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# Simultaneous use of urea and acetonitrile as organic modifiers for optimization of resolution in micellar electrokinetic chromatography

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**Abstract**

Optimization of the resolution of non-polar solutes in micellar electrokinetic chromatography (MEKC) via the simultaneous use of the modifiers acetonitrile and urea is presented. The influence of the applied modifiers on retention factors and the ratio of the migration time of the mobile phase/migration time of the micelles ($t_0/t_{MC}$), which is substantial for resolution control, is described. It is shown that with high modifier concentrations, an almost infinite elution range is approached. Applying a computer-aided optimization scheme, the separation of biogenic amines derivatized with dansyl chloride is optimized using an iterative regression strategy. © 1997 Elsevier Science B.V.

*Keywords:* Optimization; Resolution; Urea; Acetonitrile; Amines

## 1. Introduction

Micellar electrokinetic chromatography (MEKC) renders the separation of neutral and charged solutes possible, via distribution between an aqueous mobile phase and a retarded micellar phase (pseudo-stationary phase). This method was first presented by Terabe et al. [1,2] who also developed the basic equation for the resolution ($R_s$) of two adjacent peaks.

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{\bar{k}}{1+\bar{k}} \cdot \frac{1 - \frac{t_0}{t_{MC}}}{1 + \frac{t_0}{t_{MC}} \cdot \bar{k}} \quad (1)$$

where: $N$ = plate number; $\bar{k}$ = mean retention factor (for the solutes investigated); $\alpha$ = selectivity factor; $t_0$ = migration time of the mobile phase; $t_{MC}$ = migration time of the micelles. Terabe et al. [1,2] have shown that the function

$$f(\bar{k}) = \frac{\bar{k}}{1+\bar{k}} \cdot \frac{1 - \frac{t_0}{t_{MC}}}{1 + \frac{t_0}{t_{MC}} \cdot \bar{k}} \quad (2)$$

passes through a maximum if $t_{MC}$ is not infinite. Foley [3] demonstrated that the basic equation for resolution in MEKC differentiated for $\bar{k}$ has a zero value at $\bar{k} = \sqrt{t_{MC}/t_0}$, provided that the other quantities in Eq. (1) are kept constant. This assumption, however, cannot be fulfilled in practice. The retention factors can be varied by changing the phase ratio (pseudostationary phase/mobile phase) by altering the surfactant concentration or they can be decreased via addition of a modifier, thus decreasing the distribution constants of the solutes to be separated. In both cases, the ratio $t_0/t_{MC}$ and possibly $\alpha$

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are also affected, making optimization of the resolution a complex problem [4–6].

Hydrophobic solutes are eluted at migration times close to $t_{MC}$ and, therefore, they often cannot be separated by conventional MEKC. For optimization of the resolution of these non-polar solutes, the decrease of retention factors that can be achieved by decreasing the surfactant concentration, which still must be above the critical micelle concentration (CMC), is often not sufficient. For this reason, the use of modifiers is required in order to reach the optimum range of retention factors. Several publications report the utilisation of organic modifiers in MEKC and the separation of non-polar solutes made possible by this means [7–11].

For normal elution mode [12], the ideal organic modifier in MEKC has the following features:

- It does not greatly affect the CMC of the surfactant.
- It decreases $k$ and $t_0/t_{MC}$.
- It does not prolong the run time unnecessarily by increasing $t_0$.

Meeting all these requirements, urea is reported to be a versatile additive to the mobile phase [13,14]. However, if the solutes to be separated are non-polar, the addition of urea to the separation buffer does not decrease the retention factors for the solutes to a satisfactory degree. Therefore, modifiers have to be selected that have a larger impact on the retention factors for the solutes than urea.

Schwer and Kenndler [15] showed that the addition of acetonitrile to the separation electrolyte increases $t_0$ to a lower extent than alcoholic modifiers or dimethylsulfoxide. Several authors [4,7,9–11,16] have reported the successful use of acetonitrile for the separation of non-polar solutes via MEKC. Both acetonitrile and urea do not increase the CMC of the surfactant, sodium dodecyl sulfate, significantly [14,16].

In this paper, we will show that for the separation of non-polar solutes, the simultaneous use of the modifiers urea and acetonitrile can offer advantages in comparison to the use of only one modifier with respect to run time, efficiency and influence on retention factors. Dansylated biogenic amines are employed as model solutes.

In general, the optimization of separations performed with MEKC is complex due to the high number of parameters controlling the separation [6].

Computer-assisted approaches are therefore gaining more and more importance for method development in MEKC. Corstjens et al. [6] have recently published an overview on different approaches for the optimization of separations in capillary electrophoresis (CE) with emphasis on MEKC. The computer-assisted optimization procedures developed so far differ largely concerning the parameters to be optimized (i.e. surfactant concentration, pH, modifier concentration), the algorithms used and the number of necessary test runs.

Corstjens et al. [6] were the first to use an iterative regression strategy in MEKC. They optimized the pH and the surfactant (sodium dodecyl sulfate) concentration. After only seven test runs, the separation of six benzoic acids was achieved.

In the present paper, the iterative regression strategy is applied to the optimization of the separation of dansylated amines. The optimized parameters are the volume concentration of acetonitrile and the concentration of urea. The two modifiers are used simultaneously as additives to the separation electrolyte.

## 2. Experimental

### 2.1. Reagents

Most of the amines employed as standards were available at the department of chemistry (University of Marburg, Marburg, Germany). 3-Methylbutylamine and hexylamine were from Aldrich (Steinheim, Germany).

Sodium tetraborate, boric acid (Merck, Darmstadt, Germany) and sodium dodecyl sulfate (Roth, Karlsruhe, Germany), which were used for the preparation of the separation electrolytes, and dansyl chloride [(5-dimethylamino)-naphthalene-1-sulfonyl-chloride (Aldrich)] were of analytical grade. Acetonitrile was distilled. Water was twice distilled.

### 2.2. Derivatization of amines

A 10–20 μl volume of a saturated solution of dansyl chloride in acetone was added to a solution of amine in 10 ml of borate buffer [$c(Na_2B_4O_7) = 10$ mmol/l, $c(H_3BO_3) = 10$ mmol/l] dissolved in 10 ml of acetone. The solution was heated to 70°C in a

closed vessel for 30 min. After completion of the derivatization, the solution was poured into a beaker and the acetone was evaporated by heating the solution to 70°C for about 15 min.

### 2.3. Chromatographic measurements

All chromatographic measurements were carried out with a Beckman (Fullerton, CA, USA) model P/ACE CE system equipped with a UV-absorbance detector. The temperature of the capillary was controlled by liquid cooling and was maintained at 25°C. Samples were injected by application of pressure for 2 s. Detection was at 254 nm. All separations were carried out at a voltage of 25 kV. Data were recorded with the Beckman System Gold software. The sodium dodecyl sulfate (SDS) concentration was kept constant at 20 mmol/l. An homologous series of dansylated aliphatic amines was used to determine $t_{MC}$. Acetone was used as a marker of the migration time of the mobile phase.

Fused-silica capillaries (75 μm I.D., 375 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillaries was 470 mm and the length to the detector was 400 mm.

Peak identities were confirmed by spiking.

### 2.4. Software

The program used for the determination of the elution time of the micellar phase, $t_{MC}$, was written in QBASIC (Microsoft). The program used for optimization studies was written in Pascal, employing Turbo Pascal 6.0 (Borland, CA, USA).

## 3. Results and discussion

### 3.1. Determination of the migration time of the micelles

As stated above, the migration time window ($t_{MC}/t_0$) in MEKC can be extended by adding organic modifiers to the aqueous phase, allowing the separation of non-polar solutes. To quantify the influence of the simultaneous use of the two modifiers, urea and acetonitrile, on the migration time window, the migration time of the pseudostationary phase, $t_{MC}$, must be measured. In the presence of organic modifiers, determining $t_{MC}$ from the migration time of a single hydrophobic compound (i.e. sudan III) is not reliable [9]. For this reason, the determination of $t_{MC}$ using the retention times of solutes in an homologous series has been proposed [18]. In the present paper, an iterative procedure analogous to the one presented by Chen et al. [9], who applied an homologous series of alkyl phenyl ketones, was used. An homologous series of dansylated aliphatic amines (methyl-, ethyl-, propyl-, butyl-, pentyl- and hexylamine) was utilized for measuring $t_{MC}$. In this series, the retention time of hexylamine was first assumed to be equal to $t_{MC}$ and log $k$ for the other dansylated amines was calculated according to Eq. (3).

$$k = \frac{t_{ret} - t_0}{t_0 \cdot \left(1 - \frac{t_{ret}}{t_{MC}}\right)} \quad (3)$$

where $t_{ret}$ = migration time of the solute.

Log $k$ was plotted against the carbon number ($n_C$) of the alkyl group. Assuming a linear relationship between log $k$ and $n_C$, a temporary value of $k$ for hexylamine was obtained. With this $k$ value, a new value for $t_{MC}$ was obtained by transforming Eq. (3) into Eq. (4):

$$t_{MC} = \frac{t_{ret}}{1 - \frac{t_{ret} - t_0}{k \cdot t_0}} \quad (4)$$

With the new $t_{MC}$, log $k$ values were recalculated and again plotted against the carbon number, resulting in a new value for $t_{MC}$. This procedure can be repeated several times until the difference between consecutive values for $t_{MC}$ is less than 1 s (usually after 15–25 iterations). Table 1 shows the values obtained for $t_{MC}$ at various concentrations of modifier. The volume concentration of acetonitrile, $\sigma_A$, was changed from 0.0 to 0.20 in steps of 0.05 and the urea concentration, $c$(urea), was changed from 0 to 5 mol/l in steps of 1 mol/l.

At $\sigma_A = 0.0–0.15$, $t_{MC}$ increases with increasing urea and acetonitrile content. At $\sigma_A = 0.20$ and low urea content, a negative value for $t_{MC}$ is obtained, indicating that the migration velocity of the micelles is higher than the negative electroosmotic velocity, resulting in a migration of the micelles towards the

Table 1
Migration time of the micelles, $t_{MC}$, at various concentrations of acetonitrile and urea as determined using the iterative procedure

| | $t_{MC}$/min | | | | |
|---|---|---|---|---|---|
| $c$(urea)/(mol/l) | $\sigma_A = 0.0$ | $\sigma_A = 0.05$ | $\sigma_A = 0.10$ | $\sigma_A = 0.15$ | $\sigma_A = 0.20$ |
| 0 | 2.72 | 3.23 | 3.84 | 5.76 | −4.21 |
| 1 | 2.84 | 3.53 | 4.38 | 6.20 | −3.0 |
| 2 | 3.03 | 3.93 | 5.05 | 7.20 | −0.76 |
| 3 | 3.30 | 4.27 | 5.92 | 12.5 | n.c. |
| 4 | 3.69 | 4.67 | 8.25 | 19.45 | n.c. |
| 5 | 4.10 | 5.40 | 13.06 | 45.44 | n.c. |

Capillary, 365 (300) mm×75 μm I.D.; for the other parameters, see Fig. 1.
n.c. = not calculable.

anode. With high urea concentrations, the convergence criterion of the iterative procedure was fulfilled only after a large number of iteration steps with $t_{MC} = 0$ min. This result indicates that the mobility of the micelles is equal to the negative electroosmotic mobility, resulting in an infinite elution range. The usefulness of an infinite elution range was recently highlighted by Ahuja et al. [19].

### 3.2. Influence of applied modifiers on retention factors

While in the parameter space examined, the migration time of the mobile phase, $t_0$, is not increased excessively through the addition of modifiers from $t_0 = 1.1$ min [$c$(urea) = 0.0 mol/l, $\sigma_A = 0.0$] to $t_0 = 1.9$ min [$c$(urea) = 5.0 mol/l, $\sigma_A = 0.20$], the retention factors for the solutes investigated are influenced to a considerable extent. Fig. 1 shows the retention factor for dansylated hexylamine $k_{hex}$ (calculated according to Eq. (3)) plotted against $\sigma_A$ and $c$(urea). The value of $k_{hex}$ is reduced from 185 [$c$(urea) = 0.0 mol/l, $\sigma_A = 0.0$] to 1.26 [$c$(urea) = 5.0 mol/l, $\sigma_A = 0.15$], indicating that the covered range of retention factors is significantly broadened in comparison to the use of only urea or acetonitrile as the modifier [$k_{hex} = 34.3$ at $c$(urea) = 5.0 mol/l, $\sigma_A = 0.0$; $k_{hex} = 7.58$ at $c$(urea) = 0.0 mol/l, $\sigma_A = 0.15$].

### 3.3. Employed optimization strategy

The iterative regression strategy was initially developed for high-performance liquid chromatography (HPLC) by Drouen et al. [20]. Corstjens et al. [17] applied it successfully to the separation of benzoic acids by MEKC, varying the pH and the concentration of the surfactant.

In the present paper, the same principles are used to optimize the resolution of eleven dansylated amines (2-aminoethanol, methylamine, ethylamine, morpholine, 2-aminopropane, diethylamine, 1-amino-2-methylpropane, *n*-butylamine, 1-amino-3-methylbutane, *n*-pentylamine and *n*-hexylamine). The parameters to be varied are the concentrations of acetonitrile and urea in the separation electrolyte.

To use an iterative regression strategy, a computer program utilizing the following basic principles was written.

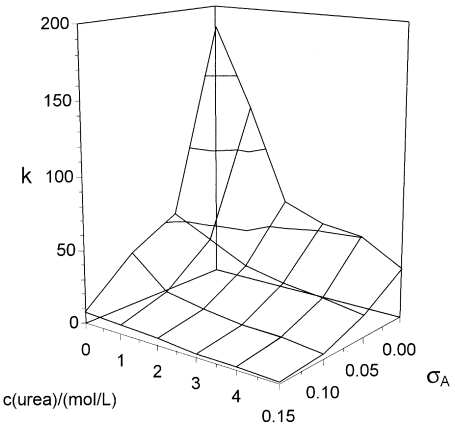


Fig. 1. Dependence of the retention factor for dansylated hexylamine on the concentrations of urea and acetonitrile. Conditions: Capillary, 465 (400) mm×75 μm I.D.; buffer, $c(Na_2B_4O_7) = 10$ mmol/l, $c(H_3BO_4) = 10$ mmol/l, $c$(SDS) = 20 mmol/l; voltage, 25 kV; temperature, 25°C; injection, pressure 2 s; detection, photometric, 254 nm.

The retention times for the solutes to be separated can be described as a function of the parameters to be varied, assuming a linear dependence of the retention times on these parameters.

$$t_{ret} = f(\sigma_A, c(\text{urea})) = a + b \cdot c(\text{urea}) + d \cdot \sigma_A \quad (5)$$

where $a$, $b$, $d$ are constants.

The simplifying assumption of a linear dependence of retention times on acetonitrile and urea content is made in order to minimize the number of necessary test runs.

In order to determine the constants in Eq. (5), three test runs at different urea and acetonitrile contents are necessary, forming a triangle in the parameter space. With the data from these test runs, a linear equation system consisting of three equations is achieved, which can be solved to give values for the constants $a$, $b$ and $d$ for every solute. Using these data and a user-defined estimated value for the plate number, the resolution for every possible peak pair at any acetonitrile and urea concentration within the parameter triangle can be calculated using Eqs. (6) and (7).

$$w = \frac{4}{\sqrt{N}} \cdot t_{ret} \quad (6)$$

where $w$ = peak width and $N$ = plate number.

$$R = \frac{\Delta t_{ret}}{\frac{1}{2}(w_1 + w_2)} = \frac{\Delta t_{ret}}{\bar{w}} \quad (7)$$

where $R$ = resolution, $w_1$ = peak width for solute 1, $w_2$ = peak width for solute 2 and $\bar{w}$ = mean peak width.

Using the software, in the first step, the retention times of the solutes are entered for three different electrolyte compositions [varied $c$(urea) and $\sigma_A$], forming a triangle that covers the area of interest. The user selects the minimum resolution that has to be reached for the worst resolved peak pair and the plate number that is approximated to be constant for all electrolyte compositions. The step-width of the underlying algorithm can also be fixed by the user [$\Delta c$(urea), $\Delta\sigma_A$]. Optionally, the user can label some solutes as 'impurities'. The resolution of labeled solutes from each other is not taken into account in the optimization procedure.

Now the resolution for every possible peak pair can be calculated (as described above) and compared with each other. The program suggests an optimum electrolyte composition, where the resolution of the worst separated peak pair exceeds the user-defined threshold value and the retention time of the last-eluting solute is minimum. Thus, the program performs an adjustment of the electrolyte composition for a minimum analysis time. Optionally, the electrolyte composition can be calculated where the resolution of the worst separated peak pair, $R_{min}$, is the highest regardless of analysis time. In the final step, the program generates a list of retention times predicted for the optimized electrolyte composition and a simulated chromatogram.

In practice, the predicted optimum diverges from the real optimum, indicating that the underlying linear functions are not able to describe the retention behaviour accurately. The selected parameter area can then be divided into smaller segments in order to reduce the difference between predicted and measured retention times. This further segmentation can be performed in an iterative manner until a satisfactory separation is achieved.

For the separation of eleven dansylated amines, the parameter area [$c$(urea) = 0–5 mol/l, $\sigma_A$ = 0–0.15] was divided into four triangles, which are marked as I–IV in Fig. 2.

Five test runs were performed. The compositions of the separation electrolytes correspond to the coordinates of the corners of triangles I–IV. Re-

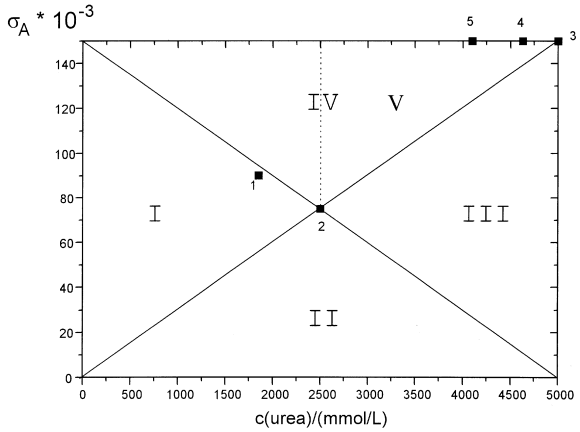


Fig. 2. Partition of parameter area and predicted optima.

tention data were measured using the following compositions of the separation electrolyte:

1. $c$(urea) = 0.0 mol/l, $\sigma_A = 0.0$.
2. $c$(urea) = 5.0 mol/l, $\sigma_A = 0.0$.
3. $c$(urea) = 0.0 mol/l, $\sigma_A = 0.15$.
4. $c$(urea) = 5.0 mol/l, $\sigma_A = 0.15$.
5. $c$(urea) = 2.5 mol/l, $\sigma_A = 0.075$.

In all cases, the concentrations of $Na_2B_4O_7$ and $H_3BO_3$ were each 10 mmol/l and the concentration of SDS was 20 mmol/l. The optimization procedure was performed for each of the four triangles. The estimated value for the plate number is $N = 150\,000$. The optimum conditions achieved and the corresponding values of $R_{min}$ are listed in Table 2. In general, good resolutions seem to be obtained both at a high urea concentration and a high acetonitrile content. The highest value for $R_{min}$ is reached in triangle IV. In order to improve the accuracy of the prediction, triangle IV was halved and a new test run with $c$(urea) = 2.5 mol/l and $\sigma_A = 0.15$ was performed. For the resulting triangle, V, a new optimization procedure was carried out, which resulted in an optimum electrolyte composition of $c$(urea) = 4.1 mol/l and $\sigma_A = 0.15$. The corresponding value of $R_{min}$ was 2.97. As this value differs only slightly from the one obtained in triangle IV and since the optimum conditions are at the border of the parameter space, further segmentation of the parameter space is not useful. At the calculated optimum conditions, all solutes are baseline separated (see Fig. 3a). Fig. 3b shows the separation performed without adding modifiers to the separation electrolyte, with most solutes eluting close to $t_{MC}$. In Table 3, predicted and measured retention times are listed for the solutes to be separated at $c$(urea) = 4.1 mol/l and $\sigma_A = 0.15$. Predicted retention times and retention time differences are in good agreement with the measured data.

Table 2
Optimum conditions for segments of the parameter space and corresponding values for $R_{min}$

| Triangle | Optimum concentrations of modifiers (mol/l) | $R_{min}$ |
|---|---|---|
| I | $c$(urea) = 1.9<br>$\sigma_A = 0.09$ | 1.72 |
| II | $c$(urea) = 2.5<br>$\sigma_A = 0.07$ | 1.43 |
| III | $c$(urea) = 5.0<br>$\sigma_A = 0.15$ | 2.59 |
| IV | $c$(urea) = 4.63<br>$\sigma_A = 0.15$ | 2.81 |
| V | $c$(urea) = 4.1<br>$\sigma_A = 0.15$ | 2.97 |

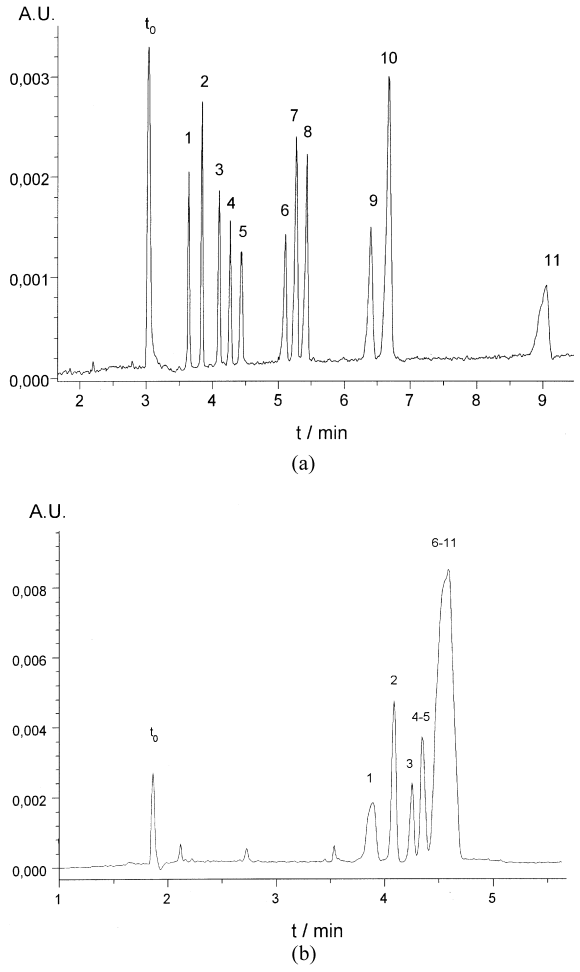


Fig. 3. Separation of dansylated biogenic amines (a) under optimized conditions, $\sigma_A = 0.15$; $c$(urea) = 4.1 mol/l, (b) without the use of modifiers, $\sigma_A = 0.0$; $c$(urea) = 0.0 mol/l. Peak identification: 1 = 2-Aminoethanol, 2 = methylamine, 3 = ethylamine, 4 = morpholine, 5 = 2-aminopropane, 6 = diethylamine, 7 = 1-amino-2-methylpropane, 8 = *n*-butylamine, 9 = 1-amino-3-methylbutane, 10 = *n*-pentylamine and 11 = *n*-hexylamine. For other experimental conditions, see Fig. 1.

Table 3
Predicted ($t_{pred}$) and measured ($t_{meas}$) retention times under optimized conditions

| Solute | $t_{pred}$/min | $t_{meas}$/min | $\Delta t_{pred}$/min | $\Delta t_{meas}$/min |
|---|---|---|---|---|
| 2-Aminoethanol | 3.620 | 3.640 | 0.189 | 0.197 |
| Methylamine | 3.809 | 3.837 | 0.250 | 0.263 |
| Ethylamine | 4.059 | 4.100 | 0.166 | 0.168 |
| Morpholine | 4.225 | 4.268 | 0.150 | 0.167 |
| 2-Aminopropane | 4.375 | 4.435 | 0.642 | 0.668 |
| Diethylamine | 5.017 | 5.103 | 0.149 | 0.169 |
| 1-Amino-2-methylpropane | 5.166 | 5.272 | 0.152 | 0.156 |
| *n*-Butylamine | 5.318 | 5.428 | 0.853 | 0.972 |
| 1-Amino-3-methylbutane | 6.171 | 6.400 | 0.240 | 0.275 |
| *n*-Pentylamine | 6.411 | 6.675 | 2.125 | 2.368 |
| *n*-Hexylamine | 8.536 | 9.043 | – | – |

$c$(urea) = 4.1 mol/l, $\sigma_A = 0.15$.
For experimental conditions, see Fig. 1.

## 4. Conclusions

The simultaneous use of acetonitrile and urea as modifiers in MEKC is useful for the separation of non-polar solutes. With the help of a computer-aided iterative regression strategy, it is possible to rapidly optimize the separation of a complex mixture of solutes. With this strategy, retention times of solutes can be predicted for a given $c$(urea)–$\sigma_A$ parameter space, facilitating method development and ruggedness testing.

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