137.41	94.77	$p < .001$
B (MeOH)	9.84	1	9.84	6.79	$p < .05$
A × E	22.73	1	22.73	15.68	$p < .005$
A × B	12.16	1	12.16	8.37	$p < .025$
E × F and B × C	8.25	1	8.25	5.69	$p < .05$
E × F and C × E	9.35	1	9.35	6.45	$p < .05$
C (NaCl)	2.04				
A × E	1.18				
C × F and B × E	2.06				
E (temp.)	0.44	9†	1.45†		
A × F	0.04				
F (potential)	0.26				
Error (dummy)	7.00				

* The critical *F* value is 22.86 at 99.9% confidence, 13.61 at 99.5% confidence, 7.21 at 97.5% confidence ,and 5.12 at 95.0% confidence ($n_1 = 1$, $n_2 = 9$).
† Insignificant effects of factors (C, E, and F) and interactions (A × C, A × F, C × F & B × E) were pooled with the errors for *F* test evaluation.

ated by comparing the values K_1 and K_2, and the importance of factors or interactions between them could be compared with differences (Δ) between levels within the same column.

Table II shows that the level difference of factor A was the largest, indicating that buffer pH was the most important factor of those considered. The MeOH concentration (B) was also important, but the NaCl concentration (C), the capillary temperature (E), and the applied potential (F) were of less importance. The interactions between the factors, pH and temperature (A × E) as well as pH and organic modifier (A × B) were significant. E × F mixed with B × C and B × F mixed with C × E were also significant but less so, indicating that less attention could be paid to factors C, E, and F and the interactions between them. The results also show that pH 2.5 (level 1) was better than pH 3.5 (level 2), and the buffer with the organic modifier (level 2) offered a better performance than that without methanol (level 1) in terms of separation and resolution efficiency.

An analysis of variance (ANOVA) was carried out to test the significance of these effects. The results are shown in Table III.

In the OA_{16} matrix (Table II), one of the columns was assigned to factor D, a dummy used for the error estimation. In the calculation, insignificant effects and interactions were pooled with the errors so that ANOVA could be implemented.

Table III shows that factor A was the most significant factor ($p < .001$). Although factors such as C, E, and F were not as important as factors A and B, the effects of interactions between factors A and E, E and F (together with B and C), and B and F (together with C and E) were at the same level as factor B or interactions between A and B.

To maintain the trial numbers within a reasonable size, it was not necessary to distinguish the difference in these mixed effects. However, the optimal combination of these factors could still be obtained by calculation, and the results were A_1B_2 (i.e., pH 2.5 and 35% MeOH), A_1E_2 (i.e., pH 2.5 and 25°C), B_2C_2 (i.e., 35% MeOH and 30mM NaCl), E_1F_1 (i.e., 35°C and 20 kV), B_2F_1 (i.e., 35% MeOH and 20 kV), and C_2E_1 (i.e., 30mM and 35°C).

There was a contradiction in the selection of temperature. The combination of A_1E_2 indicated that 25°C was better than 35°C, but when factor E was considered with other factors such as C and F, 35°C was more favorable. Considering the greater influence of factor A and of the interaction effect between factors A and E, and the small differences for the interaction effects of E_1F_1 and E_2F_2 and C_1E_1 and C_1E_2, the conditions of 25°C, 20 kV, and 30mM NaCl were chosen, whereas pH and MeOH concentration remained to be adjusted further in the second experiment.

The adjustment of buffer pH changes the EOF, solute charge, and solute mobility. To prevent elution of a solute before separation, a reduction in EOF is necessary. In the case of weak acids or bases, the degree of ionization depends on the pH of the solution, which in turn gives rise to differences in electrophoretic and electroosmotic mobilities. Because HCAs are bases, they will be protonated at low pH.

Table IV. Assignment of the Factors and Their Levels for the Second Experiment

Factor	Symbol	Level 1	Level 2	Level 3
pH	A	2.0	2.3	2.6
MeOH (%)	B	40	30	20

Table V. Assignment of the Factors and Corresponding Levels for the Second Experiment by Using an OA_9 (3^4) Matrix along with Their Responses

	pH	MeOH (%)	Dummy	Response*
Symbol	*A*	*B*	*C*	
1	1	1	1	1.08
2	1	2	2	8.33
3	1	3	3	3.99
4	2	1	2	2.67
5	2	2	3	4.39
6	2	3	1	2.03
7	3	1	3	1.78
8	3	2	2	3.88
9	3	3	1	0.51
K_1†	13.40	5.53	3.62	28.66
K_2†	9.09	16.60	14.88	
K_3†	6.17	6.53	10.16	
Δ‡	7.23	11.07	11.26	

* Response is equal to electrophoretic response function minus 54.00.
† *K* is the sum of responses at each level.
‡ $\Delta = K_{max} - K_{min}$.

The addition of an organic modifier such as methanol improves the solubility of HCAs in the buffer, increases the pK of the surface silanol groups, and produces a corresponding decrease in the electroosmotic velocity. In addition, the alcohol interacts strongly with the capillary wall and reduces the chance of interaction between the solutes and the wall surface (46).

The ionic strength has significant effects on solute mobilities and separation efficiency. It has been observed that mobility depends inversely on buffer concentration, and the addition of NaCl reduces EOF by decreasing the thickness of the double layer (47). The addition of NaCl in the present study, however, had no appreciable effect on the resolution of HCAs within the selected variable range. It was likely that the effect has been masked by other electrolytes such as Na_2HPO_4 and citric acid in the buffer system. The addition of NaCl increased ionic strength and resulted in competition between Na^+ and protonated amines for cation-exchange sites on the silica surface, thus reducing the adsorption of HCAs on the wall.

The resolution and separation efficiency are also influenced by the capillary temperature and the applied potential because of the viscosity changes of water and the variation in electric field strength. However, the present study showed that these factors had no significant effects. This might be due to the small variation in the level selection of capillary temperature and applied potential, which were restricted by other factors and the instrument itself. However, they had strong interactions with other factors; the significance of these two parameters and interactions could be analyzed, and the optimal combinations could be obtained.

Having identified the most significant parameters in the initial experiment, we used the second experiment to concentrate on the two most important factors: buffer pH and methanol concentration. The two factors were further studied to determine the most suitable conditions, whereas levels of other factors were set according to the first experiment. Table IV shows the assignment of two factors at three levels. The results given in Tables V and VI show that when pH range was narrow, the methanol concentration became more important. The optimal combination obtained was A_1B_2, (i.e., buffer pH of 2.0 and an MeOH concentration of 30%).

Thus, from the two experiments, the most favorable conditions for the CZE analysis of HCAs might be proposed as follows: 30mM NaCl, 30% methanol, pH 2.0, capillary temperature at 25°C, potential of 20 kV (current, 58 µA), 50mM Na_2HPO_4, and 20mM citric acid. Figure 1 shows an electropherogram of the 12 HCAs obtained under these conditions. Compared with the HPLC techniques (19–21), the CZE analysis of HCAs required only half the time (less than 12 min), yet peak resolution was not compromised.

Table VI. An Analysis of Variance Table for the Second Experiment

Source of variance	Sum of square	Degrees of Freedom	Mean square	*F* value	Significance*
A (pH)	8.82	2	4.41	1.70	$p < .25$
B (%MeOH)	24.99	2	12.50	4.81	$p < .10$
Pooled error	10.4	4	2.60	–	–

*The critical *F* value is 4.32 at 90% confidence and 2.00 at 75.0% confidence ($n_1 = 2$, $n_2 = 4$).

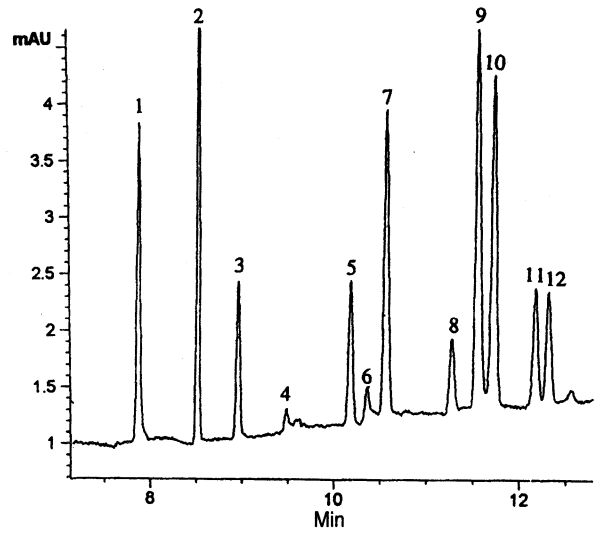

Figure 1. Electropherogram for standard mixture solution of 12 HCAs. Peaks: 1, Iso-IQ; 2, IQ; 3, NH; 4, Glu-P-1; 5, H; 6, Trp-P-1; 7, Glu-P-2; 8, AaC; 9, Trp-P-2; 10, MeIQx; 11, DiMeIQx; and 12, PhIP.

Conclusion

To the best of our knowledge, this work represents the first time optimization of a CE separation was carried out in its entirety by OAD. The usefulness of a simple systematic multivariate optimization scheme for the CZE separation of HCAs has been demonstrated. Although systematic optimization is conditional and the optimal conditions are restricted by the experimental conditions available, the most influential factors can be established using the OAD technique. The optimal combination of these factors can be selected, their effects can be evaluated, and finally, they can be applied to the analysis of actual samples.

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