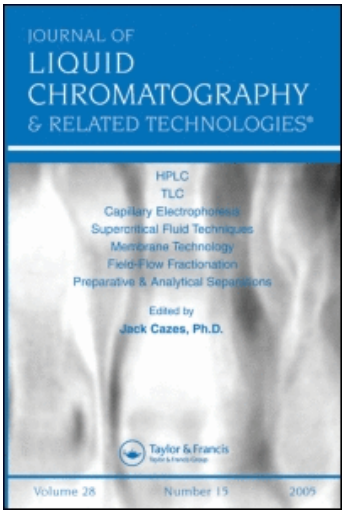

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ANALYSIS OF AMPICILLIN AND ITS DEGRADATION PRODUCTS BY CAPILLARY ELECTROPHORESIS

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ABSTRACT

Capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) have been employed for the separation of ampicillin and its degradation products, especially the oligomers of ampicillin which are readily formed during storage and in aqueous solution. Both CZE and MECC are suitable for the separation of the oligomers of ampicillin. MECC is, however, more suitable for the separation of other degradation products. Electrophoresis conditions used for the analysis of ampicillin and its degradation products were as follows: length of fused (uncoated) silica capillary 44 cm (36 cm effective length), 50 µm i.d.; voltage, 15 kV; temperature, 25°C; detection wavelength, 215 nm; separation buffer, 40 mM sodium dihydrogen phosphate, and 100 mM sodium dodecyl sulfate (SDS) adjusted to pH 7.5. The influence of various parameters on the separation such as SDS concentration and the pH of the buffer were investigated. The method shows good repeatability, linearity and sensitivity. The robustness of this method was examined by applying a full factorial design to test the influence of the pH of the buffer, the SDS concentration and the buffer concentration.

INTRODUCTION

Ampicillin is a semi-synthetic β-lactam antibiotic. Many methods for the analysis of ampicillin have been developed such as microbiological assay,[1] mercurimetric titration[2] and chromatographic analysis.[3-6] Liquid chromatography (LC) is suitable not only for the assay but also for the determination of impurities and degradation products of ampicillin.[7-9]

Recently, capillary electrophoresis (CE) has proven to be a significant and versatile technique for the analysis of β-lactam antibiotics.[10-15] Among these CE methods, micellar electrokinetic capillary chromatography (MECC) is quite often used especially for the separation of neutral and weakly ionic molecules, such as penicillins.

In this paper we report the method development of a micellar system using SDS in a phosphate buffer of pH 7.5, for the separation of ampicillin and its related substances. The robustness of this method was examined by applying a full factorial design to test the influence of the pH of the buffer, the SDS concentration and the buffer concentration.

EXPERIMENTAL

Instrumentation

CE experiments were carried out on a Spectra Phoresis 1000 (Thermo Separation Products, Fremont, CA, USA), which was driven by CE software (version 3.01) operating under IBM OS/2TM (version 1.2). The vacuum system of the instrument applies a constant negative pressure of 5.17 kPa for the injection. Hydrodynamic injection was performed for 1 s during the selectivity study and 3 s during method validation study. Fused silica capillaries were from Polymicro Technologies (Phoenix, AZ, USA): 44 cm x 50 µm I.D. and 36 cm effective length. UV detection was set at 215 nm. The capillary was washed at the beginning of the day with 0.1 M NaOH for 5 min followed by a water wash for 5 min. Before every analysis the capillary was washed for 5 min with running buffer.

Reagents and Samples

Milli-Q water (Millipore, Milford, MA, USA) was used throughout. Reagents were of analytical grade (Merck, Darmstadt, Germany or Acros Organics, Geel, Belgium). The running buffer for MECC was prepared by

dissolving SDS in sodium dihydrogen phosphate buffer, the pH of the buffer was adjusted using 5 M NaOH. For the selectivity study, a 0.05 % dimethyl sulphoxide (DMSO) solution was used as a solvent, with DMSO functioning as a neutral marker.

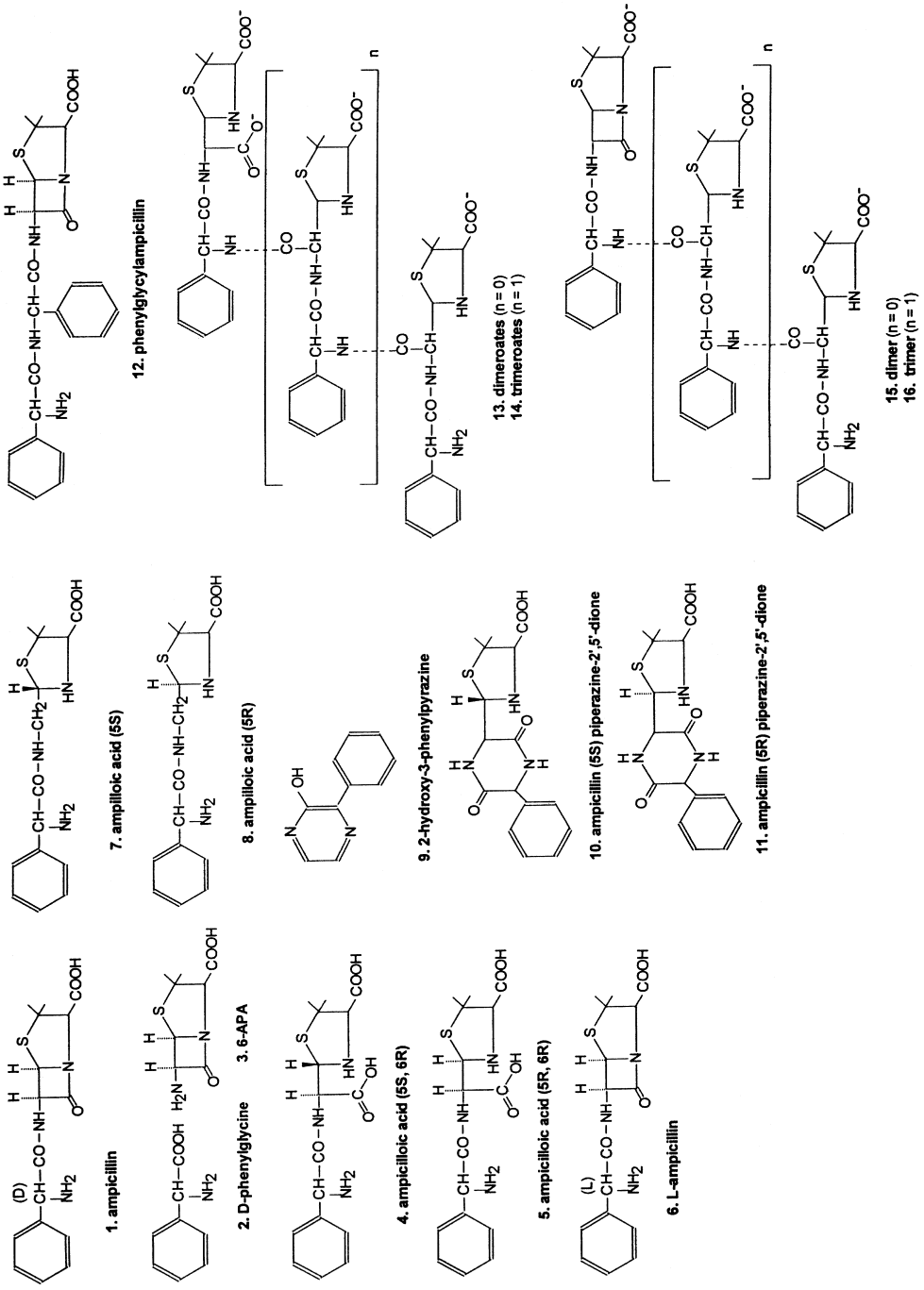
Related substances originate from semi-synthesis and from degradation. Ampicillin is commercially available (Gist-Brocades, Delft, The Netherlands). The structures of the available related substances are shown in Figure 1. D-Phenylglycine (**2**) (Acros Organics) and 6-aminopenicillanic acid (6-APA) (**3**) (Gist-Brocades) are the basic constituents of ampicillin. L-Ampicillin (**6**) and phenylglycylampicillin (**12**) can arise from the semi-synthesis. The other related substances are degradation products. Compound **6** was obtained from Antibioticos S. A., Barcelona, Spain. Ampicilloic acid (5S, 6R) (**4**) and ampicilloic acid (5R, 6R) (**5**) were prepared as described by Munro.[16] The preparation of ampilloic acid (5S) (**7**) and ampilloic acid (5R) (**8**) was performed as described by Zhu.[17] 2-Hydroxy-3-phenylpyrazine (**9**) was prepared as described by LeBelle.[18] Ampicillin (5R) piperazine-2', 5'-dione (**11**) was prepared as described by Bundgaard.[19] Epimerization of **11** to ampicillin (5S) piperazine-2', 5'-dione (**10**) was performed in a similar way as described by Haginaka for amoxicillin[20] Related substance **12** was made as described by Grant.[21] The oligomeroates (**13**, **14**) and oligomers (**15**, **16**) were prepared as described by Bundgaard[22] and Roets.[23]

RESULTS AND DISCUSSION

Selectivity Study

In order to develop a method for the separation of ampicillin and its related substances, first phosphate buffer was applied in a concentration of 40 mM at pH 7.5 using free solution capillary electrophoresis. Although all the compounds migrated within 10 min, ampicillin could not be completely separated from other compounds. So sodium dodecyl sulfate (SDS) was added to the running buffer for the separation of ampicillin and its related substances. Under the condition of 40 mM sodium phosphate buffer (pH 7.5) containing 100 mM SDS, ampicillin and its related substances were well separated from each other. The influence on the separation of the buffer pH and the concentration of SDS was investigated.

The pH is critical for the separation. Experiments were performed using sodium phosphate (40 mM) - SDS (150 mM) buffer. The applied voltage was 15 kV and the temperature 25°C. The pH was varied between 6.5 and 8 with steps of half a pH unit. The influence of the electrolyte pH on the relative migration times (migration time divided by migration time of neutral marker)



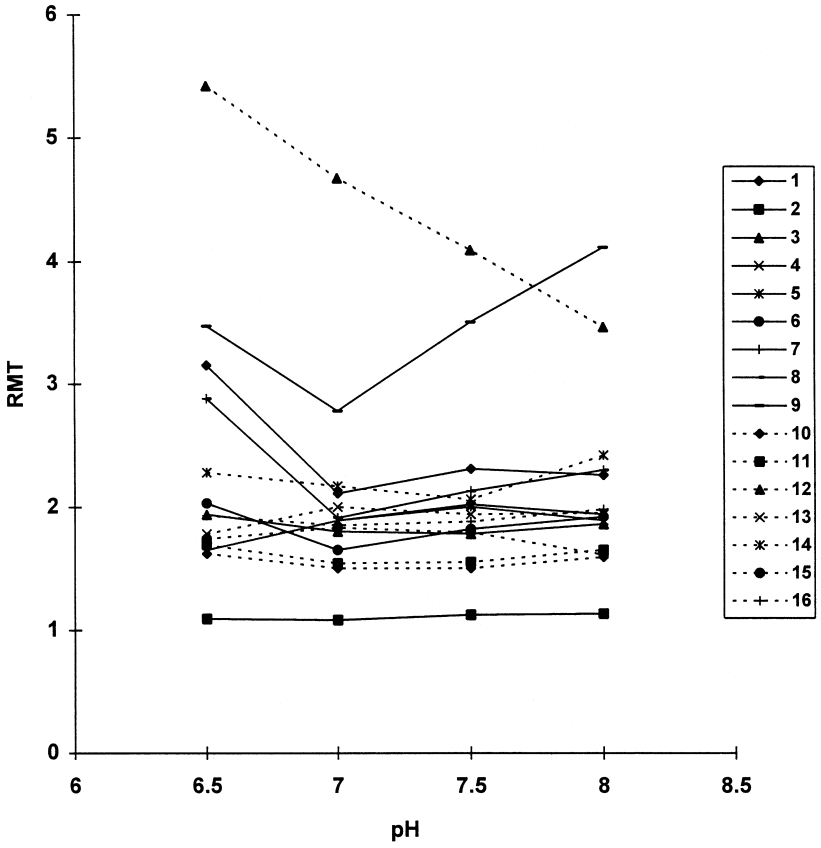


Figure 2. Influence of pH on relative migration time (RMT) of ampicillin and its related substances. Capillary: fused silica, L = 44 cm, *l* = 36 cm, I.D. = 50 µm; background electrolyte, sodium phosphate (40 mM) - SDS (150 mM) buffer; voltage, 15 kV; temperature, 25°C.

(RMT) is shown in Figure 2. From pH 6.5 to 8 the mobility of compounds **9** and **12** were influenced much more than that of other compounds. The behavior of the mobility of **9** can be explained by its pKa value, which is 8.6. Indeed, on increasing the pH, the negative charge of the molecule increases and the

Figure 1 (left). Structures of ampicillin and its related substances.

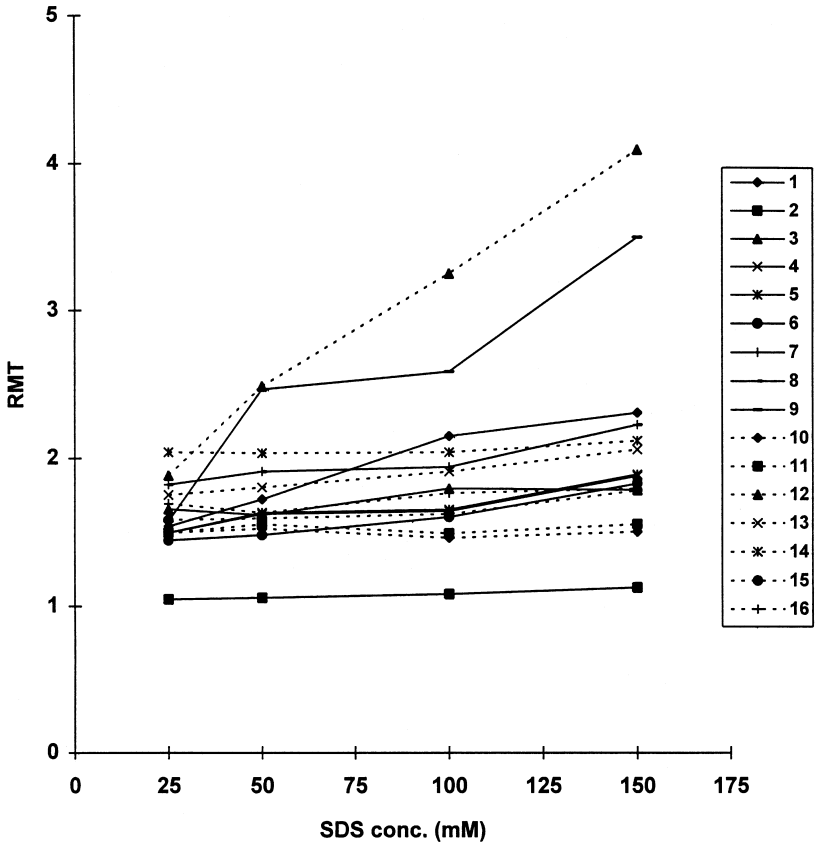


Figure 3. Influence of SDS concentration on RMT of ampicillin and its related substances. Capillary: fused-silica, L = 44 cm, *l* = 36 cm, I.D. = 50 µm; background electrolyte, sodium phosphate (40 mM) - SDS (*x* mM) buffer, pH 7.5; voltage, 15 kV; temperature, 25°C.

electrophoretic mobility decreases. The behavior of **12,** with pKa_1 = 3.4 and pKa_2 = 7.0, can not be explained by a simple mechanism. Finally, pH 7.5 was chosen as it gave the best selectivity and the shorter running time. The concentration of SDS in the buffer was also investigated. It was varied between 25 and 150 mM, keeping the phosphate concentration at 40 mM and the pH at 7.5.

The influence of SDS on the RMT of different compounds is shown in Figure 3. SDS has more influence on the mobility of substances **9** and **12** than

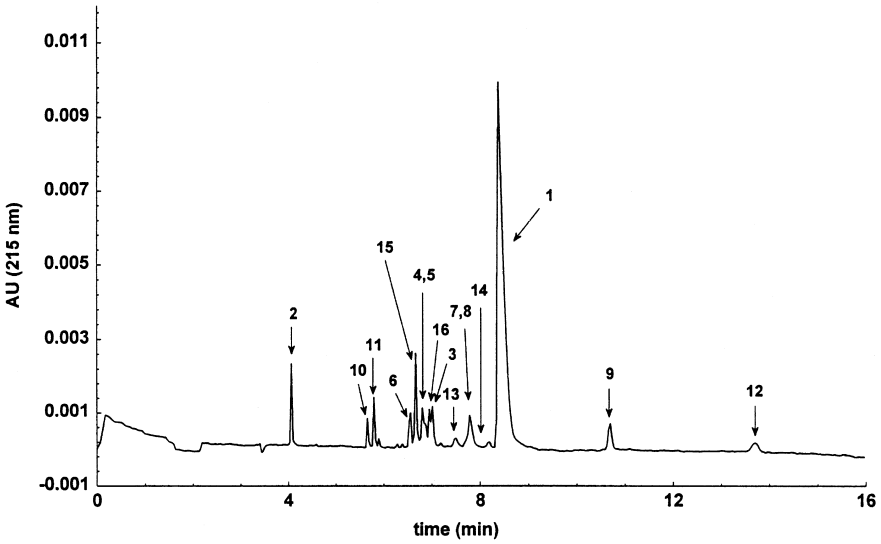


Figure 4. Electropherogram of a mixture of ampicillin and its related substances. Capillary: fused silica, L = 44 cm, *l* = 36 cm, I.D. = 50 µm; background electrolyte, sodium phosphate (40 mM) - SDS (100 mM) buffer, pH 7.5; voltage, 15 kV; temperature, 25°C.

on other substances This can be explained by a hydrophobic effect. They are less polar than other compounds and interact more with SDS while its concentration increases, which leads to their migration time increasing fast. As a good compromise between the resolution and running time 100 mM was selected.

Figure 4 shows an electropherogram of a mixture of ampicillin and its related substances.

Quantitative Performance

The quantitative aspects of this method were examined and the data are shown in Table 1. In the limit of detection (LOD) and limit of quantification (LOQ) tests, a solution of ampicillin (20 mg/20.0 mL) was diluted gradually. The solutions corresponding to 0.02 % and 0.08 % were found to correspond to the LOD and LOQ, respectively.

Table 1

Quantitative Performance*

Parameter	Ampicillin
Within-day repeatability (n=6)	
Migration time	R.S.D. = 0.8%
Corrected area	R.S.D. = 0.8%
Day-to-day repeatability (n=3)	
Migration time	R.S.D. = 2.1%
Corrected area	R.S.D. = 1.9%
Linearity y = corrected area	$y = 1502x$-116
x = concentration in mg/mL	$r = 0.9971$
range = 0.72-1.33 mg/mL	$S_{y,x} = 22$
number of concentrations = 5	
total number of analyses = 16	
LOD (S/N = 3)[a]	3.4 pg
R.S.D. = 18% (n=5)	0.02%
LOQ (S/N = 10)[a]	13.6 pg
R.S.D. = 5% (n=5)	0.08%

* Fused silica capillary, L = 44 cm, ℓ = 36 cm, I.D. = 50μm; background electrolyte, sodium phosphate (40mM) - SDS (100 mM) buffer, pH 7.5; temperature, 25°C; voltage, 15 kV; hydrodynamic injection, 1 s corresponding to an injection volume of about 1.7 nL.
[a] Injection time 10 s.

Robustness of the Method

The robustness of the method was examined by applying a full factorial experimental design.[24-25] Robustness is an important aspect of method validation.[26] One evaluates the influence of small changes in the operating or environmental conditions (variables) of the analytical procedure on measured or calculated responses. The changes introduced when performing a robustness test reflect the changes that can occur when a method is transferred between different laboratories, different experiments, or using different equipment.[27]

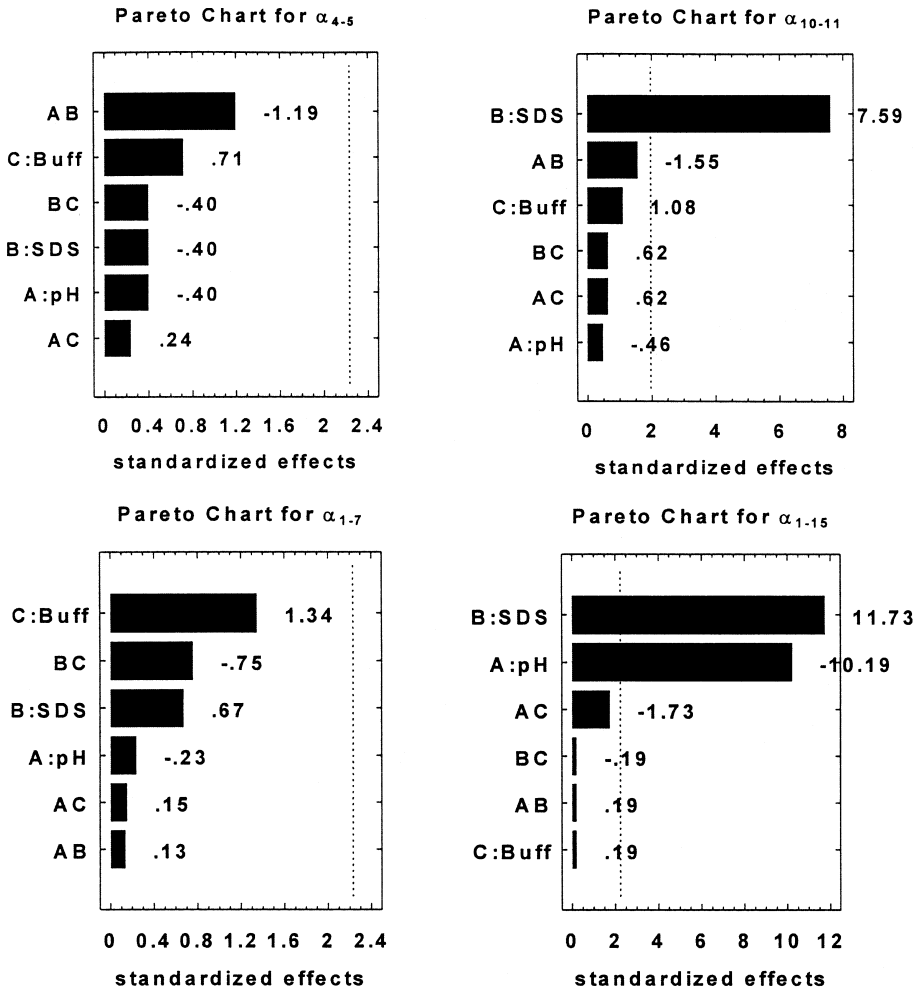


Figure 5. Standardized pareto charts for the selectivity of related substances **4** and **5** ($\alpha_{4\text{-}5}$), **10** and **11** ($\alpha_{10\text{-}11}$), ampicillin and **7** ($\alpha_{1\text{-}7}$) and ampicillin and **15** ($\alpha_{1\text{-}15}$).

In this study, three important parameters which could affect the separation were examined (low and high values are mentioned in parentheses): the pH of the buffer (7.25, 7.75); the SDS concentration in the buffer (75, 125 mM) and the phosphate buffer concentration (35, 45 mM). The measured responses were the relative migration time (RMT) of **1**, **4**, **5**, **7**, **10**, **11** and **15**. The selectivities ($\alpha = k'_1/k'_2$) between **4** and **5**, **10** and **11**, **1** and **7** and **1** and **15** were calculated. These related substances were selected because they are main degradation

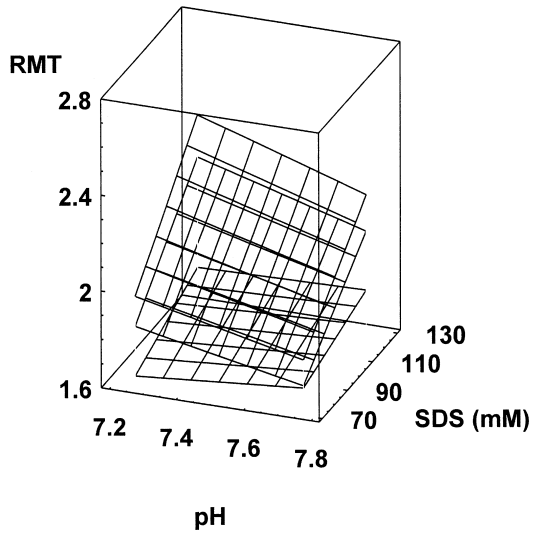


Figure 6. Estimated response surface plots for ampicillin dimer (**15**) (lower plane), ampilloic acid (5S) (**7**) (middle plane) and ampicillin (**1**) (upper plane). The concentration of phosphate buffer, 40 mM; voltage, 15 kV; temperature, 25°C. RMT = Relative migration time.

compounds of ampicillin. The application of this factorial design, analysis of the measured response variables and multivariate regression calculation were supported by the statistical graphic software system 'STATGRAPHICS', version 6.0 (Manugistics, Rockville, MD, USA). It enabled us to obtain estimated parameters for main effects, analysis of variance (ANOVA) tables, standardized pareto charts for each compound and response surface plots. Standardized pareto charts for the selectivity of **4** and **5** ($\alpha_{4\text{-}5}$), **10** and **11** ($\alpha_{10\text{-}11}$), **1** and **7** ($\alpha_{1\text{-}7}$) and **1** and **15** ($\alpha_{1\text{-}15}$) are shown in Figure 5. The codes A, B, and C indicate the effect of pH, SDS concentration and phosphate buffer concentration on the α values for **4-5**, **10-11**, **1-7** and **1-15**, respectively. The combination of two codes means the interaction between the two parameters. The standardized pareto chart consists of bars that are displayed in size order of the effects, and of a vertical line at a critical *t*-value ($\alpha = 0.05$). Parameter effects for which the bars are smaller than the critical *t*-value are considered as not significantly affecting the response variables. It can be seen that the separation of **4** and **5** or **1** and **7** is not significantly influenced by the parameters. The selectivity between **10** and **11** is only significantly influenced by the SDS concentration. An increase in SDS concentration leads to increased selectivity. For the selectivity between **1** and **15**, the SDS concentration and the pH of the buffer have a significant influence. An increase in the SDS concentration leads to an

increased selectivity, but an increase in buffer pH leads to a decreased selectivity. Interaction effects do not have an important influence on any of the separations examined.

In order to visualize better the influence of the most influent parameters, which are shown by the pareto charts, response surface plots were constructed. They show the evolution of the relative migration times as a function of two variable parameters. In Figure 6 estimated response surface plots show how the relative migration times of ampicillin and two closely migrating related substances, ampilloic acid (5S) (**7**) and ampicillin dimer (**15**) vary as a function of the buffer pH and the concentration of SDS. The concentration of the phosphate buffer was kept at 40 mM. Figure 6 confirms that no parameter has a significant influence on the separation of **1** and **7**. It is also clear that an increase in the pH of the buffer negatively influences $\alpha_{1\text{-}15}$, but an increase in the concentration of SDS improves the separation of **1** and **15**. It is observed that in all the conditions examined no overlapping of the planes occurred, which indicates that small changes in the parameters do not jeopardize the separation of ampicillin from its related substances.

CONCLUSION

The CE method discussed here is suitable for the separation of ampicillin and its related substances. It also shows good repeatability, linearity and sensitivity. The method is robust and can be used for the assay and purity control of ampicillin.

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