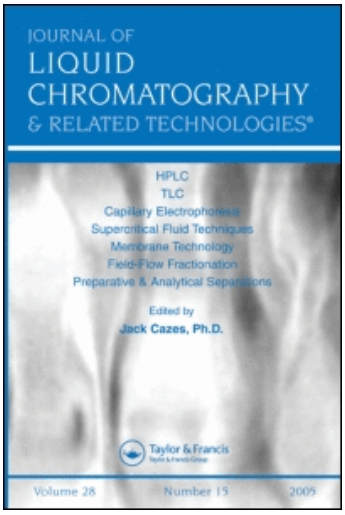

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Optimization of Micellar Electrokinetic Chromatographic Separation of Aporphine Alkaloids By Overlapping Resolution Mapping

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OPTIMIZATION OF MICELLAR ELECTROKINETIC CHROMATOGRAPHIC SEPARATION OF APORPHINE ALKALOIDS BY OVERLAPPING RESOLUTION MAPPING

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ABSTRACT

Based on previous work using the uni-variate approach to optimize the separation of nine Lauraceous aporphine alkaloids, lindcarpine, laurolitsine, *N*-methyllindcarpine, boldine, norpredicentrine, isocorydine, laurotetanine, *N*-methyl-laurotetanine, and isoboldine, by micellar electrokinetic chromatography, a three-parameter overlapping resolution mapping (ORM) scheme was employed to optimize the separation of these compounds. With the other conditions preoptimized, three buffer parameters, i.e., the concentrations of sodium borate, sodium dodecylsulfate, and sodium heptanesulfonate were chosen to carry out the optimization process. Conditions were identified that allowed base-line separation of the nine aporphines and the internal standard within eight minutes. These results show that ORM is an efficient optimization procedure, although some primary separation data is first required.

INTRODUCTION

Micellar electrokinetic chromatography (MEKC) has been increasingly used in the separation of both neutral and ionic molecules since its introduction by Terabe et al.[1] MEKC has been employed for the analysis of various pharmaceuticals, natural products, and biomolecules. Compared with capillary zone electrophoresis (CZE), MEKC produces a better resolution in many applications by providing a (pseudo)stationary phase with which the solutes can interact. Many modifiers, such as cyclodextrines,[2] organic solvents,[3] urea,[4] and ion-pairing agents[5] can be added to the electrophoretic media to increase separation selectivity.

Many strategies used to optimize high-performance liquid chromatography (HPLC) may be employed to perform multi-parameter optimization of MEKC. To date, several chemometric approaches to the systematic optimization of MEKC separations have been reported, including the Plackett-Burman design,[6] overlapping resolution mapping,[7-9] the simplex algorithm,[10] and central composite design.[11,12] The Plackett-Burman design, as well as other complete or fractional factorial designs, cannot determine the exact conditions for optimum separation. It is, however, suited as a screening tool to identify the influence of each parameter and to monitor possible interactions between large numbers of factors. The simplex algorithm and overlapping resolution mapping do not require a model to describe the migration behavior and can be employed once the working ranges of the parameters have been fixed. However, local rather than global optima are often obtained from the simplex algorithm. Central composite design can provide a response surface for prediction of areas of optimal performance, but it requires more experiments compared with overlapping resolution mapping.

Aporphine alkaloids, widely present in Lauraceous plants,[13] possess many pharmacological activities, such as the choleretic and smooth-muscle relaxing actions of boldine,[14,15] and the hypotensive and hyperlipidemia-reducing actions of dicentrine.[16,17] An MEKC method has been developed in our laboratory[18] to facilitate the study of such alkaloids in the Lauraceous plants. Five parameters of the electrophoretic media, including the background electrolyte (sodium tetraborate) concentration, surfactant (SDS) concentration, organic modifier (acetonitrile) percentage, ion-pairing agent (sodium heptanesulfonate) concentration, and pH value, were found to be relevant to the separation. These parameters were optimized by the simple uni-variate approach in which one parameter was varied at a time. This tedious step-by-step process, however, did not identify conditions producing satisfactory base-line separation.

Overlapping resolution mapping (ORM) was therefore employed in this study to optimize the separation of nine aporphine alkaloids by MEKC. Three buffer parameters, *i.e.*, sodium tetraborate concentration, SDS concentration, and sodium heptanesulfonate concentration were examined in a triangular ORM scheme.

EXPERIMENTAL

Apparatus

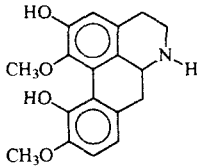
Separations were performed using a CE system consisting of a Lauer Labs' Prince programmable injector, a 30-kV high-voltage supplier (Emmen, the Netherlands), and a Dynamax UV-C Absorbance Detector (Rainin, Emeryville, CA, USA) for UV detection. The electropherograms were recorded with a EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA) on a 486 DX2 66 PC with an appropriate ADC card and interface.

A fused-silica capillary of 50 μm I.D. and 375 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA) with total length of 59 cm and detection length of 43 cm was used. A Mettler delta 320 pH meter with an InLab 410 combination electrode (Essex, England) was employed for measuring pH values.

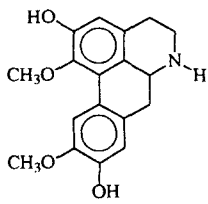
Chemicals and Reagents

Boldine, apomorphine (internal standard), and sodium dodecylsulfate (SDS) were from Sigma (St. Louis, MO, USA). Lindcarpine, laurolitsine, *N*-methyllindcarpine, norpredicentrine, laurotetanine, isocorydine, *N*-methyllaurotetanine, and isoboldine were isolated from the Lauraceous plants *Litsea cubeba*[19] and *Dehaasia triandra* Merr.[20] The identities of the alkaloids were verified by UV, IR, ^{1}H NMR, and mass spectrometry. Sodium tetraborate was from Merck (Darmstadt, Germany). Sodium heptanesulfonate was from Fluka (Buchs, Switzerland).

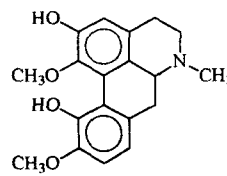
The pH of the buffer media was adjusted with 0.1 M hydrochloric acid (Carlo Erba, Milan, Italy) and 0.1 M sodium hydroxide (Fluka). Acetonitrile (chromatographic grade) was from Mallinckrodt (Paris, KY, USA). Water was obtained from a Barnstead water purification system (Dubuque, IA, USA).



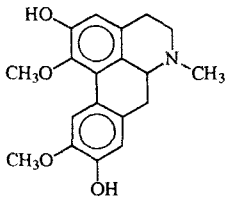
Lindcarpine
(2)



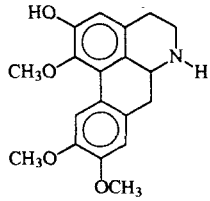
Laurolitsine
(3)



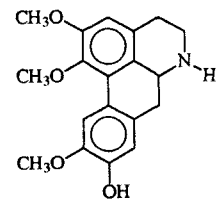
N-methyllindcarpine
(4)



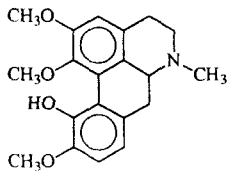
Boldine
(5)



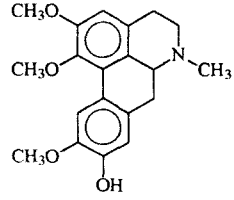
Norpredicentrine
(6)



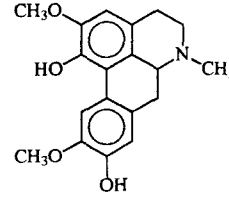
Laurotetanine
(7)



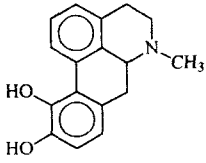
Isocorydine
(8)



N-methyllaurotetanine
(9)



Isoboldine
(10)



Apomorphine
(1)
Internal Standard

Figure 1. Structures of the aporphine alkaloids studied in the optimization.

The running buffer solutions were prepared by mixing stock solutions of sodium tetraborate, SDS, and sodium heptanesulfonate. After adding acetonitrile, water was added to volume. Following adjustment of the pH, the buffer solutions were filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA) before use.

Stock solutions of the nine aporphine alkaloids, lindcarpine, laurolitsine, *N*-methyllindcarpine, boldine, norpredicentrine, laurotetanine, isocorydine, *N*-methyllaurotetanine, and isoboldine, along with the internal standard apomorphine, were prepared at 1 mg/mL in methanol. A working solution containing each of the compounds at 100 μg/mL was prepared by mixing aliquots of the stock solutions and then diluting with methanol/water (40:60).

Electrophoretic Conditions

The experiments were conducted at 30 kV at room temperature (23 ± 2°C). The detection wavelength was set at 214 nm. Samples were injected hydrodynamically at 40 mbar for 3 sec. New capillaries were flushed with 1.0 M sodium hydroxide for 10 min, followed by 0.2 M sodium hydroxide for 10 min. Between runs, the capillary was flushed with deionized water for 3 min, 0.2 M sodium hydroxide for 3 min, water for 3 min, and the running buffer for 4 min. The run-to-run relative standard deviations of the actual migration times for all the analytes and of the relative migration times for the nine aporphines with respect to apomorphine were within 1.4 % and 0.8 % (n = 7), respectively.

RESULTS AND DISCUSSION

The nine Lauraceous aporphine alkaloids, lindcarpine, laurolitsine, *N*-methyllindcarpine, boldine, norpredicentrine, laurotetanine, isocorydine, *N*-methyllaurotetanine, and isoboldine, along with the internal standard apomorphine, are phenolic with one or two hydroxy groups on the benzene rings (Figure 1). Several of these alkaloids differ only in the position of hydroxy (as positional isomers) or methyl (*O*- or *N*-) groups. As previously described, these alkaloids could not be separated by CZE. MEKC with SDS micelles in contrast, allowed partial separation with the addition of sodium heptanesulfonate and acetonitrile; the last three peaks, however, were not completely resolved.[18] A three-parameter ORM optimization scheme was employed in the present work to improve the separation of these alkaloids. The first step was to choose three appropriate parameters for the separation.

Choice of Parameters

As shown in the uni-variate optimization process,[18] the selectivities of the alkaloids changed dramatically with many reversions of elution-order in the pH range of 8.5~10.0. A constant pH value of 9.3 was therefore employed for the optimization process in this work.

Sodium borate in the electrophoretic media was responsible for the pH stability of the solution. Its useful pH range (8.0~11.0) matches the pK_a 's of the aporphines. The migration time window (the difference between the elution times of the first and last analyte) were expanded as the concentration of sodium borate was increased.

SDS micelles acted as the pseudostationary phase in this electrophoretic system. Terabe *et al.* reported that the capacity factor in MEKC is directly proportional to the micelle concentration,[1] hence the migration time can be easily varied by adjusting the SDS concentration, as previously shown.[18] However, because SDS by itself does not provide sufficient selectivity, modifiers are usually added. Sodium heptanesulfonate, ordinarily used as an ion-pairing agent in reverse-phase HPLC, has been found useful for improving selectivity. Like sodium borate and SDS, addition of sodium heptanesulfonate to the buffer media widened the migration time window. Whether this is due to the formation of ion-pairs[3] remains to be investigated.

Addition of acetonitrile as an organic modifier also enhanced selectivity. The effect of acetonitrile on the migration times of the aporphines was more complex than the effects of sodium borate, SDS, and sodium heptanesulfonate.[18] This might be attributed to changes in micellar nature (size, shape, or critical micellar concentration) and re-distribution of solutes between aqueous and micellar phases. Sodium borate, SDS, and sodium heptanesulfonate primarily affect the ionic strength of the electrophoretic media. Their effects on the migration times of the solutes are thus more simple, suggesting that peak cross-overs would not occur over a large working range. These three parameters were therefore chosen as the variables to conduct the ORM optimization. The concentration of acetonitrile was maintained at 5% (v/v) throughout the optimization process.

Overlapping Resolution Mapping Scheme

In the triangular ORM scheme employed in this work, seven experiments were carried out at selected points in the triangle and the results were used to evaluate the entire selectivity region. The positions of the seven points are

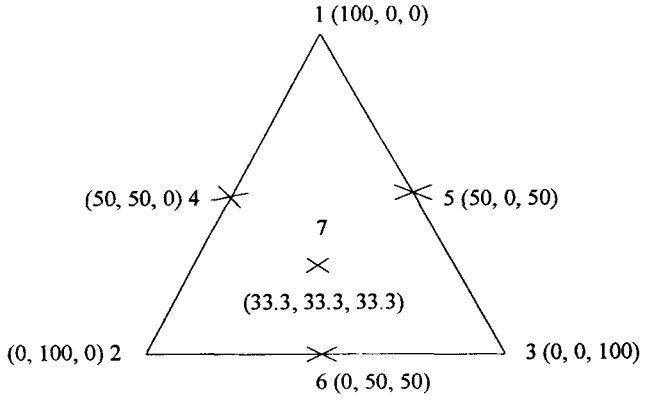


Figure 2. Experimental design of the seven ORM experiments.

Table 1

Experimental Conditions for the Seven Experiments

Expt.*	Na Borate (mM)	SDS (mM)	Na Heptane-Sulfonate (mM)
1	90	20	5
2	70	40	5
3	70	20	15
4	80	30	5
5	80	20	10
6	70	30	10
7	76.7	26.7	8.3

* All experiments were performed at pH 9.3 and with 5% acetonitrile (v/v).

shown in Figure 2. The first step in the ORM scheme was to define the working range of each parameter. The ranges were determined by the requirement of a satisfactory overall resolution within a reasonable analysis time, preferably less than 10 min.

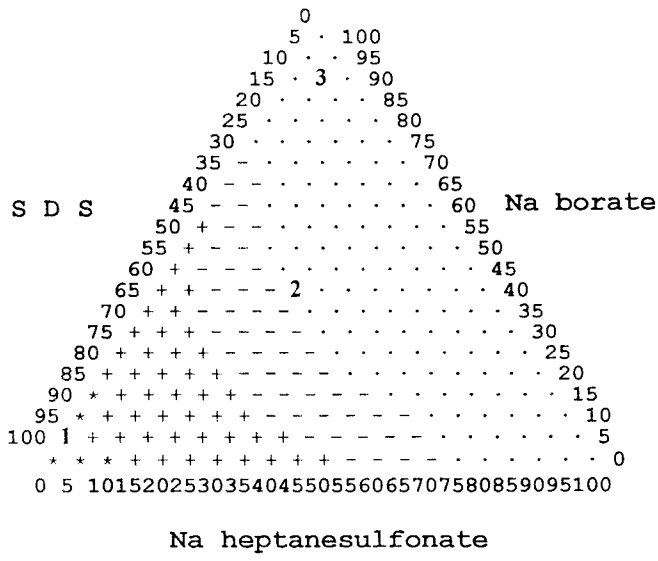


Figure 3. Overlapped resolution map for the nine pairs of peaks. Notation: (•) $Rs < 0.9$; (-) $0.9 \leq Rs < 1.4$; (+) $1.4 \leq Rs < 1.9$; (*) $Rs \geq 1.9$.

Table 2

Resolution, Rs, Between Adjacent Peaks Obtained from the Seven Experiments in Table 1

	Resolution, Rs for Peak Pair								
Exp	**1-2**	**2-3**	**3-4**	**4-5**	**5-6**	**6-7**	**7-8**	**8-9**	**9-10**
1	11.24	11.49	6.49	8.05	5.78	6.77	0.73	4.01	2.05
2	13.66	11.92	7.67	7.36	6.56	6.31	1.48	4.16	2.76
3	3.93	7.51	5.13	7.08	4.29	4.83	0.00	3.78	1.72
4	26.22	12.98	8.37	7.67	7.42	6.52	1.91	3.79	2.42
5	5.57	8.02	4.36	7.08	5.33	4.52	0.00	4.29	2.10
6	5.90	9.85	5.73	6.88	5.70	5.47	0.00	5.70	5.47
7	15.53	10.19	7.08	8.69	7.16	7.29	1.51	4.34	2.71

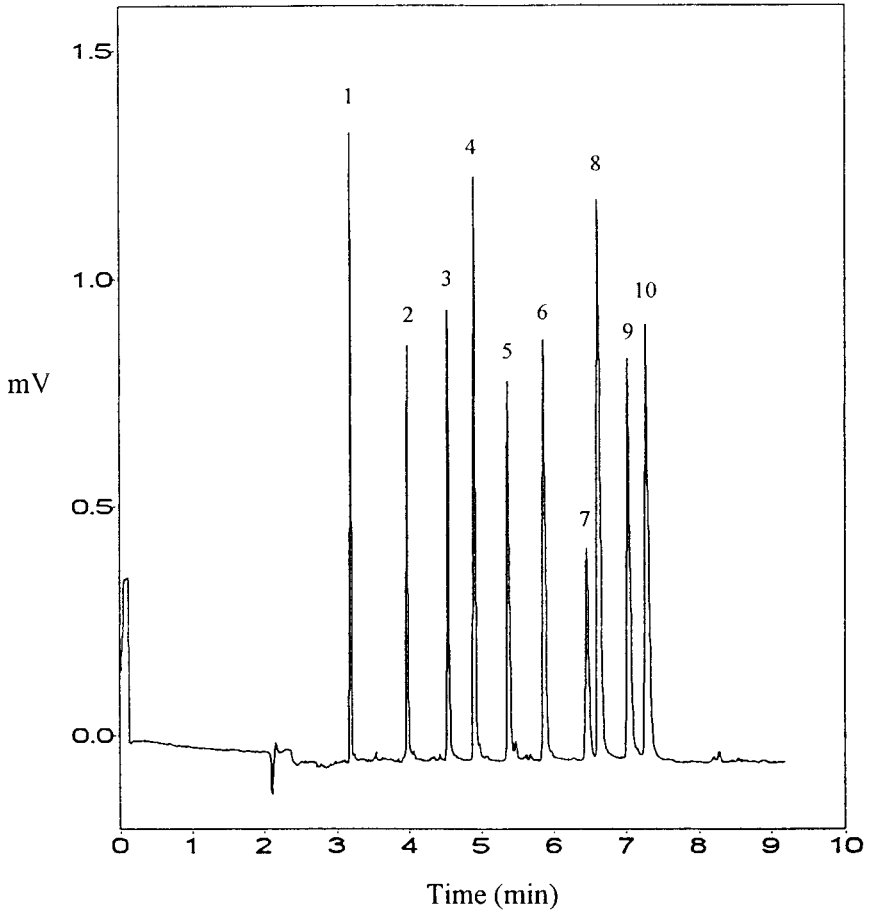


Figure 4. Electropherogram of the ten aporphines using the optimum conditions corresponding to point 1 in Figure 3. Electrophoretic conditions: 71 mM sodium borate, 39 mM SDS, 5 mM sodium heptanesulfonate, 5% acetonitrile (v/v), pH 9.3; capillary, fused silica, 50 μm I.D., detection length 43 cm, total length 59 cm; injection time, 3 sec; voltage, 30 kV; temperature, 23°C; UV detection, 214 nm.

On the basis of previous results,[18] seven preplanned experiments were designed as depicted in Table 1. From the seven electropherograms acquired from these experiments, the resolution, *Rs*, between adjacent peaks was calculated by the equation

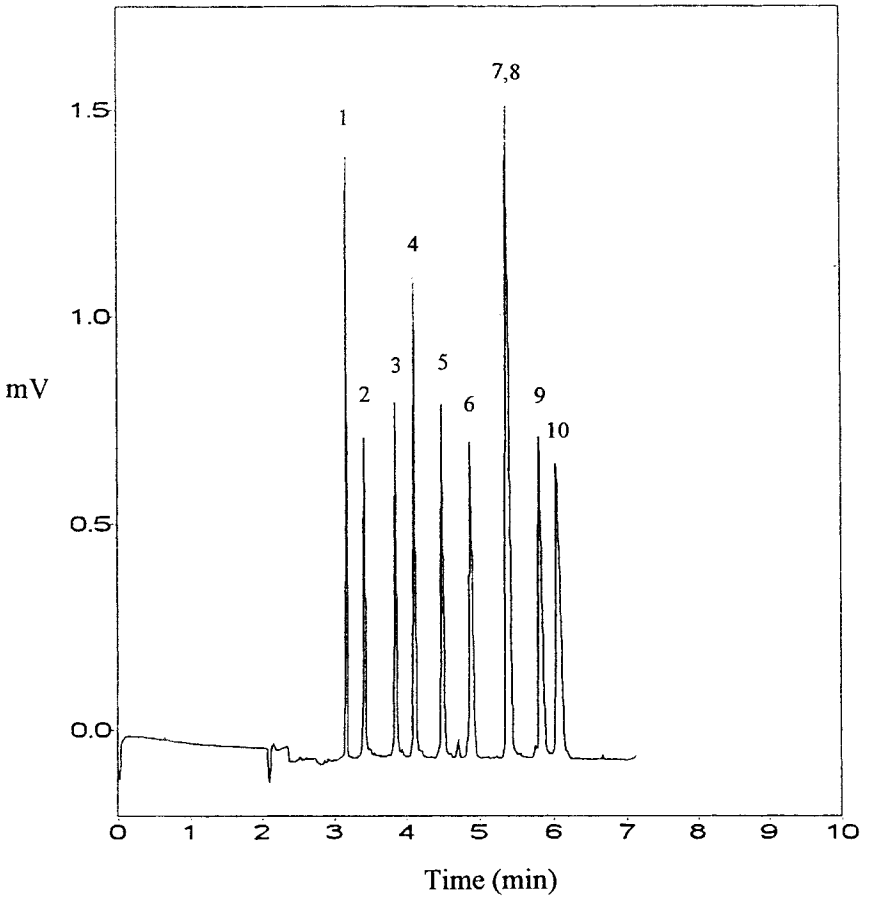


Figure 5. Electropherogram of the ten aporphines using the conditions corresponding to point 2 in Figure 3. Electrophoretic conditions: 78 mM sodium borate, 27 mM SDS, 7.5 mM sodium heptanesulfonate, other conditions as in Figure 4.

$$RS = \frac{1.18(t_2 - t_1)}{W_{1/2_1} + W_{1/2_2}} \quad (1)$$

where t_1 and t_2 are the migration times and $W_{1/2_1}$ and $W_{1/2_2}$ are the half-height peak widths of two adjacent peaks, respectively. The calculated *Rs* values for all the peak pairs are shown in Table 2. These resolution values were then fitted with a polynomial equation:

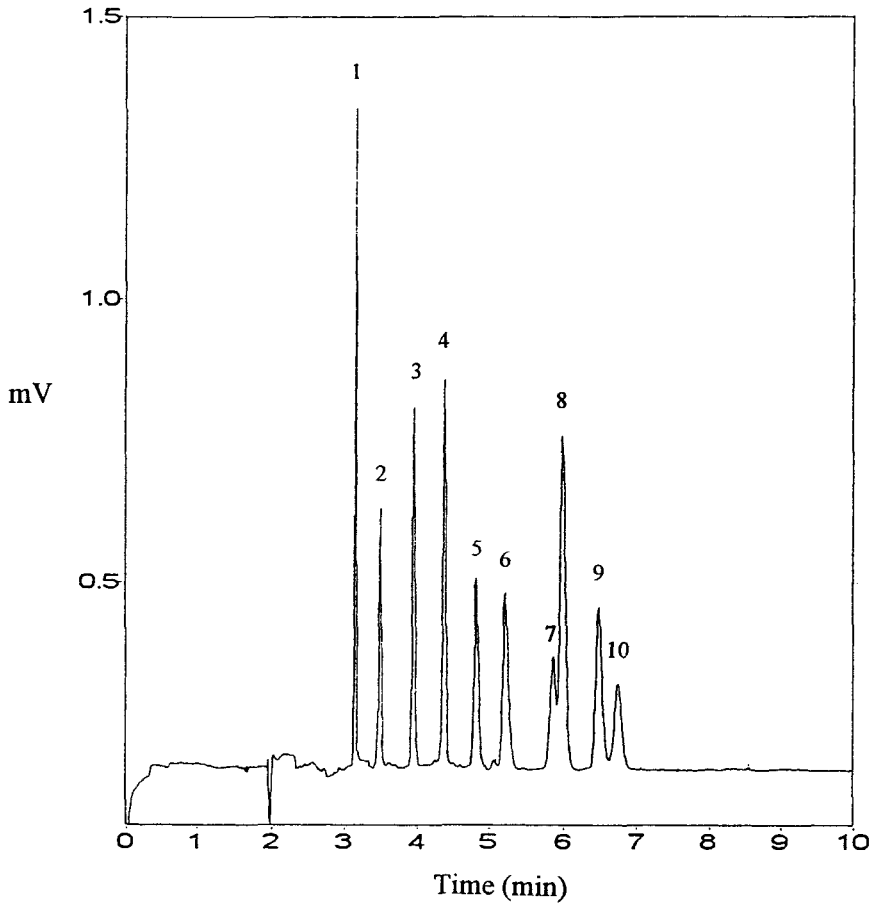


Figure 6. Electropherogram of the ten aporphines using the conditions corresponding to point 3 in Figure 3. Electrophoretic conditions: 88 mM sodium borate, 21 mM SDS, 5.5 mM sodium heptanesulfonate, other conditions as in Figure 4.

$$Rs = a_1X_1 + {}_a a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{123}X_1X_2X_3 \quad (2)$$

where a_i are the coefficients and X_i are the percentages of each parameter as defined in Figure 2. The values of a_i for each adjacent pair of peaks were determined using the BASIC program developed by Berridge.[21] From equation (2) the resolutions for each adjacent pair of peaks could be calculated for any

Table 3

Experimental and Predicted Resolutions of Adjacent Peak Pairs Under Optimum Conditions

Peak Pair	Resolution	
	Expt.	Pred.
1-2	27.14	24.80
2-3	15.23	13.02
3-4	8.97	8.38
4-5	8.95	7.36
5-6	8.77	7.40
6-7	8.47	6.52
7-8	2.12	1.91
8-9	5.02	3.86
9-10	2.70	2.52

composition of the three parameters in the triangle. Venn diagrams of each adjacent pair of compounds were then generated in which the various symbols represented the specified resolution levels.[21] By overlapping all nine Venn diagrams and then plotting the symbols representing the lowest resolution among all the individual diagrams, areas defining the composition of buffer which would give the desired resolution among all the peaks in the aporphine mixture were established. The resulting diagram for the ten aporphines is shown in Figure 3. The regions marked by the symbol * should give a minimum resolution of 1.9 between all the adjacent peak pairs. It can be seen that the conditions represented by the area near the lower left corner of the triangle appeared optimal for aporphine separation. To test the validity of this optimization scheme, the conditions represented by several points on the diagram were used to conduct the experiments. Point 1 (as the optimum), 2, and 3 represent three different predicted resolution levels, i.e., greater than 1.9, between 0.9 and 1.4, and less than 0.9, respectively. The three electropherograms (for example, point 1 containing 71 mM sodium borate, 39 mM SDS, and 5.mM sodium heptanesulfonate) are shown in Figures 4, 5, and 6, respectively. The electropherogram obtained with the optimum conditions predicted at point 1 shows that all ten peaks were base-line resolved in a total analysis time of less than 8 min (Figure 4). Comparisons between the experimentally determined resolutions and those predicted from equation (2) are listed in Table 3 and except for two peak pairs, the deviations were within 20 %.

CONCLUSIONS

Following the many successful applications of ORM to MEKC by Li et al.,[7-9] the effectiveness of this optimization strategy was demonstrated in this investigation. Three buffer parameters, i.e., the concentrations of sodium borate, SDS, and sodium heptanesulfonate were simultaneously optimized. Nine aporphine alkaloids from Lauraceous plants along with apomorphine were baseline separated within 8 minutes. Although this approach is straightforward and rapid, the parameters to be optimized and their working ranges require careful selection before undertaking the experiments. In this respect, fractional factorial designs such as the Plackett-burman design[22] and the orthogonal array design[23] should be helpful. The univariate approach, although sometimes tedious and time-consuming, can also help in these decisions.

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