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# Combination of orthogonal array design and overlapping resolution mapping for optimizing the separation of heterocyclic amines by capillary zone electrophoresis

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**Abstract**

This paper describes the application of combined orthogonal array design and overlapping resolution mapping to the optimization of capillary zone electrophoresis for the separation of heterocyclic amines. The factors affecting resolution, such as buffer pH, organic modifier, concentration of buffer solution, capillary tubing temperature, and electric field strength, were studied in two steps. In the first step, orthogonal array design was used to determine the most important factors and interactions. The experiments were carried out according to an $OA_{16}$ ($2^{15}$) matrix through sixteen pre-designed trials. Based on the results of the first step, the second set of experiments was performed according to a three-dimensional overlapping resolution mapping scheme, in which eleven pre-planned trials were executed and global optimum conditions for the separation within a reasonable analysis time were obtained. The proposed conditions were successfully applied in the separation of thirteen heterocyclic amines.

## 1. Introduction

Since 1976, a series of mutagens and carcinogens, heterocyclic amines (HCAs), have been isolated and identified from heated amino acids or cooked meat products. Later, some of them have also been found in environmental samples [1–4]. These compounds have been found to be carcinogenic in rodents [5], non-human primates [6], and potentially carcinogenic to humans [7–13]. The knowledge of the distribution and contents of HCAs in environmental samples is essential for human health risk assessments and several methods have been developed for quantitating HCAs in various matrices. The commonly used ones are negative chemical ion GC–MS [14,15], LC–MS [16,17], HPLC [18–21], and immunoaffinity chromatography [22], but these techniques either require sophisticated and costly equipment which is beyond the reach of routine laboratories or are restricted to the determination of a selected group of HCAs.

Capillary electrophoresis (CE) has now been established as a powerful separation tool. Compared with HPLC, the technique is capable of

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achieving higher separation efficiency, uses less organic solvents, and requires small amounts of samples. However, the migration behavior of ionized compounds is less well characterized than their retention behavior in HPLC. For the separation of compounds with very similar mobilities, such as isomers and analogues, more than one parameter (e.g. modifier concentration, pH, electrolyte concentration, nature of capillary tubing, capillary temperature, electric field strength, etc.) need to be incorporated in the optimization strategy to achieve an adequate separation of such complex mixtures.

Strategies for the systematic optimization of CE are few and most of the optimum separation conditions have been achieved using simple univariate optimization procedures. These techniques have been proven to be ineffective in locating the true optimum and are time-consuming.

The simultaneous multivariate optimization approach is usually the preferred method, but the application of it is often limited by the large trial size and the requirement of a sophisticated statistical background to implement. To alleviate these problems, systematic approaches are necessary.

The Plackett–Burman statistical design [23] has been used to optimize the resolution of testosterone esters. But there were no fixed rules for the selection of levels of factors and further optimization needed to be executed before the exact experimental conditions providing optimum separation could be determined. Other techniques also suffer from the limited number of parameters which can be varied, or from the difficulty in calculating the response function.

In our previous work, orthogonal array design (OAD) has been successfully applied in the optimization of analytical procedures such as chromatographic separation [24,25], solid-phase extraction [26], and others [27–31]. Orthogonal array design has some advantages over other optimization techniques in that as one kind of factorial design it can perform simultaneous multivariate optimization. Furthermore, since it is a fractional factorial design approach, the number of trials may be reduced greatly compared to the normal factorial designs by implementing a well-balanced assignment of all the factors and the interactions among them. In that way, when the effect of a factor is calculated, the influence of the other factors is canceled out. Therefore the term "orthogonal" means "balanced", "separable", or "not mixed" in this approach. A common mathematical procedure can be used to independently extract the main effects from factors and interactions amongst them quantitatively. The number of factors investigated can be up to thirty-one [32], which is decided by the size of the trials, the complexity of the system, and to what extent one wants the information. It can deal with both continuous and discrete factors. However, in the case of continuous factors the optimum conditions determined by OAD designs are limited to some discrete points, and in some cases the global optimum level lies between these points so that subsequent optimization steps may be necessary.

One of the mixture designs, the overlapping resolution mapping (ORM) scheme, has been adapted for the optimization of CE separations [33–35]. Although the considered number of factors by the two-dimensional ORM scheme is only two [33,34] and by the three-dimensional scheme three [35], ORM is capable of locating the global optimum within the selected range of experimental conditions instead of levels or points as in other approaches, and hence is faster in locating the optimum conditions.

The purpose of the present work is to demonstrate the advantages of combining the OAD design with the ORM scheme in optimizing the separation of eleven heterocyclic amines and two co-mutagenic $\beta$-carbolines (Table 1) by capillary zone electrophoresis (CZE).

## 2. Experimental

### 2.1. Chemicals

Chemicals and solvents were of HPLC or analytical grade. Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). IQ, MeIQx, 4,8-Di-

Table 1
Heterocyclic amines used in this investigation

| Chemical name | Abbreviation |
|---|---|
| 2-Amino-3-methylimidazo[4,5-*f*]quinoline | IQ |
| 2-Amino-4-methylimidazo[4,5-*f*]quinoline | Iso-IQ |
| 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline | MeIQx |
| 2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline | 4,8-DiMeIQx |
| 2-Amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline | 4,7,8-TriMeIQx |
| 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine | PhIP |
| 3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole | Trp-P-1 |
| 3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole | Trp-P-2 |
| 2-Amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole | Glu-P-1 |
| 2-Amino-dipyrido[1,2-*a*:3′,2′-*d*]imidazole | Glu-P-2 |
| 2-Amino-9*H*-pyrido[2,3-*b*]indole | AαC |
| 1-methyl-9*H*-pyrido[3,4-*b*]indole, β-carboline | H |
| 9*H*-pyrido[3,4-*b*]indole, β-carboline | NH |

MeIQx, and 4,7,8-TriMeIQx were kindly provided by Dr. K. Wakabayashi, National Cancer Center Research Institute, Japan. Iso-IQ, H, NH, PhIP, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, and AαC were purchased from Toronto Research Chemicals (Downsview, Ont., Canada). The compounds were dissolved in methanol at 250 ng/μl as stock solution, and a mixture solution containing 10 ng/μl of each compound in methanol was prepared from the stock and used as the working solution. Stock solutions of 1 *M* $Na_2HPO_4$, 1 *M* citric acid, and 1 *M* NaCl were obtained by dissolving the appropriate salts in water, and the buffer solutions were freshly prepared before use. The pH of the buffer was adjusted by adding $H_3PO_4$.

### 2.2. Instrumentation

The analysis was performed using a BioFocus 3000 automated capillary electrophoresis system (Bio-Rad, Hercules, CA, USA) with a multi-wavelength UV detector. The uncoated silica tubing was 51 cm in length and the effective length was 46.4 cm. Constant applied voltage was 18 kV (current 38 μA). UV absorbance was monitored at 190, 220, 240, and 263 nm. The injection was by the pressure mode, 1 p.s.i. s (1 psi = 6894.76 Pa) of a standard mixture solution (10 ng/μl). The capillary temperature was 25°C and the autosampler temperature was 15°C.

## 3. Results and discussion

### 3.1. OAD scheme

The assignment of factors and their levels for the first experiment is given in Table 2 according to an $OA_{16}$ ($2^{15}$) matrix. The interaction between factors was also indicated as an independent factor in the table. Interaction is believed to occur between factors when the state or value of one factor influences the state or value of the other factor. After implementing sixteen predesigned experimental trials and obtaining the corresponding electropherograms, for each trial the resolution between each adjacent pair of peaks was calculated according to

$$R = 2(t_2 - t_1)/(w_2 + w_1) \quad (1)$$

The sums of all resolutions were calculated and are also listed in Table 2 as responses. The sums of responses at each level were computed as $K_i$ in Table 2. Taking factor B as an example, $K_1$ is the sum of responses at level 1 (trials 1, 2, 3, 4, 9, 10, 11, and 12) and $K_2$ is the sum of responses at level 2 (trials 5, 6, 7, 8, 13, 14, 15, and 16). The sum of squares for each effect was obtained by using

$$Q_A = (K_1 + K_2)/a \quad (2)$$

where $Q_A$ is the sums of squares of factor A, $a$ is

Table 2
Assignment of the factors and their levels of the first experiment by using an $OA_{16}$ ($2^{15}$) matrix along with the results of the effects of selected variables on the responses

| | *A* pH | *B* | *A* × *B* | *C* NaCl | *A* × *C* | *E* × *F*<br>*B* × *C* | *D*[a] | *C* × *F*<br>*B* × *E* | *A* × *E* | *E* Temp. | *A* × *F* | *F* kV | *B* × *F*<br>*C* × *E* | *R*[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2.5 (1) | 0 (1) | (1) | 50 (1) | (1) | (1) | (1) | (1) | (1) | 20 (1) | (1) | 20 (1) | (1) | 6.06 |
| 2 | 2.5 (1) | 0 (1) | (1) | 50 (1) | (1) | (1) | (2) | (2) | (2) | 30 (2) | (2) | 15 (2) | (2) | 10.91 |
| 3 | 2.5 (1) | 0 (1) | (1) | 0 (2) | (2) | (2) | (1) | (1) | (1) | 20 (1) | (2) | 15 (2) | (2) | 8.05 |
| 4 | 2.5 (1) | 0 (1) | (1) | 0 (2) | (2) | (2) | (2) | (2) | (2) | 30 (2) | (1) | 20 (1) | (1) | 8.20 |
| 5 | 2.5 (1) | 30 (2) | (2) | 50 (1) | (1) | (2) | (1) | (1) | (2) | 30 (2) | (1) | 20 (1) | (2) | 14.12 |
| 6 | 2.5 (1) | 30 (2) | (2) | 50 (1) | (1) | (2) | (2) | (2) | (1) | 20 (1) | (2) | 15 (2) | (1) | 8.28 |
| 7 | 2.5 (1) | 30 (2) | (2) | 0 (2) | (2) | (1) | (1) | (1) | (2) | 30 (2) | (2) | 15 (2) | (1) | 12.19 |
| 8 | 2.5 (1) | 30 (2) | (2) | 0 (2) | (2) | (1) | (2) | (2) | (1) | 20 (1) | (1) | 20 (1) | (2) | 13.36 |
| 9 | 3.5 (2) | 0 (1) | (2) | 50 (1) | (2) | (1) | (1) | (2) | (1) | 30 (2) | (1) | 15 (2) | (2) | 3.84 |
| 10 | 3.5 (2) | 0 (1) | (2) | 50 (1) | (2) | (1) | (2) | (1) | (2) | 20 (1) | (2) | 20 (1) | (1) | 5.13 |
| 11 | 3.5 (2) | 0 (1) | (2) | 0 (2) | (1) | (2) | (1) | (2) | (1) | 30 (2) | (2) | 20 (1) | (1) | 0.66 |
| 12 | 3.5 (2) | 0 (1) | (2) | 0 (2) | (1) | (2) | (2) | (1) | (2) | 20 (1) | (1) | 15 (2) | (2) | 2.67 |
| 13 | 3.5 (2) | 30 (2) | (1) | 50 (1) | (2) | (2) | (1) | (2) | (2) | 20 (1) | (1) | 15 (2) | (1) | 3.65 |
| 14 | 3.5 (2) | 30 (2) | (1) | 50 (1) | (2) | (2) | (2) | (1) | (1) | 30 (2) | (2) | 20 (1) | (2) | 0.32 |
| 15 | 3.5 (2) | 30 (2) | (1) | 0 (2) | (1) | (1) | (1) | (2) | (2) | 20 (1) | (2) | 20 (1) | (2) | 7.35 |
| 16 | 3.5 (2) | 30 (2) | (1) | 0 (2) | (1) | (1) | (2) | (1) | (1) | 30 (2) | (1) | 15 (2) | (1) | 2.09 |
| K1 | 81.17 | 45.52 | 46.63 | 52.31 | 52.14 | 60.93 | 55.92 | 50.63 | 42.66 | 54.55 | 53.99 | 55.2 | 46.26 | 106.88 |
| K2 | 25.71 | 61.36 | 60.25 | 54.57 | 54.74 | 45.95 | 50.96 | 56.25 | 64.22 | 52.33 | 52.89 | 51.68 | 60.62 | |
| Δ[c] | 55.46 | 15.84 | 13.62 | 2.26 | 2.60 | 14.98 | 4.96 | 5.62 | 21.56 | 2.22 | 1.10 | 3.52 | 14.36 | |

[a] Factor D was a dummy.
[b] Response = $R - 15.00$.
[c] $\Delta = |K_1 - K_2|$.

the number of levels, and $K_1$, $K_2$ are the sums of responses at level 1 and level 2, respectively.

Factor *D* is a dummy and was used for error estimation. During calculation, the sum of squares of the errors along with those of insignificant factors and interactions were combined and treated as the estimation of pooled error results so that the analysis of variance (ANOVA) could be conducted for data analysis.

The ANOVA results are shown in Table 3. It shows that within the selected range, buffer pH (*A*) was the most important factor (large sum of squares), and pH 2.5 was better than pH 3.5. The concentration of organic modifier (methanol) was the next important factor, and a buffer with the organic modifier (*B*) was better than one without it.

Although factors such as the concentration of supporting electrolyte (*C*), the capillary temperature (*E*), and applied voltage (*F*) were not as important as *A* or *B*, the effects of interactions between factor *A* and *E*, *E* and *F* plus *B* and *C*, *B* and *F* plus *C* and *E* are within the same order of magnitude as those of factor *B* or the interaction between *A* and *B*. In order to limit the trial number to a reasonable size, it was not necessary to distinguish the differences amongst these mixed effects. By some simple calculations, the optimum combination of these factors at certain levels could still be computed and the results of two single interactions between factors *A* and *B* along with *A* and *E* are shown in Tables 4 and 5.

The electrophoretic buffer is of key importance in CE because its composition basically determines the migration behavior of the analytes. The adjustment of pH changes electroosmotic flow (EOF), as well as the solute charge

Table 3
An ANOVA table for the first experiment

| Source of variance | Sum of square | Freedom | Mean square | $F$-value | Significance[a] |
|---|---|---|---|---|---|
| $A$ (pH) | 192.38 | 1 | 192.38 | 125.4 | $p < 0.001$ |
| $B$ (MeOH) | 15.68 | 1 | 15.69 | 10.2 | $p > 0.01$ |
| $A \times B$ | 11.59 | 1 | 11.59 | 7.6 | $p > 0.01$ |
| $E \times F$ & $B \times C$ | 14.03 | 1 | 14.03 | 9.2 | $p > 0.01$ |
| $A \times E$ | 29.05 | 1 | 29.05 | 19.0 | $p < 0.005$ |
| $B \times F$ & $C \times E$ | 12.89 | 1 | 12.89 | 8.4 | $p > 0.025$ |
| $C$ (NaCl) | 0.32 | | | | |
| $A \times C$ | 0.42 | | | | |
| $C \times F$ & $B \times E$ | 1.97 | | | | |
| $E$ (temp.) | 0.31 | 9[b] | 1.53[b] | | |
| $A \times F$ | 0.08 | | | | |
| $F$ (voltage) | 0.77 | | | | |
| Errors | 9.94 | | | | |

[a] The critical $F$-value is 22.86 at 99.9% confidence, 13.61 at 99.5% confidence, 10.56 at 99.0% confidence, and 7.21 at 97.5% confidence.
[b] The insignificant factors ($C$, $E$ and $F$) and interactions ($A \times C$, $A \times F$, $C \times F$ & $B \times E$) were pooled with the errors ($D$, dummy) for $F$-test calculation.

Table 4
The optimum combination of levels for buffer pH and capillary temperature of the first experiment ($A_1E_2$)

| | $E_1$ | $E_2$ |
|---|---|---|
| $A_1$ | 35.75 | **45.42**[a] |
| $A_2$ | 18.80 | 6.91 |

[a] This value was the best response.

and solute mobility. In CZE, both the electrophoretic mobility and electroosmotic mobility contribute to the migration velocity. To prevent elution of solutes before separation, a reduction of EOF may be necessary in certain cases. In the case of weak acids or bases, their degree of ionization depends on the pH of the solution.

Table 5
The optimum combination of levels for buffer pH and concentration of methanol of the first experiment ($A_1B_2$)

| | $B_1$ | $B_2$ |
|---|---|---|
| $A_1$ | 33.22 | **47.95**[a] |
| $A_2$ | 12.30 | 13.41 |

[a] This value was the best response.

which gives rise to differences in electrophoretic and electroosmotic mobilities. HCAs are a group of weak bases, hence they are converted to protonated species at low pH. That is why pH 2.5 provided a better separation.

The existence of the organic modifier, methanol, reduced the EOF significantly and improved the separation of HCAs to some extent. In addition, methanol improved the solubility of HCAs in the buffer, interacted strongly with the capillary wall, and therefore reduced the chance of interaction between solutes and the wall [36].

The results of both Table 2 and 3 show that factors such as concentration of buffer ($C$), applied voltage ($F$), and capillary temperature ($E$) are of less importance compared to pH ($A$) and concentration of organic modifier ($B$) in the selected experimental ranges, although all of them affected the resolution. At the same time, there were some strong interactions among these factors and therefore some of the effects were combined with each other.

Anions and cations present in the electrolyte system may affect the current and hence the electroosmotic flow, the heat generated, the interaction of analytes with the wall of the capillary, and the mobilities of the ions. The

ionic strength has significant effects on solute mobilities and separation efficiency. It has been found that addition of NaCl reduced EOF by decreasing the thickness of the double-layer [37]. In the present study, the existence of NaCl had no significant effect on the separation of HCAs within the selected variable ranges. The sum of resolutions of a buffer with 50 m*M* NaCl was even smaller than that of one without NaCl. It is likely that the effect was masked by relatively high concentrations of other electrolytes, such as $Na_2HPO_4$, in the system. But the addition of NaCl resulted in a competition between $Na^+$ and amines for cation-exchange sites on the silica surface and therefore reduced the adsorption of HCAs on the wall. The adverse effect of NaCl was that the increase of the ionic strength caused heat accumulation within the capillary tubing and therefore restricted further increment of the applied voltage.

The capillary temperature affects the resolution in two ways. First, the nature of the buffer medium will be affected by changes in temperature. As the temperature increases, the viscosity decreases and both the EOF and electrophoretic mobility increase. The mobility of most ions increases about 2%/°C [38]. Second, if the temperature gradients are steep enough, density gradients in the electrophoresis buffer can be induced, which in turn cause natural convection. A convection will remix separated sample zones and reduces separation performance severely. The results show that there is no significant difference between 20 and 30°C. However, the combinations of 20°C with other factors at certain levels were better than those of 30°C with the same factors.

The efficiency of separation is directly proportional to the capillary length, provided the field strength is kept constant. In other words, for a certain length of tubing, a high field strength, i.e. a high voltage, results in better resolution. In practice, the voltage is limited by the design of the instrument and the heat produced within the capillary.

In this work, voltage was not the most critical parameter in the resolution of the HCA mixture, according to the OAD. Rather, after pH and concentration of organic modifier, it was the third most important factor. However, it is acknowledged that this study considered only one specific category of compounds, and this observation may not be applicable to other compound classes.

### 3.2. ORM scheme

Based on the results of the first experiment, the optimum combination of minor conditions was selected. In the second set of experiments, emphasis was placed on the use of the three-dimensional ORM to optimize the important factors, namely pH and the concentration of methanol, along with another factor, the concentration of $Na_2HPO_4$, which had not been taken into consideration in the first experiment.

Since the principle and optimization strategy of ORM have been discussed in detail previously [33–35], only a brief description of the main steps of the method is given here. The ORM scheme consisted of a number of steps. First, the criteria for the optimum conditions were set. The criteria used were: (1) the resolution between all adjacent pair of peaks should be greater than unity and (2) the maximum analysis time should not exceed 20 min. Secondly, a set of trial experiments were chosen within the desired experimental ranges for the three variables, pH from 2.0 to 3.0, MeOH concentration from 15 to 35%, and concentration of $Na_2HPO_4$ from 20 to 60 m*M*, as shown in Table 6.

After implementing the eleven trials, the resolution between each adjacent pair of peaks for each trial was calculated (Eq. 1). The results are shown in Table 7. Subsequently, the *R*-values of each pair of peaks were fitted to the third-order polynomial equation

$$R = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_1X_2 + a_5X_2X_3 + a_6X_1X_3 + a_7X_1^2 + a_8X_2^2 + a_9X_3^2 + a_{10}X_1X_2X_3, \quad (3)$$

where $a_i$ are coefficients and the $X_i$ are the proportion of each variable between the maximum and minimum values expressed in percentages. With the aid of a modified version of

Table 6
Conditions of the eleven trials in the ORM scheme

| Trial[a] | pH | MeOH (%) | $Na_2HPO_4$ (m*M*) |
|---|---|---|---|
| 1 | 2.0 | 15 | 20 |
| 2 | 2.0 | 35 | 20 |
| 3 | 2.0 | 15 | 60 |
| 4 | 3.0 | 15 | 20 |
| 5 | 2.0 | 35 | 60 |
| 6 | 3.0 | 35 | 20 |
| 7 | 3.0 | 15 | 60 |
| 8 | 3.0 | 35 | 60 |
| 9 | 2.0 | 25 | 20 |
| 10 | 2.0 | 15 | 40 |
| 11 | 2.5 | 15 | 20 |

[a] Trials performed with 20 m*M* citric acid, 50 m*M* NaCl, 18 kV voltage, and capillary temperature at 25°C.

the BASIC program given in Ref. [39], the coefficients for each pair of peaks were determined. The *R*-values at other experimental conditions besides the eleven trial experiments were calculated using the above equation. These *R*-values were used to construct resolution plots. As there were twelve adjacent pair of peaks, twelve individual resolution plots were obtained. By overlapping all the resolution plots and retaining the minimum resolution values, the conditions which could provide resolution equal to or greater than unity for the twelve pair of peaks in the mixture could be determined.

Fig. 1 is the final overlapped resolution plot for all twelve pairs of peaks, where the region marked with "*" represents experimental conditions which provide resolution greater than unity for all peak pairs within the desired analysis time of 20 min. It indicates that the increase in concentration of the electrolyte, $Na_2HPO_4$, did not result in improvement in separation. The optimum region was found to lie in the pH range from 2.0 to 2.1, and concentration of methanol from 25% to 100%. The maximum response was 1.1936 at the point $A = 0$, $B = 80\%$, and $C = 0$ (marked as "1" in Fig. 1), which corresponds to pH 2.0, methanol concentration of 31%, and 20 m*M* of $Na_2HPO_4$.

To confirm the validity of the ORM scheme, experimental conditions corresponding to points 1 and 2 in Fig. 1 were chosen from the regions represented by the symbols "*" and "·", respectively. High resolution ($R > 1.0$) was expected for the condition represented by point 1 whereas poor resolution ($R \leq 0.5$) was expected for the condition represented by point 2. Typical electropherograms corresponding to these two conditions are shown in Figs. 2 and 3. Just as expected, not all peaks in Fig. 3 are well separated. In the case of Fig. 2, all the peaks are

Table 7
Resolution (*R*) between adjacent peaks calculated by using migration times obtained for the trials listed in Table 6

| *R* | Trial | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| 1 | 2.55 | 4.95 | 3.65 | 3.33 | 10.3 | 7.29 | 5.64 | 2.02 | 4.20 | 3.47 | 6.93 |
| 2 | 16.0 | 3.60 | 3.60 | 17.5 | 4.32 | 1.16 | 2.51 | 4.84 | 11.3 | 14.6 | 5.49 |
| 3 | 3.95 | 8.03 | 0 | 6.20 | 1.64 | 0 | 3.15 | 3.99 | 5.91 | 5.78 | 0 |
| 4 | 2.10 | 1.33 | 0 | 4.56 | 4.95 | 0 | 2.94 | 2.02 | 5.84 | 4.25 | 4.73 |
| 5 | 0 | 2.83 | 2.24 | 1.44 | 2.44 | 2.24 | 0 | 0.39 | 1.99 | 0.97 | 1.27 |
| 6 | 2.08 | 8.49 | 0 | 1.90 | 0 | 0 | 1.18 | 0.67 | 1.47 | 0.91 | 2.36 |
| 7 | 0 | 0.79 | 0 | 1.21 | 0 | 1.72 | 6.26 | 1.88 | 4.51 | 1.47 | 2.22 |
| 8 | 1.20 | 0.93 | 0.93 | 2.34 | 1.07 | 2.19 | 0 | 1.11 | 1.26 | 0 | 1.06 |
| 9 | 1.50 | 3.35 | 2.73 | 1.94 | 2.73 | 0 | 1.52 | 1.21 | 0 | 1.57 | 1.03 |
| 10 | 1.59 | 1.94 | 2.66 | 1.24 | 2.66 | 1.8 | 2.34 | 1.57 | 3.47 | 1.47 | 6.70 |
| 11 | 0 | 2.82 | 1.25 | 1.10 | 0 | 2.82 | 2.78 | 1.04 | 1.15 | 0.73 | 0 |
| 12 | 1.06 | 0.52 | 1.36 | 1.74 | 2.16 | 2.57 | 0.71 | 0.78 | 2.77 | 2.27 | 9.01 |

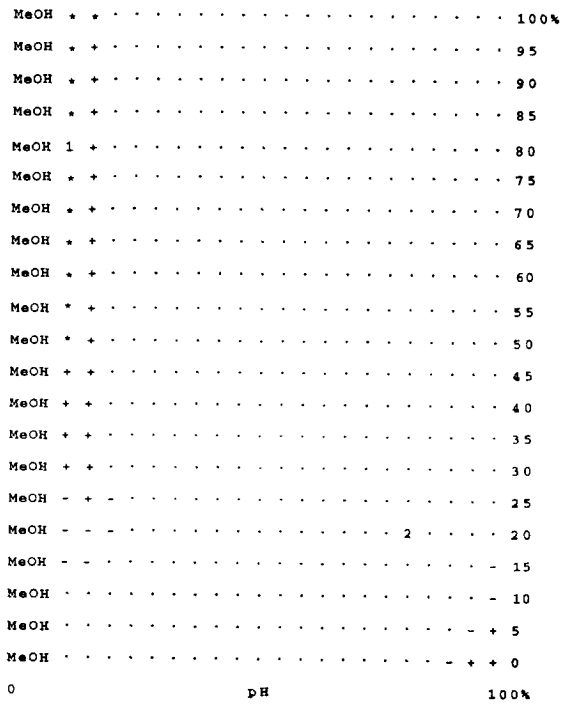


Fig. 1. Overlapped resolution diagram for the twelve pairs of HCA peaks. The minimum values of resolution among all the fifteen individual resolution diagrams are plotted in this overlapped diagram. The highest value of resolution ($R$ = 1.1936) was obtained at point 1, which corresponds to $x_1$ = 0%, $x_2$ = 80%, and $x_3$ = 0%. Point 2 represents a typical set of conditions which give poor resolution. Notation: (·) $R \leq 0.5$, (−) $0.5 < R \leq 0.7$, (+) $0.7 < R \leq 1.0$, (*) $1.0 < R$.

baseline separated and the analysis time is within a reasonable range ( < 20 min).

## 4. Conclusions

In this paper, the combination of the orthogonal array design and the overlapping resolution mapping scheme for the optimization of separation of a group of heterocyclic amines was demonstrated. The orthogonal array design method was used to perform preliminary screening to identify the important factors affecting resolution. Subsequently, the overlapping resolution mapping scheme was used to determine the global optimum conditions within the experimental ranges of the variables under consideration. The combination of the two methods overcomes the disadvantages of each individual method when used alone, and provides a powerful approach which can be utilized for the optimization of separation of complex mixtures.

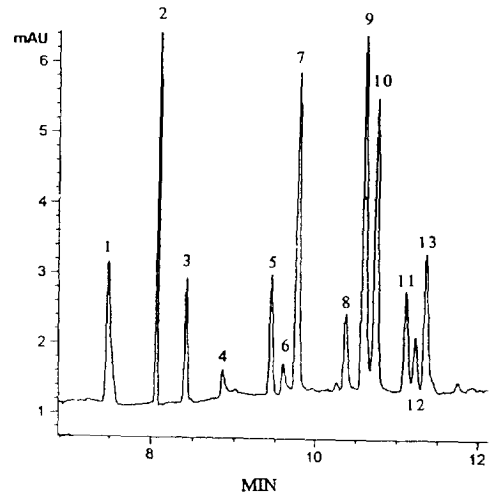


Fig. 2. Optimized electropherogram of the separation of the thirteen HCAs at point 1 ($x_1$ = 0%, $x_2$ = 80%, and $x_3$ = 0%) in ORM scheme. Electrophoretic conditions are given in the Experimental section. Peak identifications: 1 = Iso-IQ, 2 = IQ, 3 = NH, 4 = Glu-P-2, 5 = H, 6 = Glu-P-1, 7 = Trp-P-2, 8 = A$\alpha$C, 9 = Trp-P-1, 10 = MeIQx, 11 = DiMeIQx, 12 = PhIP, 13 = TriMeIQx (see Table 1).

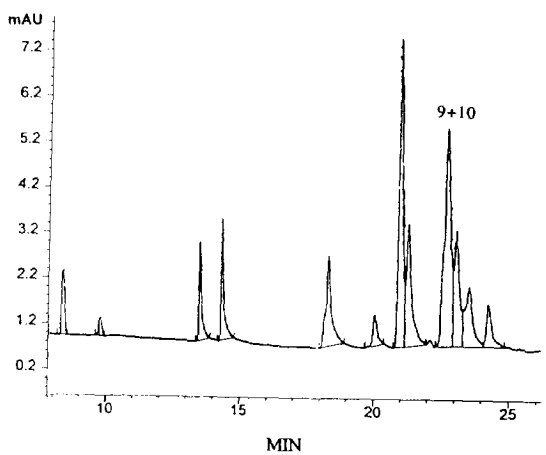


Fig. 3. Electropherogram of the separation of the thirteen HCAs at point 2 ($x_1$ = 80%, $x_2$ = 20%, and $x_3$ = 0%) in ORM scheme. Electrophoretic conditions are given in the Experimental section. Peak identifications are as given in Fig. 2.

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