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Multivariate evaluation of organic modifier effects on the separation performance of peptides in micellar electrokinetic chromatography

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Abstract

Resolution in micellar electrokinetic chromatography is highly complex and describes a non-linear function of the experimental variables. Four experimental variables; the surfactant concentration, the percentage of organic modifier, the applied temperature and the ionic strength of the buffer were studied via response surface modelling and related to resolution, retention factor and migration time window utilizing partial least-square regression. The effect of acetonitrile on the separation performance between enkephalin-related peptides was studied at four different domains, 0–5, 5–10, 10–15 and 15–20% (v/v), while keeping the other variables at constant intervals. Determination of the critical micellar concentration for sodium dodecyl sulfate, showed that micelles were formed even at high levels of acetonitrile and temperature. Principal component analysis of the responses revealed that the different parameters such as efficiency, migration time window and retention factor exert strong influence on the resolution, and could not be independently controlled. Furthermore the results revealed very different influence of the experimental variables in each domain and the effect of acetonitrile was highly non-linear and dependent on the temperature used. The resolution increased by increasing the temperature and acetonitrile in the low level domain, while it decreased in the high level domain. There seems to be a change in the thermodynamics of the system at high levels of acetonitrile, since increased temperature resulted in higher association of the peptides to the micelles.

Keywords: Principal component analysis; Background electrolyte composition; Response surface modelling; Temperature effects; Peptides; Enkephalin

1. Introduction

Organic solvents are often used as additives to the aqueous buffer in micellar electrokinetic chromatography (MEKC) to reduce the retention factors of strongly bound solutes to the micelles, to extend the migration-time window and/or enhance selectivities. Different types of organic modifiers such as methanol [1,2], acetonitrile [1,3,4], dimethyl sulfoxide (DMSO) [5], acetone [5], and dimethylformamide [1,6] have been used to improve the resolution in MEKC. Several groups have studied the effect of adding acetonitrile to the background electrolyte (BGE). The mechanism for the separation is not clear, some authors assume that the distribution between the aqueous phase and micellar phase is the underlying principle for the separation [7,8], while others argue that at higher organic solvent contents,

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the critical micelle concentration (CMC) is strongly increased so micelles are not formed, and a solvophobic interaction between the analyte molecules and individual surfactant ions instead is taking place [9–11]. Very hydrophobic compounds tend to be completely distributed to the micelles and selectivity is lost because the compounds migrate with the same mobility as the micelles. Addition of acetonitrile to the BGE will decrease the polarity of the aqueous phase and alter the retention mechanism, generally by reducing the retention factors [12–18]. Hydrophobic and ionic interactions were found to be weaker in the presence of acetonitrile giving lower retention factors [19]. Contradictory results can be noted in the literature about the effect of acetonitrile on the migration time window. Kaneta el al. [20] reported that the migration time window was not extended by addition of acetonitrile to the BGE, but Sepaniak et al. [21] reported a moderate extension of the migration time window, due to small changes in the electroosmotic flow. Other groups reported that the addition of acetonitrile extended the migration time window, giving higher resolution, but at the expense of longer analysis time [14,22]. A computer-aided method was used to optimize simultaneously the concentration of sodium dodecyl sulfate (SDS) and acetonitrile on the basis of four test runs. The result showed that with high SDS concentrations and high acetonitrile content a nearly infinite elution range was approached [23]. Solvent gradient can provide a compromise between enlarged elution range and long analysis time. Balchunas and Sepaniak [13] were first to report the use of gradient elution in MEKC. They also reported the construction of an apparatus for continuous gradient elution [21]. However, stepwise gradients with commercial capillary electrophoresis instrumentation was not a powerful tool for resolution optimization due to extreme band broadening [24]. Previous studies at commonly used levels of acetonitrile (5–15%, v/v) in the BGE showed large variations in separation performance and a change in migration pattern of the peptides going from low to high level of acetonitrile. Partial least-square (PLS) regression of the responses revealed that the experimental domain was too large and complicated to be explained by the central composite design (CCD) used [25].

The aim of this study was to evaluate the influence of organic modifier, together with surfactant concentrations, temperature and the ionic strength of the buffer, on the separation performance in MEKC. A central composite design at four different ranges of acetonitrile was applied.

2. Experimental

2.1. Chemicals

The BGE consisted of phosphoric acid (analytical-reagent grade) and sodium hydroxide (analytical-reagent grade), Titrisol from Merck (Darmstadt, Germany) diluted with water purified in a Milli-Q Water system (Millipore, Bedford, MA, USA), to give pH 6.00. The given pH value has to been seen as an operational value, and the apparent value is probably higher when acetonitrile Chromosolv from Riedel-de Haën (Seelze, Germany) was used as organic modifier. The surfactant used was SDS obtained from ICN Biomedicals (Aurora, OH, USA). The enkephalin-related peptides from Chemicon (Stockholm, Sweden), were dissolved in BGE without micelles and filtered through 0.2 μm Ministart filters from Sartorius (Göttingen, Germany). The characteristics of the enkephalin-related peptides are shown in Table 1.

2.2. Apparatus

Experiments were performed on a Hewlett-Packard ^{3D}Capillary Electrophoresis system (Hewlett-Packard, Waldbronn, Germany), with a capillary electrophoresis (CE) unit with a diode-array detection (DAD) system and a ChemStation for system control, data collection and data analysis. Absorption detection was carried out at 200 nm. The applied voltage was kept constant, at 20 kV. The samples were injected by pressure at 40 mbar for 3 s. Separation was performed on fused-silica capillaries of 48.5 cm (effective length 40 cm)×50 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA). New capillaries were flushed with 1 *M* NaOH for 20 min, 0.1 *M* NaOH for 5 min and water for 5 min. Between runs the capillary was rinsed with water, 0.1 *M* NaOH and water for 5 min, respectively. Before each injection the capillary was rinsed with

Table 1
Characteristics of the enkephalin-related peptides

No.	Peptides	Amino acid sequence	M_r	Isoelectric point (p*I*)
1	TGG	Tyr–Gly–Gly	295	6.1
2	DTLE	Gly–Gly–Phe–Leu	393	6.1
3	DTME	Gly–Gly–Phe–Met	411	6.1
4	DMME	Tyr–Gly–Gly–Phe	443	6.1
5	LE	Tyr–Gly–Gly–Phe–Leu	556	6.1
6	ME	Tyr–Gly–Gly–Phe–Met	574	6.1
7	LEA	Tyr–Gly–Gly–Phe–Leu–Arg	712	9.8
8	MEA	Tyr–Gly–Gly–Phe–Met–Arg–Phe	877	9.8
9	LEAA	Tyr–Gly–Gly–Phe–Leu–Arg–Arg	868	12.5

BGE for 15 min and conditioned for 5 min at 20 kV. The amounts injected were 67–100 pg of each peptide. The disturbance in the baseline caused by acetonitrile was used as marker for the electro-osmotic flow.

2.3. Determination of critical micellar concentrations

The CMC for SDS in a phosphate buffer at pH 6, (ionic strength, $I=0.05$) was determined at different concentrations of acetonitrile (0–20%, v/v) in the BGE and at two different temperatures, 25 and 35°C, with CE. Jacuier and Desbène [26] have shown that it is possible to use CE to determine CMC of SDS in solution containing both buffer salts and organic modifier. Such solutions are rarely compatible with the more commonly used methods, like conductivity or solubility measurements, due to the presence of organic modifier.

2.4. Experimental design

Four experimental factors; SDS concentration, acetonitrile concentration, temperature and ionic strength of the buffer were studied via central composite (2^4+5+8) design and related to resolution, retention factor and migration time window utilizing PLS regression. The levels of the factors were based on previous experimental design [27]. The concentration of acetonitrile was divided into four different ranges, 0–5, 5–10, 10–15 and 15–20% (v/v), leading to four blocks of experiments. The other experimental variables were in the same intervals in the four domains, according to Table 2. The low and high level of the SDS concentration

Table 2
The experimental domain used in the central composite design

Variable parameters	Experimental domain				
	Axial point	(−) level	(0) level	(+) level	Axial point
SDS (m*M*)[a]	15	30	45	60	75
SDS (m*M*)[b]	20	30	40	50	60
Acetonitrile (%, v/v)					
A	0	0	2.5	5	7.5
B	2.5	5	7.5	10	12.5
C	7.5	10	12.5	15	17.5
D	12.5	15	17.5	20	22.5
Temperature (°C)	17.5	25	32.5	40	47.5
Ionic strength[c]	0.01	0.02	0.03	0.04	0.05

[a] In block A.
[b] In blocks B–D.
[c] The value refers to the ionic strength of the neat phosphate buffer.

were set at 30 and 60 m*M*; the temperature at 25 and 40°C and the ionic strength of the phosphate buffer at 0.02 and 0.04 *M*. In the first block of experiments, in the range 0–5% (v/v) of acetonitrile, 25 experiments were needed. In the next block the eight experiments at high level of acetonitrile from the first block were set as the low level of acetonitrile, and were augmented with eight experiments at higher level of acetonitrile and with experiments at the axial points. This was than repeated for the other blocks as well. The experiments within each block were carried out in a random order to avoid systematic long-term influence of a particular factor. Every experiment was performed at least two times. The different blocks of experiments were performed with increasing percentage of acetonitrile. In the first block of experiments, when temperature was at low level and the other three factors at high level, the migration time of the peptide, LEAA was unstable, therefore the high level of SDS concentration was lowered to 50 m*M* in the following block of experiments. The selected responses were the migration time for the electroosmotic flow, the migration time of the peptides DTLE, DMME, LE, LEA and LEAA, the resolution between the peptide pairs, DTLE and DMME and MEA and LEAA. The programs used in the model building process were CODEX (SumIT System, Solna, Sweden) and MATLAB (MathWorks, Natick, MA, USA).

3. Results and discussion

3.1. Effect of temperature and acetonitrile content on the critical micellar concentration

The CMC was measured by studying the effective mobility of the peptides, LEAA and DTLE, in the presence of increasing concentration of the surfactant. The evaluation of the effective mobility of DTLE, as a function of the total surfactant concentration showed a sharp change in the slope at the CMC, with phosphate buffer as BGE. However, after addition of acetonitrile to the BGE the change in the slope became more diffuse, especially at higher content of organic modifier. Therefore, the effective mobility of the highly charged peptide LEAA, was studied instead. At surfactant concentrations under the CMC, the peptide had an effective mobility towards the cathode, increasing the surfactant concentration above the CMC, micelles were formed and the effective mobility of the peptide changed direction, due to strong association with the micelles. This is shown in Table 3, at low surfactant concentrations the effective mobility of LEAA was almost identical to that in neat phosphate buffer; between 2.0–3.0 m*M* SDS, the effective mobility became very low, probably due to association of the peptide with the SDS monomers. A drastic change of the effective mobility was shown at 5.0 m*M*, which was given as the CMC value under the experimental conditions studied.

Table 3
The electroosmotic mobility and the effective mobility of peptides as a function of SDS below and close to the CMC value

SDS (m*M*)	μ_{eff} (10^{-4} $cm^2/V\ s$)		
	EOF	DTLE	LEAA
0	5.74	−0.145	1.49
0.5	5.94	−0.162	1.54
1.0	5.90	−0.175	1.43
2.0	5.89	−0.130	0.038
3.0	5.91	−0.129	0.037
5.0	5.97	−0.307	−4.49

BGE: Phosphate buffer, pH 6.0 ($I=0.05$) with 5% (v/v) CH_3CN at 35°C.

The advantages of using CE to determine the CMC is that the micelle formation is studied under electrophoretic conditions identical to those used in separation. The CMC values measured at different contents of acetonitrile in the BGE and at two different temperatures are listed in Table 4. The CMC values were lower than that reported in water

Table 4
The influence of acetonitrile content and temperature on CMC

CH_3CN (%, v/v)	CMC (m*M*)	
	Temperature (°C)	
	25	35
0	3	4
5	4	5
10	4	5
15	5	6
20	7	9

BGE: Phosphate buffer, pH 6.0 ($I=0.05$).

at 25°C, 8.1 m*M* [28], which is in accordance to theory since electrolytic salts will reduce the CMC of most surfactants [29]. Increasing the percentage of acetonitrile in the BGE raised the CMC at both temperatures, as indicated in Table 4. The CMC values increased somewhat by raising the temperature from 25 to 35°C. Similar effects of acetonitrile were reported by Jacquier and Desbène [26], but in a different buffer system. To determine the CMC value more accurately more runs should be performed at a narrower concentration range of SDS around the given CMC, according to Jaquier and Desbène [30]. In conclusion the CMC values obtained, show that micelles were formed under the experimental conditions used in the experimental design.

3.2. Principal component analysis of the responses

The resolution equation in MEKC was defined by Terabe et al. [31] for uncharged compounds as;

$$R_s = \frac{N^{1/2}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k_2'}{1 + k_2'} \cdot \frac{1 - t_{EOF}/t_{mc}}{1 + (t_{EOF}/t_{mc})k_1'} \qquad (1)$$

where N is the efficiency, α is the separation factor which is equal to k_2'/k_1', k' is the retention factor, t_{EOF} and t_{mc} are the migration times for the electroosmotic flow and the micelles, respectively. The separation mechanism of the enkephalin related peptides is based on the micellar solubilization as well as electrophoretic migration. The determination of the retention factor for the peptides would require that the electrophoretic migration without micelles present should be measured at all experimental conditions studied. However, to be able to measure the k' directly from the relevant MEKC electropherogram the retention factor defined according to Khaledi and co-workers [32,33], assuming total association between the peptides and the surfactant monomers was used instead.

$$k' = \frac{t_m - t_{EOF}}{t_{EOF}(1 - t_m/t_{mc})} \qquad (2)$$

where t_m is the migration time of the peptide. The mobility of the micelles was assumed to be the same as the mobility of the peptide LEAA, as previous studies showed that this peptide was totally distributed to the micelles [27].

The mobility data given in Table 3, indicated a total association between the monomers and the peptide, LEAA, as the mobilities become very low at SDS concentrations close to the CMC. However, the peptide DTLE, had almost the same mobilities with and without SDS monomers present in the BGE and the same trend was seen at other concentrations of acetonitrile in the BGE (data not show). Therefore, calculation of the retention factor according to Eq. (2) will be somewhat overestimated.

To get an overview of how the different parameters, such as efficiency, selectivity, retention factor and migration time window are related for the enkephalin-related peptides, principal component analysis (PCA) was performed at the four different ranges of acetonitrile. The responses studied were; the migration time for the electroosmotic flow (t_{EOF}), the migration times of the peptides, DTLE, DMME, LE, LEA and LEAA, the retention factors for DTLE, DMME, LE and LEA, the resolution between the peptides pair, DTLE and DMME (R_{s1}) and between MEA and LEAA (R_{s2}) and the migration time window, defined as t_{LEAA}/t_{EOF}.

The data matrix was centered and scaled before the analysis. The loading vectors for the first and the second PC are plotted against each other in Fig. 1, for the four different blocks of acetonitrile concentrations studied (see Table 2). Over 80% of the variance was explained by the first two PCs.

In block A (Fig. 1A), the resolution between the peptide pairs, DTLE and DMME (R_{s1}) was negatively correlated to the retention factors. The migration time window and the R_{s1} were positively correlated in the second PC. Since the second PC explains less variance than the first one, the resolution will be increased mainly by decreasing the association of the peptides to the micelles, and to some extent also by enlarging the migration time window. The peptides containing arginine, LEA, MEA and LEAA, had infinite distribution to the micelles in almost all experiments, migrating with the same mobility as the micelles and were only baseline separated in one experiment. Therefore, R_{s2} and the retention factors for LEA and MEA, were not included in the modelling in this block.

Unexpectedly, the efficiency was negatively corre-

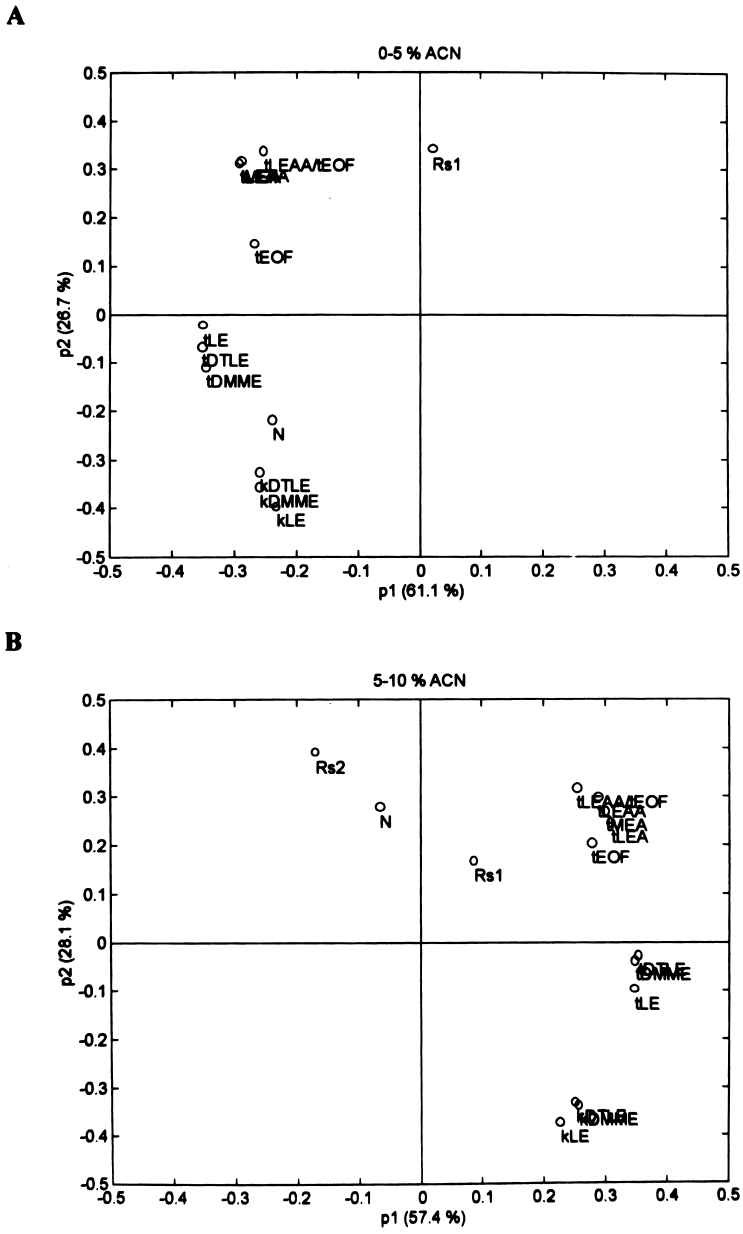

Fig. 1. Loading plots from PCA of the responses from the CCD design in Table 2. A = 0–5, B = 5–10, C = 10–15 and D = 15–20% (v/v) CH_3CN.

lated to the resolution, the reason is probably that when the efficiency increased so did the distribution to the micelles, which resulted in lower resolution.

In block B (Fig. 1B), R_{s1} was positively correlated to the migration time window and negatively correlated to the retention factor in the second PC. The R_{s2} was positively correlated to the efficiency. The efficiency was negatively correlated to the retention factors, i.e., increased association of the peptide to the micelles gave lower efficiency. The same effect was seen in the other two blocks (C and D). The efficiency was very high in all ranges of acetonitrile,

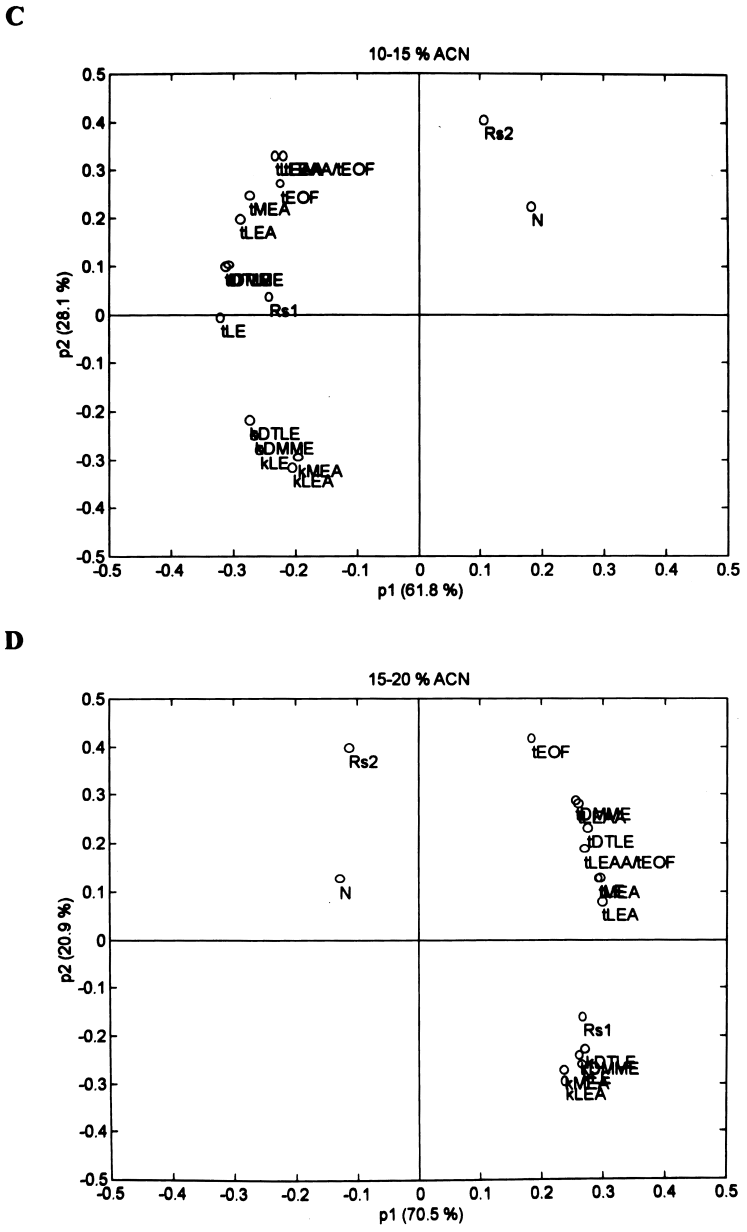

Fig. 1. (*continued*)

over 300 000 plates or more, and the efficiency was usually highest for the peptides containing arginine, although with increasing distribution the efficiency also decreased for those peptides.

In block C (Fig. 1C), R_{s2} was negatively correlated to the retention factor but not R_{s1}. Extending the migration time window will give higher R_{s1}, but for R_{s2} the picture is more complex, i.e. it is negatively correlated to the migration time window in the first PC but positively correlated in the second PC. Thus, increasing the underlying factors in the first PC that will extend the elution window will at the same time increase the retention factor, which results in lower resolution. However, in the second

PC the retention factor and the migration time window are negatively correlated and increasing the underlying factors results in enlarged migration time window as well as decreased retention factor, giving higher resolution. This is illustrated in Fig. 2, the migration time window was enlarged in both experiments but only at the experimental conditions used in Fig. 2B did the retention factors decrease at the same time (A: $k'=131$, B: $k'=22$).

In block D (Fig. 1D), the relationship between the variables had changed, especially for R_{s1}, which turned to be positively correlated to the retention

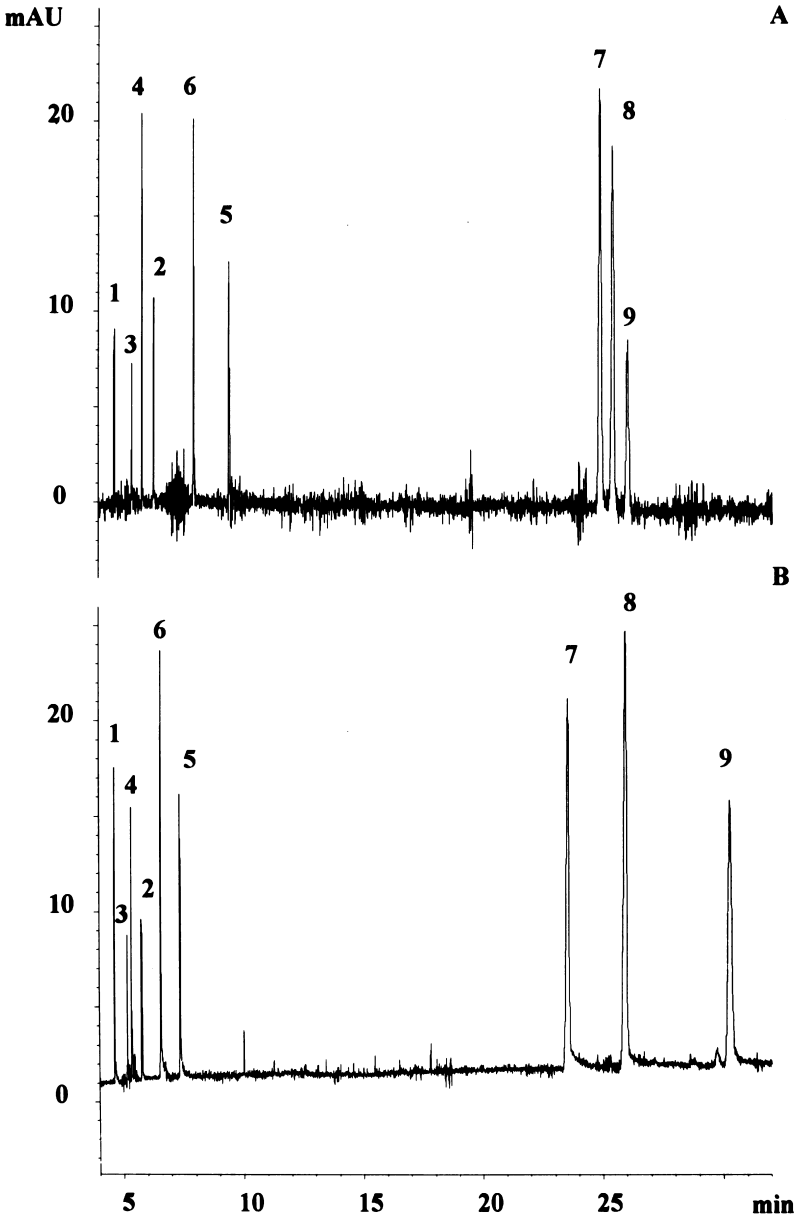

Fig. 2. Electropherogram form block C. (A) 50 m*M* SDS, $I=0.04$, 10% CH_3CN at 25°C; (B) 50 m*M* SDS, $I=0.04$, 15% CH_3CN at 25°C. Peptides numbered as in Table 1.

factors, i.e., increasing the association of the peptides to the micelles gave higher resolution. The R_{s2} was still negatively correlated to the retention factor.

The score plots from the different blocks showed similar trends between the objects, and experiments performed at conditions shown in Fig. 2B, was an extreme point in all ranges studied, with very long migration time for the peptides containing arginine, which became unstable in block D and this experiment was excluded from the modelling.

3.3. Partial least-squares regression in different ranges of acetonitrile

The responses chosen for the PLS regression were the migration time for the electroosmotic flow, the migration time of the peptides DMME, LEA and LEAA, and the resolution between DMME and DTLE (R_{s1}) and between MEA and LEAA (R_{s2}).

The results from the CCD in the four different ranges of acetonitrile are shown in Table 5. The data matrix was expanded with interaction and cross terms before the PLS analysis. The variance explained for the migration time of the electroosmotic flow and the peptide, DMME, were over 90% and three PLS components were significant. The relative standard deviation calculated by eight repeated measurements at the center point was between 1–2% for EOF and about 2.5% for the migration time of DMME. The explained variance for the migration time of the peptides, LEA and LEAA, which have the strongest degree of association to the micelles, was below 80% with two PLS components. However, in the high-level domain of acetonitrile, the explained variance was ca. 90% with three PLS significant components. The relative standard deviation was higher, about 6% for the migration time of these peptides. Two PLS components were significant for the resolution and the relative standard deviation was about 8%, but increased significantly, in the high-levels domain, due to varying efficiencies between repeated runs.

The regression coefficients from the local PLS analysis in the four blocks studied were very different, especially for R_{s1}, except for SDS which generally had a significant positive effect, as illustrated in Fig. 3. Acetonitrile and temperature had significant positive effects on the R_{s1} in blocks A and B, while in blocks C and D the effects became significantly negative. The ionic strength of the buffer had a significant positive effect on R_{s1} in blocks A, B and C, while in block D it was not significant.

3.4. Partial least-squares regression of the blocks together

Previous studies with CCD over the range, 5–15% (v/v) of acetonitrile, gave models with very low explained variance and very bad prediction ability [25]. However, PLS regression over all four blocks together gave models with high explained variance ($R^2 > 0.85$) as shown in Table 6. Therefore, by including more levels of acetonitrile in the design as well as more objects (72 experiments) it was possible to model over a rather large range of organic modifier (0–22%, v/v). The number of significant PLS components had increased compared to the local

Table 5
The result from the local PLS regression in four different ranges of acetonitrile

Responses	CH_3CN (%, v/v)											
	0–5			5–10			10–15			15–20		
	R^2	$\bar{Y}$	SD^n	R^2	$\bar{Y}$	SD^n	R^2	$\bar{Y}$	SD^n	R^2	$\bar{Y}$	SD^n
t_{EOF}	0.94 (2)	3.05	0.032	0.94 (3)	3.17	0.047	0.90 (3)	3.28	0.067	0.90 (3)	3.50	0.060
t_{DMME}	0.93 (3)	5.47	0.13	0.96 (3)	4.67	0.12	0.93 (3)	4.30	0.11	0.98 (3)	4.29	0.18
t_{LEA}	0.74 (2)	13.8	0.41	0.82 (2)	14.3	0.27	0.76 (2)	14.6	0.40	0.90 (3)	12.6	0.95
t_{LEAA}	0.74 (2)	13.8	0.41	0.83 (2)	14.8	0.26	0.78 (2)	17.0	0.50	0.87 (3)	18.5	1.3
R_{s1}	0.82 (3)	5.40	0.49	0.76 (2)	7.75	0.35	0.83 (2)	8.02	0.50	0.72 (2)	5.54	1.5
R_{s2}	–	–	–	0.93 (2)	2.63	0.24	0.84 (2)	11.2	1.5	0.57 (2)	25.9	4.3

$\bar{Y}$ = Mean response, the value in parenthesis is the number of significant PLS components, $n = 8$.

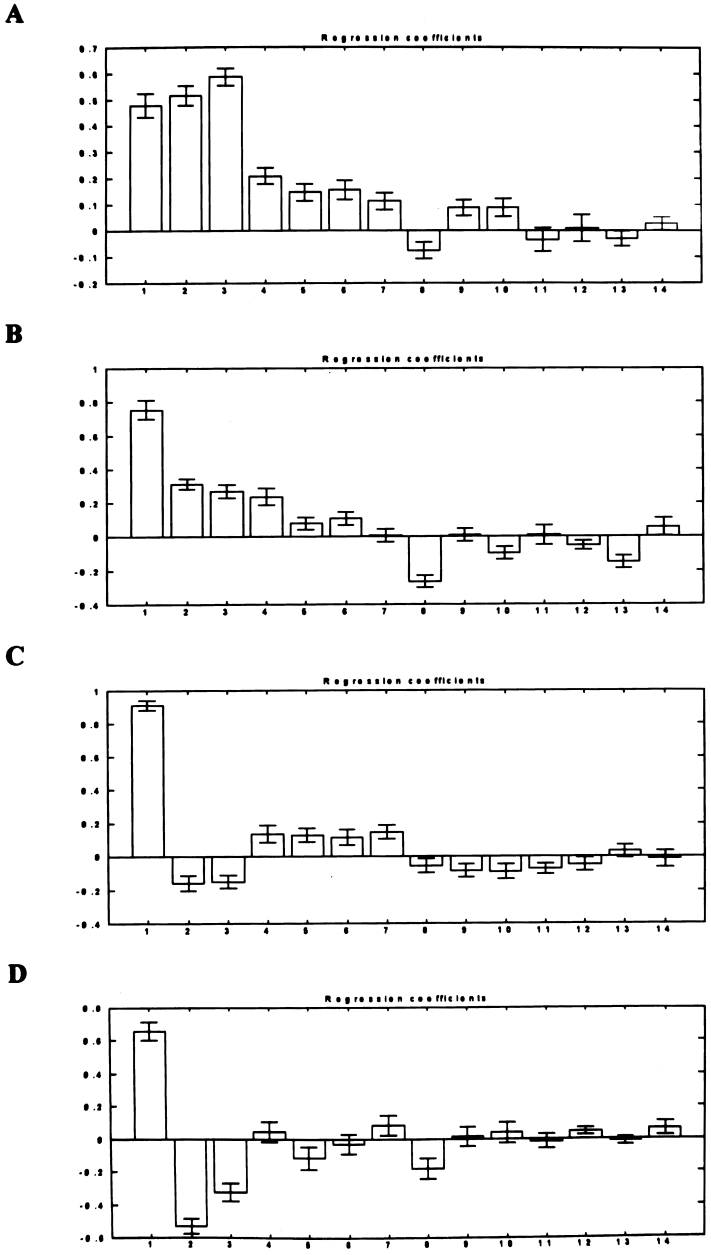

Fig. 3. The regression coefficients for R_{s1} at the different blocks of experiments. 1=SDS, 2=CH_3CN, 3=temperature, 4=ionic strength, 5=SDS×CH_3CN, 6=SDS×temperature, 7=SDS×ionic strength, 8=CH_3CN×temperature, 9=CH_3CN×ionic strength, 10=temperature×ionic strength, 11=SDS^2, 12=CH_3CN^2, 13=$temperature^2$, 14=$ionic\ strength^2$.

PLS models (see Table 5), indicating a more complicated surface.

The model was validated by a test set of 12 independent experimental runs that were not included in the model process. The prediction ability of the model was estimated by the root mean square error of prediction (RMSEP). RMSEP is defined as the square root of the average of the squared

Table 6
The result from PLS regression over all the four domains

Responses	R^2	RMSEP^n
t_{EOF}	0.96 (4 PLS)	0.156
t_{DMME}	0.97 (4 PLS)	0.554
t_{LEAA}	0.85 (3 PLS)	6.03
R_{s1}	0.86 (3 PLS)	1.03

$n=12$ experiments from a test set.

differences between predicted and measured *Y*-values of the test set:

$$RMSEP = \sqrt{\frac{\sum_{i}^{n}(\hat{y}_i - y_i)^2}{n}} \quad (3)$$

The prediction ability of the model was very good for the electroosmotic flow and for the migration time of peptides with low association to the micelles, with low RMSEP, see Table 6. The RMSEP was however, much higher for the resolution and the migration time of the peptide that was totally associated with the micelles, but at the same time these responses had much higher experimental error (see Table 5).

The effect of acetonitrile on the separation performance was highly non-linear and very complicated as will be discussed below by studying the response surface for the different responses.

3.5. Migration time window

The t_{EOF} and t_{LEAA} were positively correlated in all four blocks, as shown in Fig. 1. This means that factors that will increase t_{LEAA} will at the same time increase t_{EOF}, and the migration time window will be extended, due to a stronger effect on t_{LEAA}.

The t_{EOF} increased with addition of acetonitrile to the BGE, which is in accordance with other studies, i.e., the electroosmotic flow decreases due to decreased zeta potential at the capillary surface [14,34].

The temperature had a large effect on the t_{EOF}, which decreased with increased temperature due to decreased viscosity of the BGE.

The response surface for the migration time window is shown in Fig. 4, the SDS concentration and the ionic strength were set at the highest level since the migration time window was extended by increasing the SDS concentration and the ionic strength of the buffer.

There are strong interaction effects between acetonitrile and temperature. The migration time window increased by increasing the acetonitrile concentration at low temperatures, but at high temperatures the migration time window was not affected or was slightly decreased. This might explain the contradictory results of acetonitrile effects on the migration time window reported in the literature. Some studies concluded that the migration time window was not extended by addition of acetonitrile to the BGE [13,19,20], while others came to the opposite conclusion [21,22]. Because of the large interaction effect between acetonitrile and temperature, the effect of acetonitrile will be highly dependent on the temperature used. The temperature had a stronger effect on the migration time window at high level of acetonitrile than at low level as illustrated in Fig. 5. The migration time window was only moderately extended by decreasing the temperature at low level of acetonitrile, while at high level the migration time window was greatly extended. It is though important to note that we assumed that t_{LEAA} migrated with the mobility of the micelles and a decrease in the apparent migration time window at high level of acetonitrile might also be due to decreased association of LEAA to the micelles. Previous studies [28] at high level of acetonitrile showed that t_{LEAA} was of same magnitude as the migration time for the marker, Sudan III. However, it is known that the marker may have some partition into the bulk phase at high levels of organic modifier and therefore complimentary studies of the micellar mobility in the presence of organic modifier are under investigation.

3.6. Migration behavior of the peptides

The migration behavior in the different blocks of experiments was different as illustrated by an electropherogram at different levels of acetonitrile in Fig. 6. In the first block (0–5%, v/v) the peptides containing arginine are not separated except at one experimental condition, with high levels of acetonitrile, temperature and ionic strength and low level of SDS concentration. The peptide DTLE has a lower molecular mass than DMME and lower association

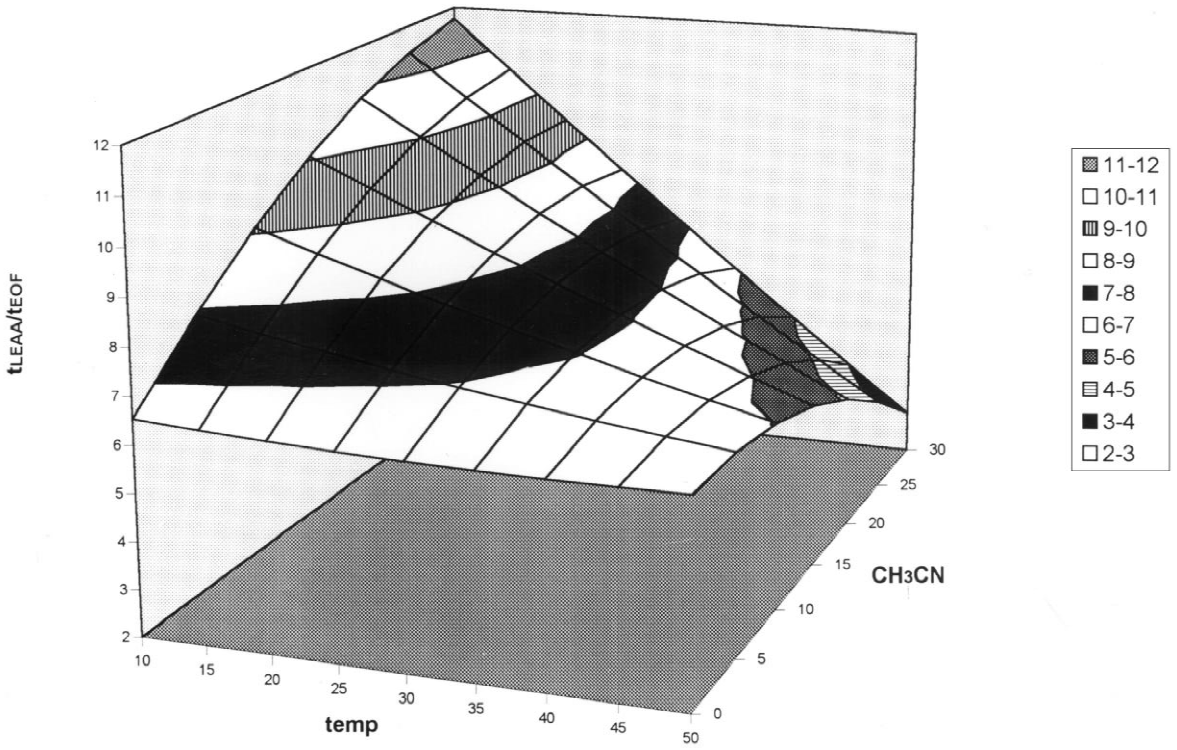

Fig. 4. Response surface plot for the migration time window, as a function of acetonitrile and temperature at 60 m*M* SDS and $I=0.05$.

to the micelles would be expected since both peptides have the same net charge, still DTLE had higher association to the micelles at most experimental conditions. The exception was that without addition of acetonitrile to the BGE the peptide DTLE migrates before DMME, at low temperature (see Fig. 5A). However, when acetonitrile was added to the BGE or the temperature was raised the migration order changed. At 5% (v/v) of CH_3CN in the BGE or higher, the migration order of the peptides was unchanged.

The SDS micelles are negatively charged and the peptides MEA, LEA and LEAA, had the highest degree of association to the micelles, due to strong electrostatic interactions. The peptides will also partition to the non-polar interior of the micelles according to their hydrophobicity. It has been reported that Leu-enkephalins are more hydrophobic than Met-enkephalins [35], even though the latter is 18 units heavier. This is in accordance with our results, the peptides containing leucine had stronger association to the micelles than peptides containing methionine.

At the highest level of acetonitrile the retention factor of the peptides containing only one arginine decreased and they migrated much faster than the peptide containing two arginine. The retention factor (k') was calculated according to Eq. (2). Peptides without arginine had much lower association to the micelles, k'_{DMME} was between 0.16–3.2, while k'_{LEA} was between 1.8 and infinity. Increasing the SDS concentration and decreasing the ionic strength of the buffer resulted in higher k'_{DMME}. The effect of acetonitrile was highly non-linear and there was a strong interaction effect between the acetonitrile and temperature. This is illustrated by the response surface of k'_{DMME} in Fig. 7.

At low temperatures the k'_{DMME} will decrease in a non-linear way with increased acetonitrile concentration, in accordance with Muijselaar et al. [22] reporting a second-order relationship for acetonitrile. One the other hand at high temperatures the k'_{DMME}

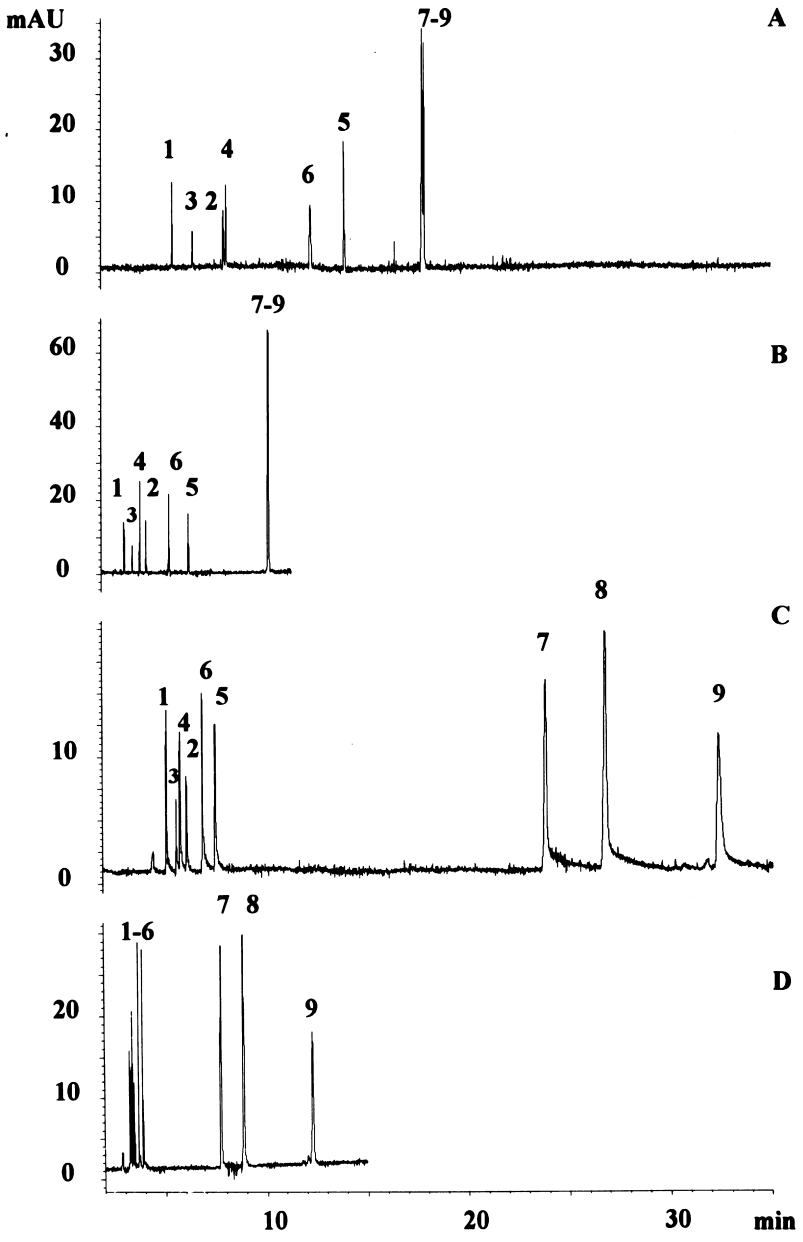

Fig. 5. Effect of temperature on the migration time window at 40 m*M* SDS and $I=0.03$. (A) 17.5°C with 2.5% (v/v) CH_3CN, (B) 47.5°C with 2.5% (v/v) CH_3CN, (C) 17.5°C with 17.5% (v/v) CH_3CN, (D) 47.5°C with 17.5% (v/v) CH_3CN. Peptides numbered as in Table 1.

will increase with increased acetonitrile concentration. The response surface for k'_{DMME} further indicates that the thermodynamics of the system changes at a certain acetonitrile concentration. At low content of acetonitrile the retention factor decreases with increased temperature, in accordance with thermodynamic studies in the literature, that is, the distribution coefficient decreased with increased temperature, giving negative enthalpy changes [36,37]. However, at $\geq$15% (v/v) CH_3CN, the slope changed

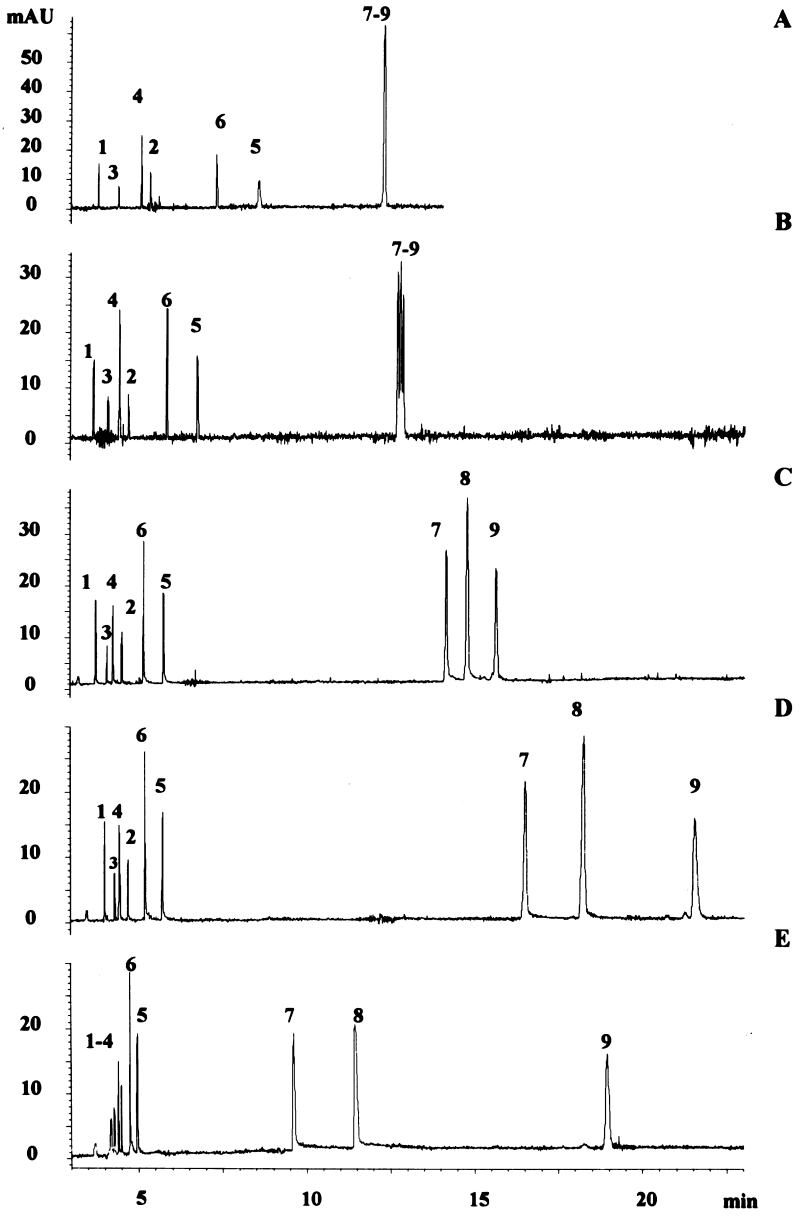

Fig. 6. Electropherogram at different levels of acetonitrile. BGE: Phosphate buffer pH 6.0 ($I=0.03$) with 40 m*M* SDS at 32.5°C. A=2.5, B=7.5, C=12.5, D=17.5 and E=22.5% (v/v) CH_3CN, respectively. Peptides numbered as in Table 1.

and the retention factor increases with increased temperature, indicating a change to an endothermic reaction. This effect was even more pronounced for peptides with higher association to the micelles.

A further evidence of different behavior in the system was that the migration time of the micelles was affected by acetonitrile in a non-linear way. The migration time increased with increasing acetonitrile concentration until it reached an optimum at about 15% (v/v) acetonitrile, then a further increase of acetonitrile concentration resulted in lower migration time (see Fig. 6). Similar effects were reported by

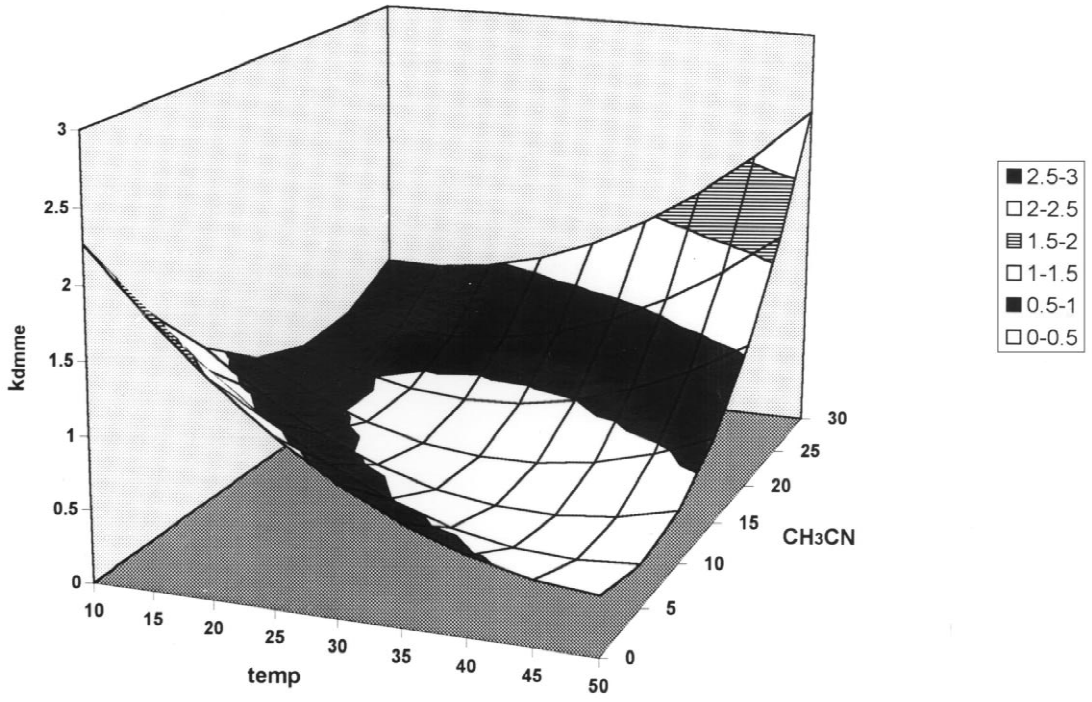

Fig. 7. Response surface plot for the retention factor of DMME, as a function of acetonitrile and temperature at 60 m*M* SDS and $I = 0.05$.

Seifar et al. [8], the mobility of the SDS micelles decreased at higher acetonitrile content. The hypothesis is that this is due either to smaller average size or to stronger binding of counterions to the micelles, as the dielectric constant of the buffer decreased. Misra et al. [38] reported a change in the micelle structure at about 16% (v/v) CH_3CN. Thus the observed decreased migration time of the micelles at high level of acetonitrile might be due to a change in the micellar structure, or that the association of LEAA to the micelles has decreased, and in that case no longer can be used as a marker for the micellar mobilities.

3.7. Resolution

The resolution between the between the peptide pairs, DMME–DTLE (R_{s1}) and between MEA–LEAA (R_{s2}) is negatively correlated in the high-level domains of acetonitrile as seen in Fig. 1. This means that factors giving increased R_{s2} will at the same time decrease R_{s1}. The peptides containing arginine were not separated in the first block, and in the other blocks R_{s2} increased continuously with increasing concentration of acetonitrile. The parameters that affect the resolution can not be controlled independently of each other as indicated in Fig. 2. Even though the migration time window was extended the resolution was only increased if the retention factor was decreased at the same time. The highest R_{s2} values resulted in lost separation between DMME and DTLE (R_{s1}). The response surface for R_{s1} is complicated (Fig. 8), the effect of acetonitrile was non-linear and there was a strong interaction effect between acetonitrile and temperature.

There is an optimum acetonitrile concentration (15%, v/v) that will give the highest R_{s1} at all temperatures. At lower acetonitrile content the R_{s1} can be optimized by increasing the temperature, mainly due to decreased retention factors. At high levels of acetonitrile the R_{s1} is optimized by decreasing the temperature, mainly due to increased migration time window. Simultaneously the retention factors decrease, which in principal decrease R_{s1} (see above). However, the effect on the migration window and efficiency dominate giving higher resolu-

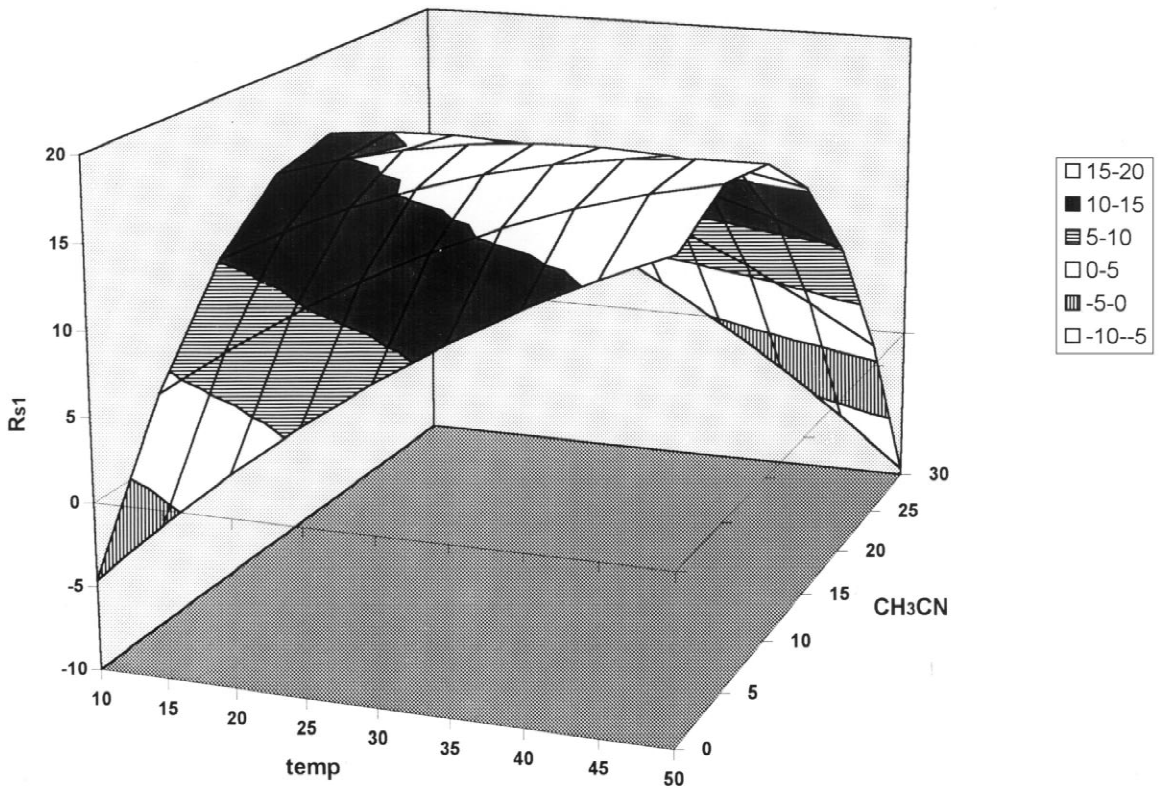

Fig. 8. Response surface plot for the resolution between DTLE and DMME, as a function of acetonitrile and temperature at 60 m*M* SDS and $I = 0.05$.

tion. This exemplifies the importance of using multivariate data analysis, since the parameters that influence the resolution can not be independently controlled.

4. Conclusions

Determination of the CMC with CE showed that the CMC values increased with acetonitrile content and increased temperature, but micelles were formed at all experimental conditions used during the CCD modelling.

The loading plots from PCA of the responses revealed that resolution, migration time window, retention factor and efficiency are correlated to each other and cannot be independently controlled.

The results from the local PLS regression at different ranges of acetonitrile showed very different influence of the experimental variables in respective domain. When studying resolution it was shown, for example, that temperature and concentration of acetonitrile were highly positively significant in the low-level domains, while they were negatively significant in the high-level domain. PLS regression of the responses over all blocks of acetonitrile together (0–20%, v/v), gave a model with high explained variance and good prediction ability for some of the responses.

The results showed that the effect of acetonitrile was highly non-linear and there was a strong interaction effect between temperature and acetonitrile. At low level of acetonitrile the migration time window was moderately affected by temperature, while at high level it was extended by decreasing the temperature.

The retention factor for peptides with moderate association to the micelles, decreased with increased

temperature at low levels of acetonitrile, while at high levels of acetonitrile the retention factor increased. Furthermore the effect of acetonitrile on the migration time of the micelles was highly non-linear, and the results indicated different behaviour in the system probably due to change in the micelles structure at high level of acetonitrile. A change of the thermodynamics from exothermic to endothermic behaviour was noticed above 15% (v/v) CH_3CN in the BGE.

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