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Orthogonal array design experiments for optimizing the separation of various pesticides by cyclodextrin-modified micellar electrokinetic chromatography

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Abstract

The potential of cyclodextrin-modified micellar electrokinetic chromatography (CD–MEKC) for the separation of complicated environmental samples of pesticides and the applicability of orthogonal array designs (OADs) for the optimization of separation in CD–MEKC were investigated. Six relevant factors were investigated: type and concentration of cyclodextrin, concentration and pH of buffer, and concentration of micelle and organic modifier. In the first experiment, five factors were examined at two levels using an OA_{16} (2^{15}) matrix, by which the effect of each factor was estimated using individual contributions as response function. Based on the results of the first experiment, three most important factors were chosen for further optimization using OA_9 (3^4) matrix to locate more exact levels for each variable. Finally, main-effect curves were used to predict approximate optimum conditions under which adequate separation of 15 pesticides was achieved within 30 min. © 1998 Elsevier Science B.V.

Keywords: Orthogonal array design; Optimization; Chemometrics; Pesticides

1. Introduction

Capillary electrophoresis (CE) as a powerful separation technique was initially applied to biochemical analyses [1–4], but in recent years its applicability in environmental analyses has been demonstrated by an increasing number of studies [5–11]. Some of these involve pesticides, such as aromatic-containing organic acids, triazines, sulfonyl ureas and urea herbicides [8–11]. Most previous studies were focused on the determination of certain classes of pesticides. However, real environmental samples are often complicated, usually involving far more than one category of pesticides.

Micellar electrokinetic chromatography (MEKC) was first developed by Terabe et al. [6] for separating neutral as well as charged compounds. Terabe also introduced cyclodextrins (CDs) to MEKC (CD–MEKC) for separating hydrophobic (e.g. polycyclic aromatic hydrocarbons) or closely related (e.g. optical isomers) compounds [8]. A number of reports on the use of CD–MEKC to effect different separation problems [12–15] are available. In view of the versatility of CD–MEKC, the technique should be suitable for screening separation of complicated environmental samples, which usually consist of neutral and charged, hydrophobic and hydrophilic,

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related and diversified compounds. In this work, we applied CD–MEKC to the separation of a mixture of various pesticides, such as aromatic-containing acids, carbamates, triazine and organophosphorous pesticides.

In CD–MEKC, a solute is partitioned among three phases, the micelle, the CD cavity and the surrounding aqueous phase [8]. As a result, numerous parameters should be incorporated in the optimization strategy in order to achieve an adequate separation of complex mixtures within an acceptable analysis time. Corstjens et al. [16] have comprehensively reviewed the strategies for the optimization of selectivities in CE, especially in MEKC. These strategies include overlapping resolution mapping (ORM) [17], Plackett–Burman statistical design [18], and physicochemical approaches [19–21].

ORM, initially developed by Glajch [22] for multi-parameter optimization in HPLC, was first adapted for the optimization of CE separations by Ng et al. [17]. In this method, the resolution of each adjacent pair of peaks is plotted against different separation conditions. The optimum separation condition can be deduced from an overlay of all the resolution plots. The advantage of this approach is that it is capable of locating the global optimum with the selected range of experimental conditions instead of level or points in other approaches. However, this approach suffers from the limited number of parameters (2 or 3) that can be tested [23,24], and is not suitable for the screening of many parameters.

Placket–Burman statistical design is a saturated fractional factorial design which can be used for the screening of many parameters within a limited number of experiments [25–27]. However, most of the applications of this design adopted two-level designs only. An exception is the work by Jones [26] who used a three level design. However, it has been proven that this design can lead to wrong conclusions due to the fact that three-level design is not well balanced, and it is impossible to extract the influence of each factor separately [27].

Orthogonal array design (OAD), a fractional factorial design, has been recently actively investigated in many fields [28–30]. The use of analysis variance in OAD instead of the Student's *t*-test in Plackett–Burman schemes and the associated triangle table for the assignment of variables and two-variable interaction are the two major advantages of OAD over Plackett–Burman schemes although both designs have the same number of experimental trials [28]. Orthogonal here means balanced, separable or not mixed. In that way, when the effect of a factor is calculated, the influence of the other factors is canceled out, and hence different effects can be extracted independently. The number of factors investigated can be up to thirty-one, which is decided by the size of the trials, the complexity of the system and to what extent one requires the information.

In our previous work, OAD has been successfully applied in the optimization of analytical procedures such as chromatographic separation [31], solid-phase extraction [32], and standard CE [33]. In the present work, we investigated the applicability of OAD for optimizing separation in CD–MEKC.

2. Experimental

2.1. Apparatus

The experiment was performed on a Prince CE system equipped with the Butler buffer exchanger (Prince Technologies, Amsterdam, Netherlands), with detection at 210 nm on a Lambda 1000 spectrophotometer (Bischoff, Leonberg, Germany) and a 63 cm (effective length 52 cm)×75 μm I.D. fused-silica capillary tube. A Chromatopac C-R6A integrator (Shimadzu, Kyoto, Japan) was used for data processing. The Prince CE system is air-cooled, and all experiments were conducted in an air-conditioned laboratory at an ambient temperature of 23°C.

2.2. Reagents

All pesticides used were analytical standards supplied by Supelco (Bellefonte, PA, USA). They were dissolved in methanol at 1 mg/ml concentration as stock solutions. A mixed solution containing 30 ppm of each pesticide in methanol–water (50:50) was prepared from the stock solution before analysis, and was used as the working solution.

Sodium tetraborate was purchased from Merck (Holtenau, Germany). Phosphoric acid was purchased from Carlo Erba (Milan, Italy). Sodium

dodecyl sulfate (SDS) and β-cyclodextrin were purchased from Fluka (Buchs, Switzerland). Hydroxypropyl-β-cyclodextrin (average molar substitution=0.8) was bought from Aldrich (Milwaukee, WI, USA).

HPLC-grade acetonitrile was bought from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). The water used for the preparation of the sample and buffers was purified by a Milli-Q system (Millipore, Bedford, MA, USA).

2.3. Preparation of background electrolyte

Buffer solutions were prepared by mixing equimolar disodium tetraborate and boric acid (molarity expressed as borate equivalents) solutions in appropriate volume ratios. pH values refer to the pH of the pure buffer solutions. SDS, CDs, and acetonitrile were added, and the volume was adjusted with deionized water. Buffer, SDS, CD and acetonitrile concentrations are reported here based on the final volume of the mixture.

Selection of parameter levels in the present study was based on our previous experience with CD–MEKC and the literature. Concentrations of SDS and borate were selected in such a way that excessively high current and long analysis times were avoided.

2.4. Optimization strategy

The experiment was designed to determine the effect of operating conditions on the separation of 15 different pesticides, and to identify good separation conditions with a limited number of experiments.

Selectivity in the separation of a complicated mixture of pesticides using MEKC is influenced by many factors, including column length and internal diameter, temperature, applied voltage, injection volume, pH value, type and concentration of buffer, surfactant, and organic solvent. In CD–MEKC, two additional parameters have significant influence on the separation, i.e. the type and concentration of CD. Consequently, numerous parameters can be optimized for separation.

The temperature affects different physicochemical parameters like viscosity, pK_a, pH, absolute mobilities and critical micellar concentration (CMC) of various surfactants, and thus, separation. As a result, an efficient temperature control is inevitable in method development. Throughout our experiments, ambient temperature was kept constant at 23°C in a well air-conditioned laboratory. The column length and its internal diameter, applied voltage and injection volume were also fixed throughout the experiments.

We initially attempted to investigate the effect of five parameters including pH, concentrations of sodium tetraborate and SDS, type of CD and concentration of acetonitrile (MeCN), which were likely to affect the quality of the separation according to our previous experience. For this purpose, a screening experiment was conducted using a two-level OAD with an OA_{16} (2^{15}) matrix. Three- or more-variable interactions were ignored, since they were much less likely to occur, and if they did exist, then they would most likely be of much a smaller magnitude than a main effect. However, it is always possible to have a two-variable interaction (e.g. 1×2, 2×6, ... where 1×2 means the interaction between parameters 1 and 2, etc.). In this case, a two-variable interaction is regarded as an independent parameter and assigned to a column in the orthogonal array matrix; the assignment of the parameters, levels and interactions are given in Table 1.

On the basis of the results obtained as shown in the table above, significant factors were identified and the most significant factors were chosen for further optimization using a three-level OAD with an OA_9 (3^4) matrix. More exact levels were selected around the superiority levels obtained from the initial examination. At this step, interactions among variables were not incorporated in the matrix and focus was placed on the main-effects of the three most important factors.

In most previous optimization strategies [17–20,32], persistent efforts were made to locate global optimum conditions. Although it is possible to obtain global optimum conditions, it is often time consuming in terms of calculation requirements, and not cost-effective in practice, especially for a complicated system, such as CD–MEKC. In many cases, it is more desirable to locate approximate optimum conditions which can essentially meet our requirements while costing much less time. This is what we have done, focusing on locating approximate op-

Table 1
Assignment of factors and levels of the first experiment by using an OA_{16} (2^{15}) matrix along with the response

Trial No.	1[a]	2[a]	3 1×2	4 2×6 8×12	5[b]	6[a]	7 1×6	8[a]	9 1×8	10 2×8 6×12	11[b]	12[a]	13 1×12	14 2×12 6×18	15[b]	IC
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3.6168
2	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	3.7227
3	1	1	1	2	2	2	2	1	1	1	1	2	2	2	2	3.3090
4	1	1	1	2	2	2	2	2	2	2	2	1	1	1	1	3.5688
5	1	2	2	1	1	2	2	1	1	2	2	1	1	2	2	3.3859
6	1	2	2	1	1	2	2	2	2	1	1	2	2	1	1	3.3859
7	1	2	2	2	2	1	1	1	1	2	2	2	2	1	12	3.4624
8	1	2	2	2	2	1	1	2	2	1	1	1	1	2	2	3.6167
9	2	1	2	1	2	1	2	1	2	1	2	1	2	1	1	3.5398
10	2	1	2	1	2	1	2	2	1	2	1	2	1	2	1	3.5397
11	2	1	2	2	1	2	1	1	2	1	2	2	1	2	2	3.2800
12	2	1	2	2	1	2	1	2	1	2	1	1	2	1	2	3.5688
13	2	2	1	1	2	2	1	1	2	2	1	1	2	2	2	3.3569
14	2	2	1	1	2	2	1	2	1	1	2	2	1	1	2	3.4628
15	2	2	1	2	1	1	2	1	2	2	1	2	1	1	1	3.3569
16	2	2	1	2	1	1	2	2	1	1	2	1	2	2		3.5398
$E1$[c]	3.5086	3.5182				3.5493		3.4135				3.5242				
$E2$[c]	3.4556	3.4460				3.4149		3.5508				3.4400				
D[d]	5.30	7.22				13.43		13.73				8.42				

Column 1: Borate concentration, 1:25 m*M*, 2:21.5 m*M*.
Column 2: SDS concentration, 1:30m*M*, 2:20m*M*.
Column 6: Type of CD (4 m*M*), 1.:β-CD, 2:HP-β-CD.
Column 8: pH value, 1:8.0, 2:9.0.
Column 12: MeCN concentration, 1:15%, 2:10%.
[a] Parameter column.
[b] Dummy column.
[c] Mean effect of factor at level 1 or 2.
[d] $D=|E1-E2|\times 10^2$.

Table 2
Assignment of the factors and levels of the second experiment using an OA_9 (3^4) matrix along with the effects of important factors on the response (IC)

Trial No.	Column No.				IC
	1	2	3	4	
1	1 (15)	1 (4.0)	1 (8.5)	1	3.4153
2	1 (15)	2 (8.0)	2 (9.0)	2	3.7264
3	1 (15)	3 (12.0)	3 (9.5)	3	3.6125
4	2 (29)	1 (4.0)	2 (9.0)	3	3.4721
5	2 (20)	2 (8.0)	3 (9.5)	1	3.6983
6	2 (20)	3 (12.0)	1 (8.5)	2	3.4827
7	3 (25)	1 (4.0)	3 99.5)	2	3.5606
8	3 (25)	2 (8.0)	1 (8.5)	3	3.5678
9	3 (25)	3 (12.0)	2 (9.0)	1	3.5214

Column 1: MeCN concentration (%).
Column 2: β-CD concentration (m*M*).
Column 3: pH value.
Column 4: unassigned.

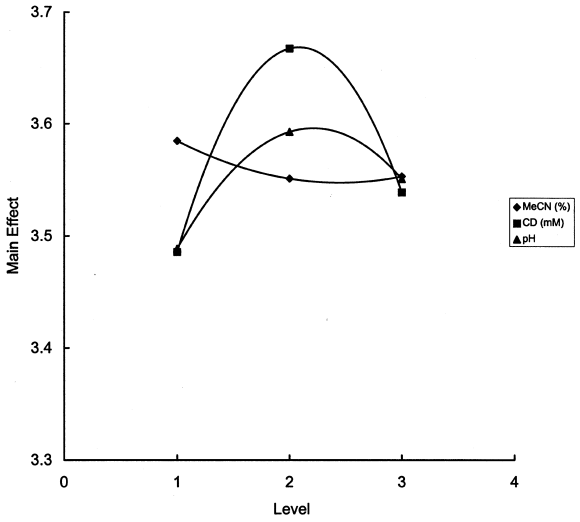

Fig. 1. Main-effect curves of the three most significant variables relative to response (IC). MeCN = acetonitrile, CD = β-cyclodextrin.

timum conditions. The best separation conditions regarded as the best combination of all influencing factors can be approximated by the best combination of the most significant factors. The latter can be further approximated by the combination of the most significant factors at their best levels, which can be determined from the main-effect curve of these factors relative to the response. The reason for this is that significant factors, especially the most significant factors, have a consistent effect even when the conditions of other factors differ, i.e. the order of superiority–inferiority levels, in particular, is not sensitive to small changes in other factors. Hence, the high reliability of the best level can be expected [34,35].

The assignment of the three most significant factors and their magnitudes are as shown in Table 2. Main effect curves of these parameters relative to response were plotted (Fig. 1), and the best separation conditions were approximated by taking the level of each factor giving the maximum individual contribution (IC). In many cases, the approximate optimal conditions produced the results meeting our requirements. Nevertheless, further fine-tuning of separation conditions within a narrow range using the simplex method is sometimes required to finalize the optimal conditions.

In the present work, a response function based on information theory [36,37] was chosen to judge the quality of the electropherogram. If a mixture of n components is separated and the resulting electropherogram is composed of k_1 singlets, k_2 doublets and k_p p-multiplets with $\Sigma\ (pk_p)=n$, the contribution of the p-multiplets to the quantity of information is given by [40]:

$$I_p = (pk_p/n)\log_2(n/p)$$

where pk_p/n is the appearance frequency and $\log_2(n/p)$ is the quantity of specific information obtained by the identification of a component in a p-multiplet. The total information from the chromatogram is the sum of the ICs of each peak group:

$$IC = \sum(pk_p/n)\log_2(n/p)$$

The value of IC varies between zero, all the peaks together ($p=n$, $k_p=1$), and $\log_2$ (n), all the peaks separated ($p=1$, $k_p=n$).

It should be noted that the classification of singlet, doublet or triplet is based on the resolution between adjacent peaks. In chromatographic analysis, we usually need to know what components are in the sample and how much there is of each component. For identification and accurate quantification of two peaks in a chromatogram, it is usually required that the resolution (R) of two adjacent peaks should be no

less than 1. When R is 1, and the ratio of peak height varies from 1/32 to 32/1, the quantitative error in terms of peak height is usually within ±3% [38]. In practice, the error in the determination of unsymmetrical peaks resulting from peak tailing or fronting should be larger than ±3% when R is 1 [39]. Hence, classification of singlets or doublets on the basis of whether R is more or less than 1 is an approximate indicator which cannot distinguish minor variation in the quality of the electropherogram. Nevertheless, two peaks were conveniently considered as two singlets if R was no less than 1, and as a doublet if R was less than 1.

3. Results and discussion

3.1. Initial experiments using OA_{16} (2^{15}) matrix

In this work, 15 pesticides were selected for study. These pesticides belong to five categories: triazines, carbamates, organophosphorus and organochlorine compounds, and phenoxy acids, all of which have been widely used against pests, weeds, bacteria, pathogens, etc. in modern agriculture. These pesticides include weak acids (e.g. mecoprop), bases (e.g. simazine) and neutral compounds (e.g. chloroneb). Some of them have closely related structures (e.g. isoprothiolane and isoprocarb). Mixtures of this composition cannot be effectively analyzed by HPLC or GC. CD–MEKC could, on the other hand, be a better choice given its versatility.

The results of initial experiments are given in Tables 1 and 2. They indicate that (Table 3) the most significant factors were the type of CD used and pH; these parameters were statistically significant at the 99% confidence level. The next most significant factors were MeCN and SDS concentration, at the 95% confidence level. The ionic strength, i.e., concentration of borate has no significant influence on the separation of the 15 pesticides as a whole.

Superiority and inferiority levels of the four significant factors were estimated by comparing the mean effect of factors at two levels. Table 1 gives the mean effect of these factors at level 1 and level 2, respectively. It is shown that high SDS (30 mM) and MeCN (15%) concentration, and high pH (9.0) will improve the IC. Additionally, the use of β-CD instead of HP-β-CD will also increase the response function.

The pH of the electrolyte solution in CE is often an important separation parameter for changing the selectivity of the system [16]. In general, variation of the pH does not influence the separation of neutral compounds, but show strong influence on the net charge and therefore the separation of weak acids (e.g. mecroprop) and bases (e.g. simazine and asulam). As one can readily see, even a small change of the pH had a dramatic effect on the resolution of the 15 pesticides, as shown by the large difference between the mean effect at pH 8 and 8.5. For this reason, it is necessary to properly select and control pH.

Cyclodextrins are able to form highly selective inclusion complexes with a wide range of compounds [8,12–15], especially those with aromatic rings and/or alkyl chains, which are possessed by most of the pesticides under study. Different cyclodextrins often show quite different selectivities. Derivatized CDs also show different selectivities as

Table 3
Variance analysis for the first experiment

Source of variation	Sum of squares×10^2	Degrees of freedom	Mean square×10^2	F-value[a]	Significance
Borate concentration	1.122	1	1.122	3.099	$p>0.05$
SDS concentration	2.086	1	2.085	5.760	$p<0.05$
Type of CD	7.525	1	7.525	20.78	$p<0.01$
pH value	7.249	1	7.249	20.02	$p<0.01$
MeCN concentration	2.835	1	2.835	7.831	$p>0.01$
Pooled error[b]	3.615	10	0.362		

[a] The critical F-value is 10.04 at 99.0% confidence, and 4.95 at 95% confidence.
[b] The sum of squares of the errors (column 5, 11 and 15) along with those of interaction among facotrs (column 3, 4, 9, 10, 13 and 14) were combined and treated as pooled error.

compared to native CDs. In this study, native β-CD showed better selectivity than the derivatized HP-β-CD.

Addition of MeCN changes the polarity of the aqueous solution, affects the partitioning of solutes amongst the SDS micelle, CD cavity and aqueous solution, and hence changes the pseudo-effective mobilities of different solutes [41,42]. Our previous experience indicates that addition of MeCN usually brings about great improvement in the resolution of more hydrophobic compounds while causing slight degradation in the separation of more hydrophilic compounds [15]. Therefore, the appropriate MeCN concentration should be sought to effect the separation of both hydrophobic and hydrophilic compounds, without a severe trade-off in the resolution of members within either compound class.

3.2. Experiments using OA_9 (3^4)

The results of the experiments designed using OA_9 (3^4) are presented in Table 2 and Fig. 1. In this experiment, borate and SDS concentration was set at 25 and 30 m*M* respectively, and the effects of three most important factors, pH value, CD and MeCN concentrations on response function were investigated in more detail using a three-level design. In this experiment, the superiority levels obtained from the first experiment were used as the starting levels for pH (8.5) and MeCN concentration (15%). β-CD (superiority) was used for HP-β-CD (inferiority), with concentration varying from 4 to 12 m*M* (our previous experience showed that increasing CD concentration might improve separation). The main-effect curves of these factors are given in Fig. 1. It is shown that the three factors imparted a different influence on IC within the selected range. Separation improved considerably when the CD concentration was increased from 4 to 8 m*M*, but decreased with further increase in the concentration of CD; separation also improved considerably when pH was increased from 8.5 to 9.0, and a further increase in pH had only a slight effect on the response; the concentration of MeCN had less effect on the separation than the other two variables, and the response function varied slightly within the range of 15–25% MeCN.

Since the three factors investigated in this experiment were shown to have a great influence on the response, i.e., their effects were not much affected by their interactions, determination of the best separation conditions could hence be approximated by the determination of the combination of MeCN and β-CD concentration, and the pH at their optimum values. We sought these values in the main-effect curves as shown in Fig. 1 by reading off the values which gave the highest IC. It is clear that the best values for MeCN and β-CD concentration are 15% and 8.5 m*M* respectively, and for pH, 9.5.

In our experiments, the IC varied from 3.2800 (trial 11 in Table 1), to 3.7264 (trial 2 in Table 3),

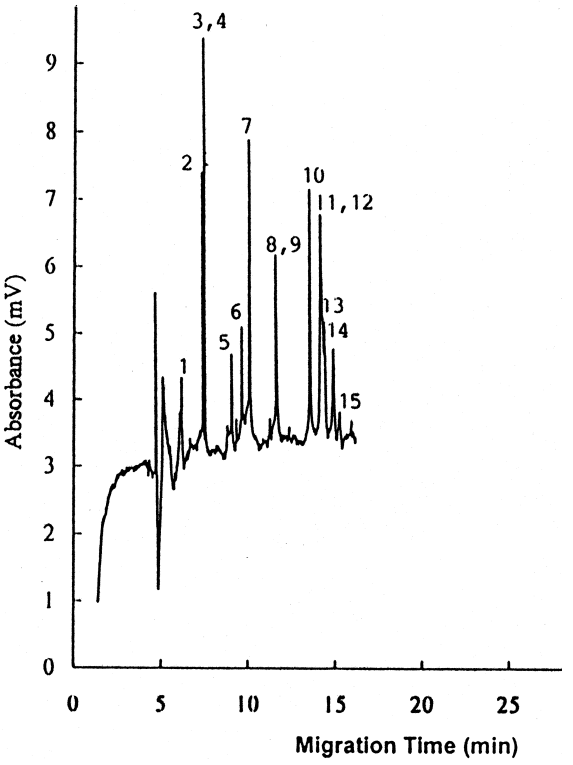

Fig. 2. Electropherogram of pesticides obtained under the worst conditions (trial 11 in Table 1) in the first experiment: fused-silica capillary (65 cm×50 μm I.D.); voltage, 25 kV; UV detection, 220 nm; hydrodynamic injection, 30×6 mbar·s; borate buffer, 12.5 m*M* (pH 8.0); SDS, 30 m*M*; MeCN, 15%; HP-β-CD, 4 m*M*. Peak identities: 1=simazine, 2=mecoprop, 3=isoprocarb, 4=asulan, 5=fensulfothion, 6=fenobucarb, 7=isoprothiolane, 8=mepronical, 9=chloroneb, 10=pencycuron, 11=isofenfos, 12=thiobencarb, 13=terbutol, 14=butamifos, 15=iprodine.

and finally 3.9072 (approximate optimum conditions), corresponding to the progressive improvements exhibited by the respective electropherograms shown in Fig. 2 [with 1 unresolved three-component band (triplet), 2 unresolved two-component bands (doublets), and 8 resolved peaks (singlets)], Fig. 3 [one poorly resolved doublet (peaks 12 and 13), and 13 resolved peaks], and Fig. 4 (15 resolved peaks). Thus, IC can be used as an optimization criterion of separation quality, and the procedure presented did significantly improve the separation of the 15 pesticides by using only a small number of experiments. Under the approximate optimum conditions (Fig. 4), all peaks were baseline-resolved except for peaks 12 and 13 (which were nevertheless adequately separated, with $R=1.06$), within 30 min. This result essentially met our requirements for unambiguous identification and accurate quantification of the analytes within an acceptable analysis time. It should be noted, however, that electropherograms even under the approximate optimum conditions show peak tailing and fronting. In CE, the general deviation from a symmetrical Gaussian peak is due to the difference in mobilities between ions of sample and co-ions of the background analytes [43]. For a strong electrolyte system, peak tailing is observed if the mobility of the sample ions is lower than that of the co-ions of the background electrolyte, while the converse is true for peak fronting. Peak shape is also affected by sample injection, sample concentration and conductivity, Joule heating, wall adsorption of sample solutes, etc. In the present work, the capillary is cooled by forced convection of air, and small volumes of sample (<20 nl) were injected. Hence,

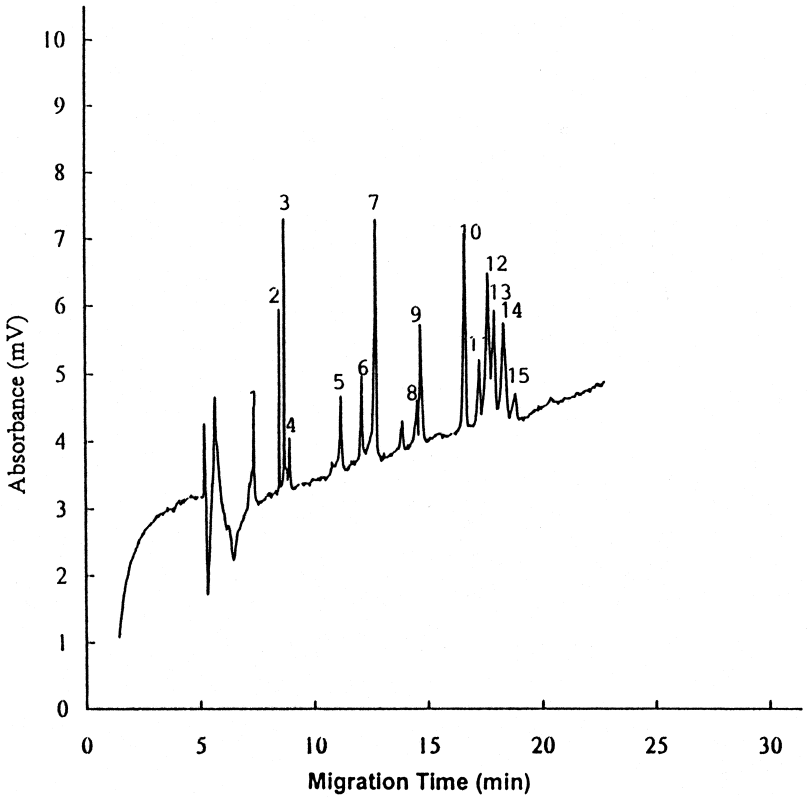

Fig. 3. Electropherogram of pesticides obtained under the best conditions (trial 2 in Table 3) in the second experiment. Borate buffer, 25 m*M* (pH 9.0); SDS, 30 m*M*; MeCN, 15%; β-CD, 8.0 m*M*. Peak identities: 1=simazine, 2=mecoprop, 3=asulan, 4=isoprocarb, 5=fensulfothion, 6=fenobucarb, 7=isoprothiolane, 8=mepronical, 9=chloroneb, 10=pencycuron, 11=isofenfos, 12=thiobencarb, 13=terbutol, 14=butamifos, 15=iprodine. Other conditions as in Fig. 2.

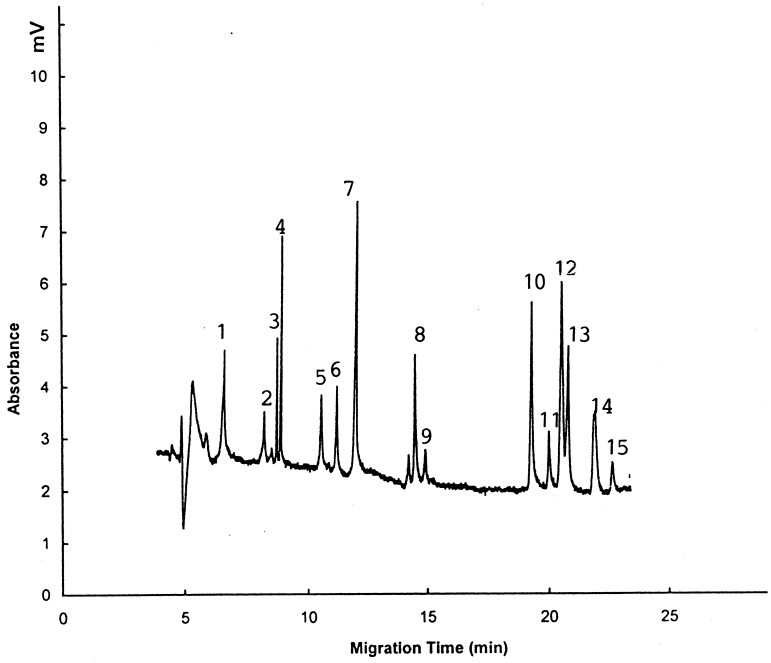

Fig. 4. Electropherogram of pesticides obtained under approximate optimum analytical conditions: borate buffer, 25 m*M* (pH 9.5); SDS, 30 m*M*; MeCN, 15%; β-CD, 8.5 m*M*. Analytes: 1=simazine, 2=isoprocarb, 3=mecoprop, 4=asulan, 5=fensulfothion, 6=fenobucarb, 7=mepronical, 8=isoproth, 9=chloroneb, 10=pencycuron, 11=isofenfos, 12=thiobencarb, 13=terbutol, 14=butamifos, 15=iprodine. Other conditions as in Fig. 2.

peak tailing and fronting may likely be due to the difference in mobilities between sample solutes and buffer co-ions, the difference in conductivity between sample solution (water–methanol=1:1) and buffer, and the adsorption of some analytes on the uncoated fused silica wall.

4. Conclusions

In this paper, the potential of CD–MEKC for the separation of a complicated mixture of pesticides and the applicability of OAD and main-effect curve for the optimization of separation in CD–MEKC were demonstrated. The results suggest that CD–MEKC is a suitable technique for screening the separation of complicated environmental samples. The application of OAD and the main-effect curve enable the optimization of a complicated mixture with a limited number of experiments.

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