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Optimisation of resolution in micellar electrokinetic chromatography by multivariate evaluation of electrolytes

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Abstract

A novel approach to multivariate evaluation of separation electrolytes for micellar electrokinetic chromatography is presented. An initial screening of the experimental parameters is performed using a Plackett–Burman design. Significant parameters are further evaluated using full factorial designs. The total resolution of the separation is calculated and used as response. The proposed scheme has been applied to the optimisation of the separation of phenols and the chiral separation of (+)-1-(9-anthryl)-2-propyl chloroformate-derivatized amino acids. A total of eight experimental parameters were evaluated and optimal conditions found in less than 48 experiments.

Keywords: Resolution; Micellar electrokinetic chromatography; Buffer composition; Chemometrics; Optimization; Phenols; Amino acids

1. Introduction

Micellar electrokinetic chromatography (MEKC) has been established over the past decade as a highly efficient separation technique [1]. The abundance of separations developed has shown the potential of MEKC for use as a complement or alternative to high-performance liquid chromatography [2]. Considering its low sample consumption, high separation efficiency and general compatibility with biological sample matrices, MEKC may even be the method of choice for many analytical separations.

MEKC is primarily a chromatographic technique, where the separation is based on the differential partitioning of analytes between a micellar phase and a surrounding electrolyte. Several experimental parameters influence the separation efficiency, the selectivity, and the retention factor of a MEKC separation system.

The separation efficiency is affected by, for example, temperature gradients in the capillary, the mobility of the ions in the background electrolyte (BGE), and concentration effects associated with the injection [3]. The parameters affecting separation efficiency have been extensively covered in the literature [4,5]. The selectivity is influenced by parameters governing the partitioning of the analytes between the micellar phase and the surrounding electrolyte. The partitioning is mainly determined by the choice of surfactant, the use of mixed micelles, the addition of organic modifiers and, if weak acids or bases are involved, the pH. Finally, the retention factor depends on the concentration of the surfactant,

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the mobility and hydrophobicity of the micelles, and the presence of organic modifiers in the BGE.

Evidently, optimising the resolution in a MEKC separation system involves evaluating the contribution from several experimental parameters. The optimisation of individual chromatographic descriptors such as separation efficiency, selectivity and retention factor in order to maximise resolution has been thoroughly studied [6]. This approach assumes that the different parameters influence only one descriptor. In many cases concerning MEKC, however, more than one chromatographic descriptor is influenced by the same experimental parameter. It is thus advantageous to optimise as many parameters as possible to avoid the risk of missing the true optimum conditions.

Several types of experimental designs can be used to reduce the number of experiments that have to be performed in order to optimise a separation while retaining a multivariate approach. A reduced factorial design [7], a D-optimal design [3] or a Plackett–Burman design [8] can all be used, for instance, to screen many experimental parameters at the same time. A full factorial design including axial and centre points [9] can subsequently be used to optimise the most significant parameters. A review article on optimisation of separation electrolytes for MEKC has been written by Corstjens et al. [10].

In this paper we present an alternative optimisation strategy, comprising an initial screening using a Plackett–Burman design with subsequent optimisation of significant parameters using factorial design. Thus, the number of experiments needed for screening significant parameters is greatly reduced. The sample used in developing the proposed strategy was a mixture of phenols. Studies were also performed on the correlation between various chromatographic descriptors and the total resolution of the separation. Finally, the strategy was used to optimise the separation of (+)-1-(9-anthryl)-2-propyl chloroformate (APOC)-derivatized amino acids [11,12].

2. Experimental

2.1. Chemicals

All amino acids (Asp, Glu, Ala, Ser, Met, Val, Tyr, Leu, Ilu and Phe) were from Sigma (St. Louis, MO, USA). The synthesis and characterisation of the (+)- and (−)-APOC reagent has previously been described in the literature [11]. Boric acid and sodium tetraborate were from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was obtained from USB (Cleveland, OH, USA) and sodium deoxycholate (SDC) from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade and all the solutions were prepared with water from an Elgastat UHQII (Elga, High Wycombe, UK).

Standard stock solutions of 100 m*M* SDS, 50 m*M* SDC, and 100 m*M* borax were used to prepare the buffers with different compositions. The experimental design determined the exact buffer composition.

2.2. Apparatus

The MEKC separation system included a Prince autosampler (Lauerlabs, Emmen, The Netherlands) and a Lambda 1000 UV detector (Bischoff, Leonberg, Germany), set at 210 nm for the phenols and 256 nm for the derivatized amino acids.

An untreated fused-silica capillary with an internal diameter of 50 μm and an external diameter of 375 μm was used for the experimental design analyses (Polymicro Technologies, Phoenix, AZ, USA). This capillary was 85 cm long, with a detection window 75 cm from the injection end. Before use the capillary was preconditioned with 0.1 *M* NaOH for 15 min, distilled water for 15 min and separation electrolyte for 20 min.

The laboratory-made laser-induced fluorescence (LIF) system used has previously been described [11]. The capillary used for the LIF experiments was an untreated fused-silica capillary with an inner diameter of 25 μm and an external diameter of 150 μm (Polymicro Technologies). The capillary had a total length of 90 cm with the detection point situated 82 cm from the injection end.

Data were collected using a personal computer in conjunction with ELDS 900 software (Chromatography Data System, Kungshög, Sweden). The experimental designs were constructed using MODDE software (Umetrics, Umeå, Sweden).

2.3. Experimental parameters

As a rule, the factors determining whether an experimental design will be successful or not are: (1)

the choice of parameters to optimise, (2) the interval within which the parameters are varied and (3) the choice of response used in the optimisation. It may not be possible to optimise some parameters that are known to influence the response, in this case the resolution, due to limitations in the experimental instrumentation. In our study the possibility of varying the dimensions of the capillary was limited by the relatively poor sensitivity of UV absorbance detectors. This parameter was therefore omitted. Further, our study was focused on the optimisation of separation conditions. Thus, experimental considerations associated with the injection [3] were not examined. It was assumed that sufficient stacking of sample components is ensured by keeping the conductivity of the sample plug several times lower than the conductivity of the BGE.

The parameters included in the study were the pH of the BGE, the concentration of the primary surfactant (SDS), the concentration of a secondary surfactant (SDC), the buffer concentration (sodium tetraborate; borax), the temperature in the capillary compartment, the amount of acetonitrile (ACN) added as an organic modifier, the concentration of urea as an organic modifier, and the separation voltage applied.

2.4. Experimental domains for the separation of phenols

The pH was varied between 8 and 10 in the initial screening experiments. This was within the effective buffering range of tetraborate. The primary surfactant used was SDS. SDS has been the most commonly used surfactant in MEKC since it has low UV absorbance, a low Kraft point, high solubility in aqueous media, and a low critical micelle concentration (CMC). SDS is also a relatively strong hydrogen bond donor as compared to most other surfactant systems [13]. As phenols exhibit hydrogen bond acceptor characteristics, despite being hydrogen bond donors, it would appear that SDS could be a reasonable first choice of surfactant. The concentration of SDS was varied between 15 and 30 m*M* in order to cater for a wide range of hydrophobicity while retaining micellar conditions. The concentration of SDC was varied between 0 and 6 m*M*. Studies on mixtures of alkyl chain surfactants and bile salts, such as SDS and SDC, respectively, have been presented in the literature [14,15]. The mixed bile salt/SDS micelles exhibit chemical properties resembling micelles consisting solely of bile salt surfactants added at relatively low percentages [15]. The interval studied in the initial experimental design was thus set between 0 and 20% of the SDS concentration. Reports of non-linearity in the resolution of enkephaline-related peptides with respect to the addition of acetonitrile [16] prompted us to limit the amount of acetonitrile added to the range of 0 to 10%. A secondary organic modifier, urea, was included in the experimental design as it may alter the selectivity of the separation system by changing the solvation of analytes in the aqueous phase [17]. The interval chosen for the addition of urea, 0 to 1 *M*, was based on our prior experience of urea as an organic modifier in MEKC. The temperature range used (20–30°C) was limited by the working range of the instrumentation. The parameters tested, and the experimental domains in which they were varied, are presented in Table 1.

2.5. Choice of response function

The choice of an appropriate response function is of prime importance in the multivariate optimisation of chromatographic systems. The response function used should ideally set a value on each chromatogram with reference to criteria set by the researcher [18]. In this study the objective was to separate as many of the phenols or derivatized amino acids as possible. The chiral separation of amino acids is still primarily an academic pursuit and thus no consideration was given to the length of time needed for the separation.

Two response functions were considered: the chromatographic resolution statistic (CRS) and the arcs tangens resolution (ATR). The ATR response function is a sum of resolution values that have been processed through a mathematical function similar to Harrington's one-sided desirability function [18]. This function is termed $F(R_i)$ in the following text. The values pertaining to the resolution between two neighbouring peaks is first calculated using the function:

$$F(R_i) = \frac{\arctan\left[a \cdot (R_i - b)\right] + \pi/2}{\pi}$$

Table 1
Plackett–Burman design; separation of phenols

Run order	pH	[SDS] (m*M*)	[SDC] (m*M*)	[ACN] (%)	[Urea] (*M*)	[Buffer] (m*M*)	Temperature (°C)	Voltage (kV)	Resolution
9	10	15	6	10	1	30	20	20	4.80
7	8	30	6	0	1	30	30	20	5.01
6	10	30	0	10	0	30	30	30	5.01
3	8	15	6	10	1	10	30	30	4.96
10	8	30	0	0	1	30	20	30	4.29
5	8	15	0	10	0	30	30	20	4.55
13	10	15	0	0	1	10	30	30	3.75
14	10	15	6	0	0	30	20	30	4.22
12	10	30	6	0	0	10	30	20	5.04
8	8	30	6	10	0	10	20	30	4.95
1	10	30	0	10	1	10	20	20	5.00
4	8	15	0	0	0	10	20	20	4.95
15	9	22.5	3	5	0.5	20	25	25	4.88
11	9	22.5	3	5	0.5	20	25	25	4.94
2	9	22.5	3	5	0.5	20	25	25	4.88

where R_i is the chromatographic resolution as calculated by:

$$R_i = \frac{1.18(\Delta t)}{(w_1 + w_2)}$$

where a is a slope value, b is the position of the inflection point of the $F(R_i)$ curve along the R_i axis, Δt is the time difference between the peaks and w is the peak width at half its height. The value of a governs how quickly the $F(R_i)$ curve increases from 0 to 1 around the value of b. The values of a and b are chosen by the researcher. The $F(R_i)$ curve acts as a cut-off filter and the researcher can decide the value of R_i below which peaks are not considered eligible for quantitation (the value b) and how strict the discrimination of these resolutions should be (the value a).

The ATR and CRS evaluations of several chromatograms were visually compared and it was found that the ATR function gave better representations of them. Further, the CRS function is unsuitable for optimisation purposes, partly because it is not monotonous in nature, and partly because it is not defined across the entire range of values that the resolution of two neighbouring chromatographic peaks can attain. The ATR response function was thus used for the optimisation experiments presented in this paper.

3. Results and discussion

3.1. Step 1: Initial screening using a Plackett–Burman design

The first step of the optimisation procedure was the screening of parameters using a Plackett–Burman design. This reduced factorial design can provide information about the main effects of each variable. However, the main effect is, in each case, confounded with interaction effects exerted by the other parameters. Three centre point separations are included to obtain an estimation of the variance in the model. The regression coefficients for the eight selected parameters are presented in Fig. 1a. All the parameters showed significant effects on the resolution except for the concentration of the buffer and the temperature of the capillary compartment. The concentration of SDS, the concentration of SDC and the concentration of ACN all showed positive effects. The pH of the separation electrolyte, the concentration of urea and the separation voltage all showed negative effects. The non-significant parameters were thus locked at the value of the respective centre points. The voltage was kept at the lower value (20 kV) in all further experiments. Considering contemporary MEKC theory, it seemed unlikely that significant interaction effects would occur between

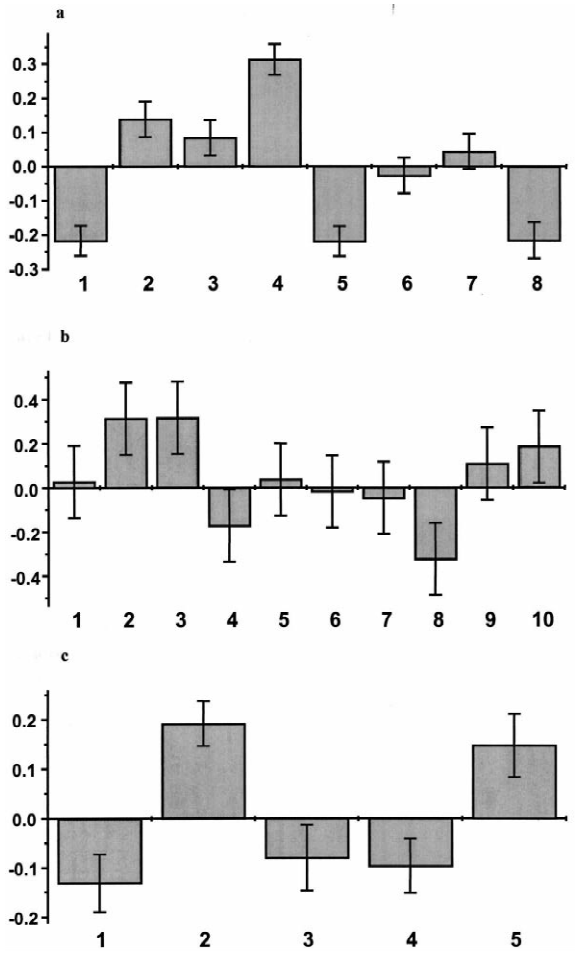

Fig. 1. (a) Plackett–Burman design; separation of phenols. The factors are: 1=pH, 2=[SDS], 3=[SDC], 4=[ACN], 5=[urea], 6=[buffer], 7=temperature, 8=applied separation voltage. (b) Full factorial design; four factors, separation of phenols. The factors are: 1=pH, 2=[ACN], 3=[SDS], 4=[SDC], 5=pH·[ACN], 6=pH·[SDS], 7=pH·[SDC], 8=[ACN]·[SDS], 9=[ACN]·[SDC], 10=[SDS]·[SDC]. (c) Circumscribed central composite design; two factors, separation of phenols. The factors are: 1=[ACN], 2=[SDS], 3=[ACN]·[ACN], 4=[SDS]·[SDS], 5=[ACN]·[SDS]. Significance level: 95% (*t*-test).

the separation voltage and the pH of the separation electrolyte, the concentration of SDS, the concentration of SDC or the concentration of ACN. However, a further reduction in the applied separation voltage would result in exceedingly long separation times.

The concentration of urea did not show any considerable difference in selectivity as compared to the concentration of ACN, so urea was excluded from the optimisation scheme. The separations with urea added to the electrolyte also took longer time, since the electroosmotic flow was slower.

Interaction effects between the parameters governing micelle formation, i.e., the concentration of SDS, addition of SDC or the addition of ACN, are likely to occur according to theory. These parameters were thus further investigated using a full factorial design. The pH of the separation electrolyte was also varied, with the experimental range shifted to 7 for the lower value and 9 for the upper value.

3.2. Step 2: Full factorial design

The second step in the optimisation process was to perform a full factorial design in order to elucidate if any interaction effects occurred. The experiments performed in the full factorial design are presented in Table 2. The calculated effects of the parameters are shown in Fig. 1b.

The pH of the separation electrolyte is not a significant parameter in the experimental domain.

Table 2
Full factorial design; four factors, separation of phenols

Run order	pH	[ACN] (%)	[SDS] (m*M*)	[SDC] (m*M*)	Resolution
2	7	0	15	0	4.30
5	9	0	15	0	4.30
1	7	10	15	0	4.88
11	9	10	15	0	4.08
16	7	0	30	0	4.99
4	9	0	30	0	5.03
6	7	10	30	0	4.97
12	9	10	30	0	4.97
9	7	0	15	6	3.23
17	9	0	15	6	3.11
15	7	10	15	6	4.89
3	9	10	15	6	4.82
14	7	0	30	6	5.02
8	9	0	30	6	5.02
19	7	10	30	6	5.00
7	9	10	30	6	5.03
10	8	5	22.5	3	4.96
13	8	5	22.5	3	4.97
18	8	5	22.5	3	4.98

Neither did any of the interaction terms involving pH show significance. The pH was thus kept at 9 for the final optimisation, since the electroosmotic flow was more stable and the analysis was faster than at pH 7.

The addition of SDC resulted in a significant negative effect, while the interaction term between SDS and SDC was significantly positive. Upon inspection of the contour surface plot of SDS and SDC it was concluded that the addition of SDC did not seem to influence the resolution much in the presence of high concentrations of SDS. The addition of SDC, however, severely diminished the resolution when the concentration of SDS was low. This led to the exclusion of SDC from further experiments.

Both the concentration of ACN and the concentration of SDS showed significant positive effects. The ACN and SDS interaction term, however, showed a significant negative effect.

3.3. Step 3: Final optimisation using circumscribed central composite design (CCC)

A full factorial design, with axial and centre point experiments, was carried out for two parameters: the concentration of SDS and the concentration of ACN. The parameters, the range over which these were varied and the ATR response values for the chromatograms are presented in Table 3. All main, quadratic and interaction terms were found to be significant (Fig. 1c). Fig. 2 shows the response surface plot for the two parameters. The data suggest

Table 3
Central composite design circumscribed; two factors, separation of phenols

Run order	[ACN] (%)	[SDS] (m*M*)	Resolution
10	2	15	4.91
1	10	15	4.32
5	2	30	5.00
8	10	30	5.00
2	0.34	22.5	4.94
4	11.66	22.5	4.95
11	6	11.90	4.47
9	6	33.11	5.02
7	6	22.5	4.98
6	6	22.5	4.96
3	6	22.5	4.94

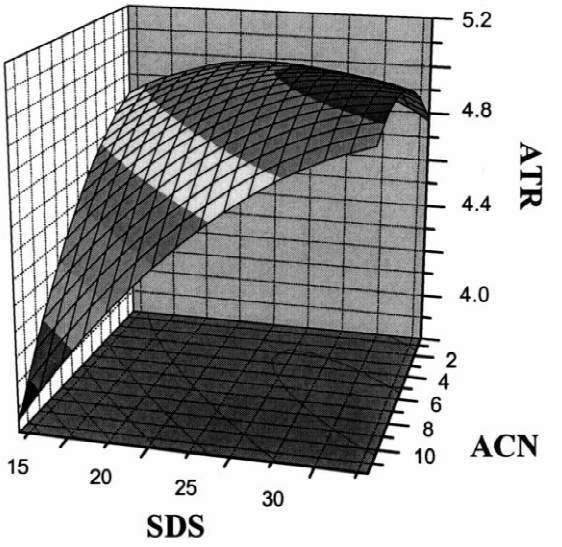

Fig. 2. Response surface plot of ATR resolution value as a function of the concentration of SDS and the concentration (in %) of ACN.

that an optimum exists within the experimental domain in an area defined through 25–32 m*M* SDS and 4–8% ACN.

At this point in the investigation of the phenols, a total of 45 experiments had been performed. The initial screening needed 15 experiments. A further 19 experiments were performed in the full factorial design, and 11 more for the final CCC design. Optimal separation conditions for the phenols can be given on the basis of the results from these experiments. The following conditions should be selected: pH 9, SDS concentration≈28 m*M*, buffer concentration=20 m*M*, ACN concentration≈6%, temperature=25°C and voltage=20 kV.

3.4. Correlation studies

According to theory there is an optimum range for the retention factor where resolution is easiest to achieve [6,8]. The ATR resolution value as a function of the median k' value of the analytes was plotted in order to verify this. The results are presented in Fig. 3a. No correlation was found in any of the three experimental designs. Terabe et al. predicted that the optimum k' values in MEKC would have values between 2 and 5 [6]. All the k'

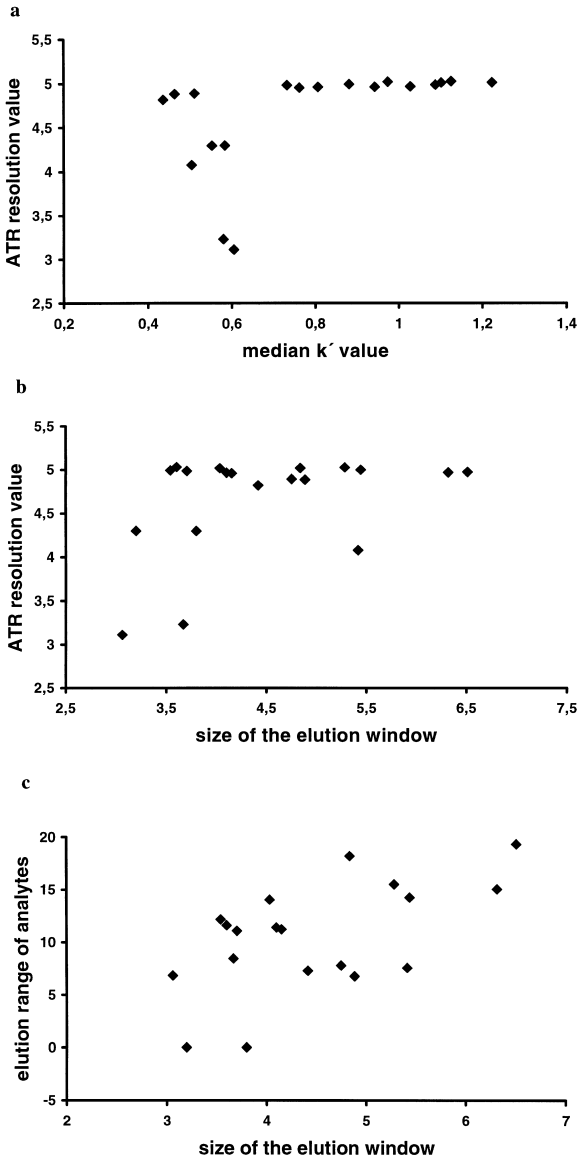

Fig. 3. Plots showing: (a) the ATR resolution value as a function of the median k' value of the phenols, (b) the ATR resolution value as a function of the size of the elution window, (c) the elution range of the analytes as a function of the size of the elution window. The k' values were calculated according to $k'=(t_R-t_0)/(t_0-t_0t_R/t_{mc})$, the elution window as $M=t_0/t_{mc}$, and the elution range as the difference, in elution time, between the last and first eluting species.

values derived in this study are, however, lower than these optimal values. A conclusion that could be drawn from the results presented here is that it is easier to achieve optimal separation conditions at k' values closer to the optimal predicted k' values, but this is not necessarily true. Some of the parameters that influence k' may also influence the separation efficiency or the selectivity. Thus, the improvement in resolution as a result of the separation system operating at optimal k' conditions may be counteracted by a reduction in separation efficiency or a change in selectivity. Similar conclusions have been drawn for the separation of enkephaline-related peptides by MEKC [3].

Data were also plotted to investigate the correlation between the size of the elution window and the ATR resolution values for the three experimental designs. Fig. 3b shows the plot for the full factorial design. No significant correlation could be found for any of the experimental designs. The conclusion drawn from the correlation plots between the size of the migration window and the elution range, i.e., the time difference between the first and the last eluted phenol, as presented in Fig. 3c were the same as that for the previous correlation plots. This implies that the selectivity of the MEKC system has a greater impact on the elution range of the analytes than the size of the elution window.

3.5. Chiral separation of (+)-APOC-derivatized amino acids

The separation of amino acids derivatized with the chiral reagents (+)- and (−)-APOC has previously been optimised for several of the parameters studied in this investigation [11,12]. However, the parameters have not all been studied simultaneously, which increases the risk that interaction effects between different parameters will be missed and that the optimum found will be a local, rather than global, optimum.

The initial Plackett–Burman design was identical to the one used for optimising the separation of phenols (Table 4), since a design incorporating as many experimental parameters as possible could act as a general optimisation scheme for separations in MEKC. The regression coefficients obtained from the Plackett–Burman design are presented in Fig. 4a.

Only the pH of the separation electrolyte, the concentration of ACN and the applied separation voltage showed significant effects. This may be due

Table 4
Plackett–Burman design; separation of amino acids

Run order	pH	[SDS] (m*M*)	[SDC] (m*M*)	[ACN] (%)	[Urea] (*M*)	[Buffer] (m*M*)	Temperature (°C)	Voltage (kV)	Resolution
15	10	15	6	10	1	30	20	20	7.20
10	8	30	6	0	1	30	30	20	6.99
11	10	30	0	10	0	30	30	30	11.55
3	8	15	6	10	1	10	30	30	6.56
2	8	30	0	0	1	30	20	30	8.81
14	8	15	0	10	0	30	30	20	11.24
12	10	15	0	0	1	10	30	30	8.90
6	10	15	6	0	0	30	20	30	8.57
13	10	30	6	0	0	10	30	20	6.86
5	8	30	6	10	0	10	20	30	13.72
1	10	30	0	10	1	10	20	20	7.99
9	8	15	0	0	0	10	20	20	10.64
4	9	22.5	3	5	0.5	20	25	25	10.66
8	9	22.5	3	5	0.5	20	25	25	10.58
7	9	22.5	3	5	0.5	20	25	25	11.25

to strong interaction effects that counterbalance the main effects of the parameter. In cases like this, parameters with a high probability of interaction should be included in a more refined study where interaction terms can be more easily detected. It is, for instance, highly probable that the concentration of SDS would affect the separation of analytes exhibiting such a wide range of hydrophobicity as the derivatized amino acids.

Parameters with a low probability of interaction may, however, be locked or discarded if the coefficients so permit. Thus, the voltage, a parameter of physical rather than chemical nature, was locked at the highest level (30 kV) that the high voltage supply could generate. The addition of urea as an organic modifier was removed as a parameter for further optimisation for the same reasons it was discarded in the optimisation of the separation of the phenols. The temperature and buffer concentration were also locked as parameters: the temperature at its lowest value and the buffer concentration at its mean value.

A full factorial design was performed with four parameters: the pH, the concentration of SDS, the addition of SDC and the addition of ACN (Table 5). The regression coefficients for the full factorial design are presented in Fig. 4b. Only the regression coefficient for ACN showed any significance for the resolution (95% level, *t*-test). As this is an unlikely result, only the pH, which is thought be less likely to be involved in interactions than the other parameters, was fixed at its highest level (which gave faster and more reproducible analyses). The other parameters were once again studied in a full factorial design (Table 6). The range of SDS was changed to an interval spanning 25 m*M* to 35 m*M* due to the large interaction term between SDS and ACN.

The regression coefficients for this factorial design are presented in Fig. 4c. None of the effects showed any significance. However, inspection of the response surface plots led to the following conclusions. The addition of ACN was advantageous to a greater extent if the concentration of SDS was high. The addition of SDC showed little effect on the resolution when the concentration of SDS was high and it decreased the resolution if the concentration of SDS was low. The addition of ACN, in turn, increased the resolution in the absence of SDC and decreased the resolution in its presence. Comparing the magnitudes of the effects it was concluded that the optimum conditions lay in the direction of higher concentrations of SDS and ACN. Scouting experiments at 40 m*M* SDS and addition of 15% ACN failed due to excess Joule heating. It was thus concluded that, for the experimental set-up used in the optimisation, the optimal separation conditions were: pH 10, SDS concentration = 35 m*M*, buffer concentration = 20 m*M*, ACN concentration = 10%, temperature = 20°C and voltage = 30 kV.

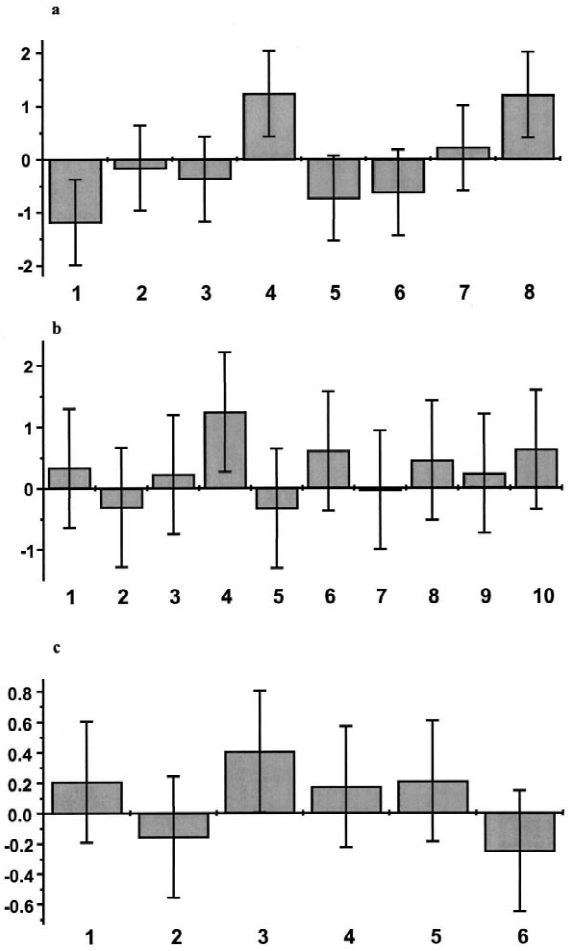

Fig. 4. (a) Plackett–Burman design; separation of amino acids. The factors are: 1=pH, 2=[SDC], 3=[SDS], 4=[ACN], 5=[urea], 6=[buffer], 7=temperature, 8=applied separation voltage. (b) Full factorial design; four factors, separation of amino acids. The factors are: 1=pH, 2=[SDC], 3=[SDS], 4=[ACN], 5=pH·[SDC], 6=pH·[SDS], 7=pH·[ACN], 8=[SDS]·[SDC], 9=[ACN]·[SDC], 10=[ACN]·[SDS]. (c) Full factorial design; three factors, separation of amino acids. The factors are: 1=[SDS], 2=[SDC], 3=[ACN], 4=[SDS]·[SDC], 5=[SDS]·[ACN], 6=[SDC]·[ACN]. Significance level: 95% (*t*-test).

In order to compare the optimised separation electrolyte of the derivatized amino acids with the separation electrolyte identified in the earlier, stepwise optimisation, buffers containing 40 m*M* SDS and various levels of ACN were prepared. These were tested using a capillary electrophoresis (CE) apparatus with an LIF detection system constructed in the laboratory. On this instrumentation, electrophoretic band-broadening is greatly reduced due to the small amounts of analyte injected and the effective heat dissipation through the use of capillaries with smaller internal diameters. The use of smaller diameter capillaries also results in a lower separation current with a subsequent reduction in Joule heating. It was found that a 10% addition of ACN gave a satisfactory resolution of the most hydrophilic amino acids (glutamate and aspartate) while retaining good resolution for the hydrophobic amino acids. Chromatograms showing the separation

Table 5
Full factorial design; four factors, separation of amino acids

Run order	pH	[SDS] (m*M*)	[SDC] (m*M*)	[ACN] (%)	Resolution
4	8	20	0	0	12.85
9	10	20	0	0	11.75
13	8	20	6	0	9.65
15	10	20	6	0	8.04
10	8	30	0	0	7.48
8	10	30	0	0	10.76
19	8	30	6	0	9.24
12	10	30	6	0	11.56
2	8	20	0	10	11.02
1	10	20	0	10	12.14
16	8	20	6	10	12.30
3	10	20	6	10	11.69
11	8	30	0	10	12.86
18	10	30	0	10	14.83
5	8	30	6	10	13.17
14	10	30	6	10	13.06
7	9	25	3	5	9.71
17	9	25	3	5	9.36
6	9	25	3	5	9.04

Table 6
Full factorial design; three factors, separation of amino acids

Run order	[SDS] (m*M*)	[SDC] (m*M*)	[ACN] (5%)	Resolution
4	25	0	0	13.47
9	35	0	0	13.50
8	25	6	0	13.69
3	35	6	0	13.65
10	25	0	10	14.74
11	35	0	10	14.85
1	25	6	10	13.20
7	35	6	10	14.75
2	30	3	5	14.44
5	30	3	5	13.69
6	30	3	5	13.70

of (+)-APOC-derivatized amino acids from hydrolyzed β-amyloid enriched human senile plaque, obtained using the optimised separation electrolytes from this investigation and the study presented earlier [12], were compared (Fig. 5). The resolution of the chromatograms is similar. The difference in elution time between the first and last eluting amino acid is larger in the separation with the electrolyte presented in this paper. The separation of hydrophilic amino acids is slightly reduced, while better resolution is achieved for the more hydrophobic amino acids. The optimised separation electrolyte presented here is probably close to a global optimum for the separation of APOC-derivatized amino acids. The electrolyte presented in previous work may, however, represent a local optimum with a focus on the separation of hydrophilic amino acids.

3.6. General remarks

The experiments described above have shown the applicability of the proposed scheme for optimising MEKC separation electrolytes. In both optimisations that were performed, significant interaction effects were found between the parameters governing micelle formation, i.e., the concentration of primary surfactant, the addition of a secondary surfactant and the addition of an organic modifier. The role of the secondary surfactant is to change the chemical environment inside the micelle and thus alter the selectivity of the separation system. The organic modifiers play a similar role, but in the aqueous phase of the separation system. A possible way of simplifying the optimisation scheme would thus involve excluding the secondary organic modifier (in our case urea) and to only have one parameter for altering the selectivity in each phase of the separation system. Urea and ACN could then be compared in a small two-factor factorial design if the initial optimisation scheme failed.

Another suggestion to speed up the optimisation could be to initially make scouting experiments to

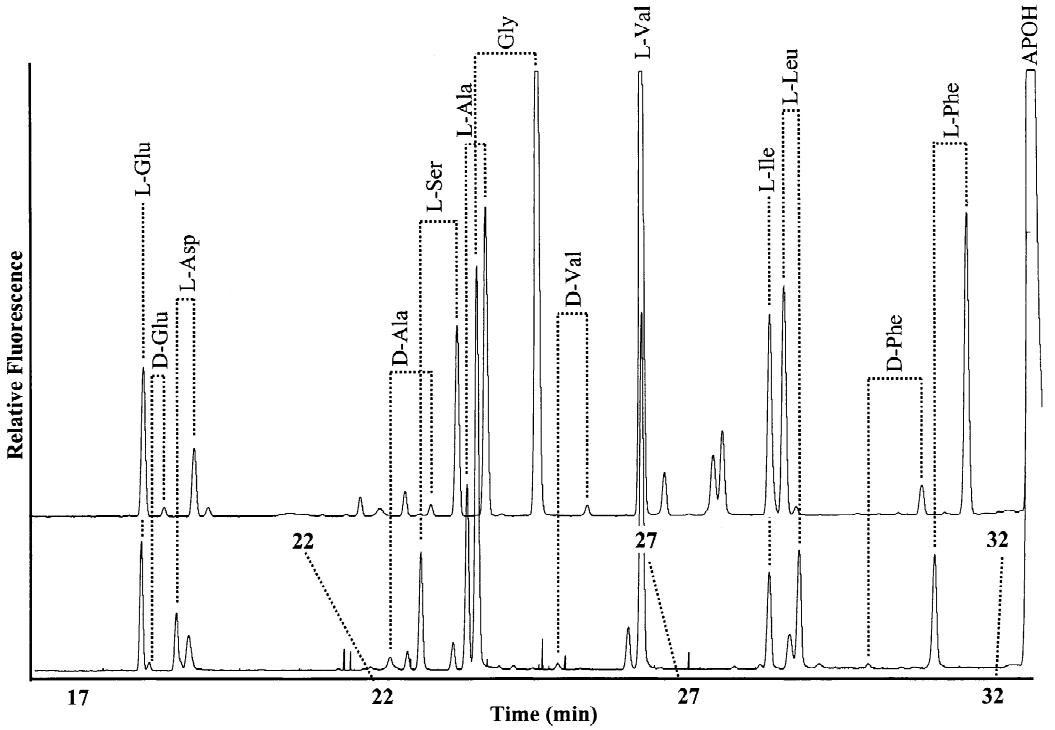

Fig. 5. Comparison of separation electrolytes optimised using two alternative approaches. The upper trace shows a separation using the composition 20 m*M* tetraborate buffer at pH 9.8 with a mixed micellar phase; 20 m*M* SDS and 7.5 m*M* SDC. The lower trace shows a separation using the electrolyte developed in this paper; 20 m*M* tetraborate buffer at pH 10 with 40 m*M* SDS and an addition of 10% ACN. The sample was in both cases (+)-APOC-derivatized amino acids from hydrolysed human senile plaque.

determine a concentration of surfactant at which the k' of the analytes are close to the optimum values presented in the literature. This would then constitute the centre point concentration for the initial screening experiment.

4. Conclusions

In this study, a method for optimising the resolution in MEKC has been developed, using experimental design.

(1) Eight factors involved in MEKC separations were identified and optimised.

(2) A strategy for performing an optimal number of experiments, needed for optimisation of the factors, was established.

(3) The strategy was based on a method using experimental design, which involved a screening step, one or two full factorial designs, which can, if needed, be augmented by the centre points and the axial points to take curvature into account and thereby complete a CCC.

(4) The initial screening steps showed, for the two applications investigated in this work, that four of the eight factors (voltage, temperature, urea and buffer concentration) could be fixed, i.e., optimised simultaneously. The following factorial design allowed the optimisation of the pH (and also SDC in the phenol case) and, finally, results from the central composite design experiments optimised the last two factors, SDS and ACN.

(5) This strategy is general, flexible and can significantly reduce the number of experiments needed for a complete multivariate optimisation of eight factors. A full factorial design with eight factors needs 256 experiments, while about 45 experiments were sufficient according to the proposed strategy.

(6) The proposed optimisation strategy was developed and tested for its utility in developing a method for separating phenolic compounds by MEKC, and subsequently validated by applying it to amino acids. It is assumed that the proposed method is general and that it can be used for optimising the resolution of other groups of analytes.

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