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Enantioselective capillary electrophoretic separation of tryptophane- and tyrosine-methylesters in a dual system with a tetra-oxadiaza-crown-ether derivative and a cyclodextrin

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Abstract

Different dual selector systems containing a cyclodextrin derivative (methyl-β-cyclodextrin and dimethyl-β-cyclodextrin) and a new diaza-crown-ether derivative (*N*-[2-(1,4,10,13-tetraoxa-7,16-diazacyclooctadecan-7-yl)propanoyl]glycine) were studied in the enantioselective separation of tryptophan-methylester and tyrosine-methylester enantiomers. This paper deals with the systematic study of the effects of changing the composition of the background electrolyte on the resolution of the D- and L- forms using an experimental design approach. It was found that the dual systems allowed a better chiral separation of the amino acid derivatives. The experimental design approach also allowed improving the separation compared to the starting conditions (center point of the design), which were adopted from a previous study.
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Keywords: Chiral separation; Dual selector systems; Diaza-crown-ether; Response surface methodology; Experimental design

1. Introduction

The enantioselective separation of chiral molecules has recently become an intensively studied field of analytical and pharmaceutical research. The fact that the different stereo isomers of a compound may result in completely different pharmacokinetic, pharmacodynamic or even toxic effects in the human body is well known, and its importance makes the study of this topic even more urgent [1]. The use of single isomer drugs, which is also strongly recommended by regulatory authorities by defining more strict requirements to patent new racemic drugs, is an additional encouragement to develop enantioselective separation methods [2]. The large-scale production of chiral pharmaceutical products arose the need for developing reliable analytical techniques, which can be useful at every stage of drug production, including the analysis of starting materials (e.g. the amino acid derivatives examined here), control of asymmetric synthesis and the quality control of the end product.

Enantioselective separation methods were developed using various techniques (gas chromatography, thin-layer chromatography, supercritical fluid chromatography, electromigration methods, liquid–liquid extraction, etc.) but the most widely used methods are high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) [3–5].

Chiral separations in CE are based on the formation of diastereomeric complexes between the enantiomers and a chiral selector, which is added to the background electrolyte. The most popular chiral selectors are cyclodextrins [6–13], since they are non-UV absorbent, commercially available, stable, environment-friendly and they are usually soluble in the background electrolyte.

Crown-ethers are successfully applied in the separation of positively charged particles, but their stereoselectivity is

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limited [14]. With the introduction of four carboxylic-acid groups they can be used as chiral selectors for the separation of enantiomers containing primary amine functional groups [15–17].

Furthermore, the use of background electrolytes combining cyclodextrins and crown-ethers—both chiral and non-chiral—has been reported for various separations [18–20]. The effect of the crown-ether on the resolution is positive in most cases, but sometimes—depending on the substrates and the cyclodextrin—no effect or a decreased enantioselectivity is observed. The three species (analyte, CD and crown-ether) form a difficult multi-complex system, where possibly a three-body complex is responsible for the enantiorecognition [14].

Iványi et al. [21] showed that newly synthesized diaza-crown-ethers individually did not have the ability to achieve chiral separation of some amino acid derivatives. Their use in dual selector systems with cyclodextrin-containing background electrolytes induced better results, than using cyclodextrin alone. They also investigated the substitution of 1,4,7,10,13,16-hexaoxacyclooctadecane (18C6) with the diaza-crown-ether in dual selector systems, and in most cases the use of diaza-crown-ether proved to be more efficient. A possible explanation given is the following: at low pH values both the N atoms of the amino-group of the diaza-crown-ether are positively charged. During the formation of the three-member complex between the cyclodextrin, the diaza-crown-ether and the amino acid, one proton leaves the complex due to its electronic overload. This would stabilize the complex, and lead to a stronger three-member interaction and enhanced enantioselectivity. The results were compared with those of Armstrong et al. [14], where cyclodextrins and 18C6 systems were used. Therefore, the same separation conditions were applied, which were not necessarily optimal for the actual dual systems [21].

The goal of this paper is to further study the newly introduced diaza-crown-ethers in CE separations. Therefore, the influence of their concentration in the background electrolyte and occasionally of their interaction with other parameters (e.g. CD concentration) on the separation of tyrosine-methylester (TyMe) and tryptophane-methylester (TrMe) isomers is evaluated from the response surfaces obtained from the execution of an experimental design. This should lead to more optimal separation conditions than those applied in [21].

TrMe and TyMe were selected as model compounds for the type of chiral substances that could be separated by the approach of Refs. [14] and [21]. The importance of these amino acid derivatives lies in their application as building blocks in combinatorial chemical processes.

A second objective is to optimize and improve the separation conditions of [21]. A three-factor central composite design [22], with the cyclodextrin concentration, crown-ether concentration and buffer concentration as factors, was executed for these purposes. The considered responses were resolution and migration time. As the focus of this paper lies in improving the already existing separation rather than to develop a method that can be used routinely for specific applications, method validation was not considered after the optimization step.

2. Materials and methods

2.1. Chemical

D,L-Tryptophan-methylester hydrochloride (TrMe), D,L-tyrosine-methylester hydrochloride (TyMe) and methyl-β-cyclodextrin (Me-βCD) were purchased from Sigma (St. Louis, MO, USA), and D-tryptophan-methylester from Aldrich (Steinheim, Germany). Hydroxypropyl-β-cyclodextrin (HP-β-CD), dimethyl-β-cyclodextrin (diMe-β CD) were gifts from Beckman (Fullerton, CA, USA). 18-crown-6 (18C6) and *ortho*-phosphoric acid were obtained from Fluka Chemie (Bornem, Belgium), NaH_2PO_4 was obtained from Merck (Darmstadt, Germany). *N*-[2-(1,4,10,13-tetraoxa-7,16-diazacyclooctadecan-7-yl)propanoyl]-glycine (*RS*-**1**) was synthesized in our laboratory. Water was purified by a Milli-Q system (Millipore, Milford, MA, USA).

2.2. Instrumentation

The pH was measured with an Orion 520A (Boston, MA, USA) pH meter.

All experiments were carried out on a SpectraPHORESIS ULTRA device with a SpectraPHORESIS vial server, UV3000 UV–vis detector and SN4000 communication interface (Thermo Separation Products, San Jose, CA). For the separation a 56.5 cm long (50.00 cm effective length), 75 μm diameter uncoated fused-silica capillary (Composite Metal Services, Ilkley, UK) was used.

Samples were injected hydrostatically using positive pressure of 0.8 psi for 2 s (injected volume is 6.7 nL). Further experimental conditions were a 15 kV analyzing voltage, 25 °C controlled temperature and 3-wavelength detection (218, 236 and 254 nm). Before each separation, the capillary was rinsed with the running electrolyte for 2 min, while at buffer change a 2 min conditioning with, consecutively, 0.1 M NaOH and MilliQ water, and a 5 min wash with the new running electrolyte were applied.

2.3. Electrolytes and sample preparation

The various phosphate buffers concentrations of pH 2.27 were prepared by adjusting the pH of a NaH_2PO_4 solution having the desired concentration with the same concentration *ortho*-phosphoric acid.

The background electrolytes were kept in an ultrasonic bath for 10 min to dissolve the chiral selectors, and then were filtered through a 0.45 μm nylon syringe filter (Chromafil, Macherey-Nagel, Düren, Germany). Before use they were placed again in the ultrasonic bath for 15 min in order to degas.

Table 1
Central composite design for three factors and resulting conditions

Experiment	Experimental design levels			Experimental design conditions		
	[CD]	[RS-**1**]	[Buffer]	[CD] (mM)	[RS-**1**] (mM)	[Buffer] (mM)
1	−1	−1	−1	20	20	30
2	+1	−1	−1	40	20	30
3	−1	+1	−1	20	40	30
4	+1	+1	−1	40	40	30
5	−1	−1	+1	20	20	70
6	+1	−1	+1	40	20	70
7	−1	+1	+1	20	40	70
8	+1	+1	+1	40	40	70
9	−1.68	0	0	13.2	30	50
10	1.68	0	0	46.8	30	50
11	0	−1.68	0	30	13.2	50
12	0	1.68	0	30	46.8	50
13	0	0	−1.68	30	30	16.4
14	0	0	1.68	30	30	83.6
15	0	0	0	30	30	50

[CD], cyclodextrin concentration; [RS-**1**], *N*-[2-(1,4,10,13-tetraoxa-7,16-diazacyclooctadecan-7-yl) propanoyl] glycine concentration; [buffer], buffer concentration.

Stock solutions of the racemic compounds were made by dissolving them in a concentration of 1 mg/ml (a small excess of the D-enantiomer of tryptophan-methylester was used) in Milli-Q water. The stock solution of TrMe was 5-fold diluted because of its higher molar absorbance. Before use, the sample solutions were filtered also through a 0.45 μm nylon syringe filter (Chromafil, Macherey-Nagel, Düren, Germany) and then degassed in an ultrasonic bath.

2.4. Experimental design

In order to examine the impact of the changes in composition of the background electrolyte on resolution and migration time, a five-level, spherical, central composite design for three factors was created, which requires 15 experiments (Table 1). The three factors were *RS*-**1** concentration, buffer concentration and cyclodextrin concentration.

The Nemrod software (LPRAI, Marseille, France) was used for the creation of the design. After its execution, data treatment and drawing response surfaces was done with the same software.

3. Results and discussion

The experimental domain examined was defined starting from the conditions used by Iványi et al [21], and the central point of the design was chosen equal to the conditions used in their studies (Table 1). The +α extreme level of the cyclodextrins was determined by their solubility limits, and hence, the +α level of *RS*-**1** was set similarly.

For the responses (resolution and migration time) the following model was determined:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad (1)$$

where y represents the response, x_1, x_2, x_3 the factors and b_{ij} the regression coefficients of the model.

Two amino acid derivatives TrMe and TyMe were chosen to study the impact of the above mentioned concentration changes. The experiments at the central point conditions of the design gave similar results to the resolutions observed by Iványi et al [21]. These conditions were possible starting points for method optimization, since in none of the described cases baseline separation was achieved.

3.1. Optimization of the separation of TrMe with the dual system Me-βCD + RS-*1*

In Fig. 1 the resolution (a) and the migration time (b) are plotted as a function of the *RS*-**1** and CD concentrations. The buffer concentration was kept constant (50 mM). The increase of both the cyclodextrin and the diaza-crown-ether concentrations results in an increased resolution (Rs), but at higher concentrations of the selectors the migration time (T_m) increases fast, which can be detrimental from a practical point of view. At low *RS*-**1** concentration T_m is only slightly affected by the cyclodextrin concentration, but when the amount of *RS*-**1** in the solution is high, the effect of the cyclodextrin concentration on T_m becomes much more important (Fig. 1b).

The shape of the response surface in Fig. 1a shows that there is no interaction between cyclodextrin and *RS*-**1** concentrations. However, for the response T_m (Fig. 1b), we observe an interaction effect between the factors, since the effect of the CD concentration is depending on the *RS*-**1** concentration and vice versa.

A minimum in migration time is observed at low *RS*-**1** and medium CD concentration. However, in this domain resolution is insufficient (below 0.5). The optimum in the experimental domain can be defined around point A (Fig. 1).

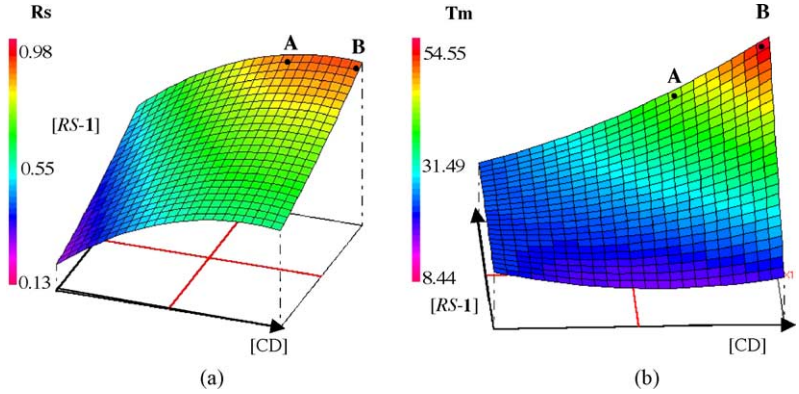

Fig. 1. Resolution (a) and migration time (b) of TrMe enantiomers as a function of *RS*-**1** and Me-βCD concentrations at constant buffer concentration (50 mM). Points A and B: see text.

In point B, we get a similar resolution (around 1), while the migration time is much longer (see Fig. 1b).

Fig. 2 represents the resolutions as a function of buffer and *RS*-**1** concentration (a) and of buffer and CD concentration (b). The third factor was kept constant. When the CD concentration is kept constant (30 mM) (Fig. 2a), the resolution slightly increases with increasing *RS*-**1** concentration, as was already noticed in Fig. 1. The influence of the buffer concentration on the resolution was found to be low. The EOF increased as a function of buffer concentration, which generates shorter migration times and a decreased resolution. This is contradictory to what one would theoretically expect [10], i.e. a decrease of EOF as a function of the ionic strength, and hence the buffer concentration.

Fig. 2b shows that at high CD concentrations there is no clear effect of the buffer concentration on the resolution, while at low CD concentrations it has a negative effect. It can, therefore, be concluded that there is an interaction effect between buffer and cyclodextrin concentration.

At constant buffer and *RS*-**1** concentrations, the Rs as a function of the cyclodextrin concentration seems to go through a maximum. This can be observed in Figs. 1a and 2b. This seems to be similar to the curves found by Wren and Rowe [23] for situations where only cyclodextrin is used in the background electrolyte and where Rs is also going through a maximum.

Table 2
Influence of different selectors on resolution and migration time of TrMe and TyMe enantiomers

Selector	Tryptophan-methylester		Tyrosine-methylester	
	Rs	T_m (min)	Rs	T_m (min)
Me-βCD + *RS*-**1**	0.99	38.6	1.15	31.42
Me-βCD	0.075	13.9	0	13.4
Me-βCD + 18C6	0	15.0	0.9	18.2

[CD, *RS*-**1**, buffer, 18C6] = 50 mM.

In summary, it can be said from the response surfaces that within the examined domain addition of *RS*-**1** in dual systems with Me-βCD improves the separation, but increases the migration time. Buffer concentration has a minor effect on the separation, at least in the parts of the experimental domain where Rs is higher. However, the best predicted Rs in the experimental domain examined still remained moderate, i.e. around one.

To test the efficiency of the Me-βCD + *RS*-**1** dual selector system compared to the single Me-βCD and the dual Me-βCD + 18C6 containing background electrolytes, some experiments were carried out in the conditions describing point B of Fig. 1. The results are given in Table 2, a typical electropherogram is shown in Fig. 3.

In the comparison of three different systems, the use of the dual Me-βCD + *RS*-**1** combination proved to be most effi-

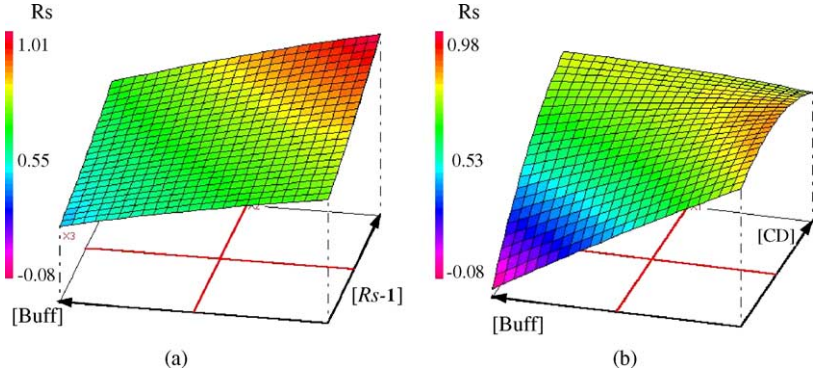

Fig. 2. Resolution of TrMe enantiomers as a function of *RS*-**1** and buffer concentrations (a) and of buffer and Me-βCD concentrations (b) at constant cyclodextrin and *RS*-**1** concentrations, respectively (30 mM).

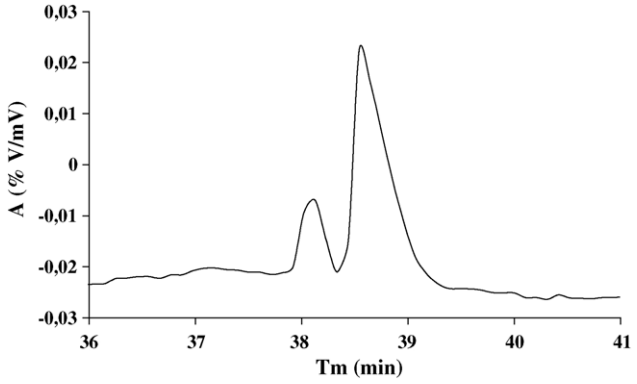

Fig. 3. Separation of the TrMe enantiomers in a dual Me-βCD and *RS*-**1** system [CD, *RS*-**1**, buffer] = 50 mM. Other experimental conditions: see text.

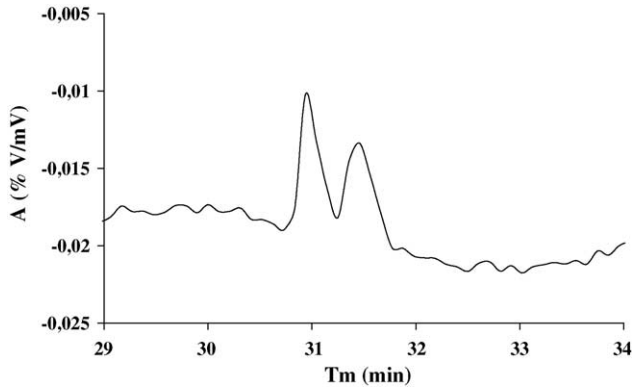

Fig. 5. Separation of the TyMe enantiomers in a dual Me-βCD and *RS*-**1** system. [CD, *RS*-**1**, buffer] = 50 mM. Other experimental conditions: see text.

cient. Without *RS*-**1** in solution, the cyclodextrin causes some separation, but the Rs is one order of magnitude less, while for the dual Me-βCD + 18C6 system even this minor separation disappeared. The separation under the optimized conditions (Rs 0.99) is better than in the center point (Rs 0.74). However, it is not better than the one reported in [21] (Rs 1.01). This difference in results at center point conditions may be due to reproducibility problems, which in CE can occur when a given method is transferred between instruments, operators or laboratories [24].

The results of experiments with single *RS*-**1** and 18C6 as chiral selector are not shown, because—in accordance with the results of Iványi et al. [21]—they do not exhibit enantiomer Rs in any condition examined in this paper.

*3.2. Optimization of the separation of TyMe with the dual system Me-βCD + RS-**1***

Using the same design to examine the separation of tyrosine-methylester, clearly different results were obtained (Fig. 4). The response surfaces of Rs as a function of *RS*-**1** and CD concentrations and as a function of buffer and CD concentrations show a minimum. The behavior of Rs as a function of the CD concentration is not similar to the curves found by Wren and Rowe. The explanation of this minimum is beyond the scope of this article, but anyway it draws one's attention to the diverse behavior of the same systems towards different substrates (see also Figs. 1 and 2).

As can be seen on Fig. 4a, there is a large difference between the Rs values in points A and B as compared to Fig. 1. Generally, the two enantiomers can be separated more efficiently here, but the system is less robust, i.e. it is more sensitive to the changes in the measuring conditions.

In summary, the occurrence of *RS*-**1** improves again separations in dual systems, especially at high CD concentrations.

In the particular case of TyMe, the outcome of the same separation conditions as executed for TrMe is summarized in Table 2. The chiral recognition ability of the 18C6 + Me-βCD system is significantly increased for this substance, while with a single cyclodextrin-containing background electrolyte system no separation was observed. The Me-βCD + *RS*-**1** selector combination still provides the best potential to separate the enantiomers (see Fig. 5). The obtained Rs (1.15) is similar to that in [21] at center point conditions, but much better than the center point result of the actual design. These differences again can be attributed to the earlier mentioned reproducibil-

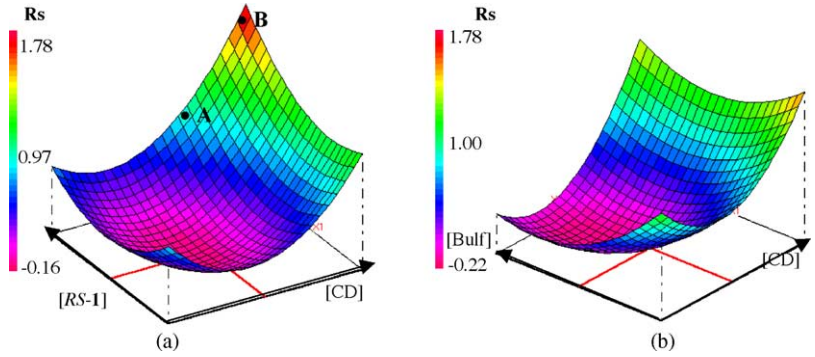

Fig. 4. Resolution of D and L isomers of TyMe as a function of *RS*-**1** and Me-βCD concentrations (a), and of buffer and CD concentration (b) at constant buffer and *RS*-**1** concentrations, respectively (30 mM).

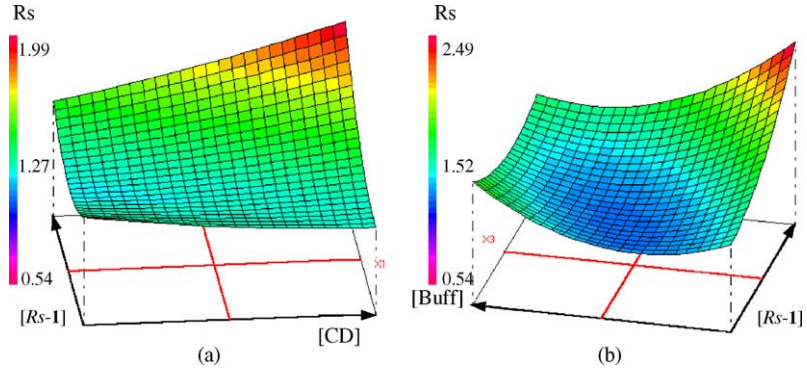

Fig. 6. Resolution of D and L isomers of TrMe as a function of *RS*-**1** and diMe-βCD concentrations (a), and of buffer and *RS*-**1** concentrations (b) at constant buffer and CD concentration, respectively (30 mM).

ity problem occurring with some CE separations [24]. The tendency for the differences observed with [21] is similar as observed in Section 3.1. However, as the main aim of this study was to evaluate the effect of the different factors on the separation, the experimental design approach allowed defining more optimal separation conditions than at center point level of the actual situation.

3.3. Optimization of the separation of TrMe with the dual system diMe-βCD + RS-1

Changing methyl-β-cyclodextrin to its dimethyl analogue alters the properties of the background electrolyte, which results itself in different Rs results (Fig. 6 versus Figs. 1 and 2). The use of diMe-βCD acts beneficial upon the separation of the TrMe enantiomers. In contrast to the use of Me-βCD the Rs values are above 1 in the whole experimental domain. At low *RS*-**1** concentrations the increasing CD concentration results in a slight loss of Rs. However, an interaction between the two selectors can be observed, because at high *RS*-**1** concentrations the higher CD concentration is favourable in order to separate the enantiomers.

The reaction conditions (derived from Nemrod) and the results achieved in the single CD, the CD + 18C6 and the CD + *RS*-**1** containing systems, are shown in Table 3. In comparison to the other studied systems, the use of the diMe-βCD + *RS*-**1** dual system is by far the most effective in the separation of TrMe enantiomers. In Fig. 7, the electropherogram of the best separation can be seen.

The Rs value (1.93) is much higher than at nominal level (1.22), and a clear baseline separation was observed.

Table 3
Influence of different selectors on resolution and migration time of TrMe enantiomers

Selector	Tryptophan-methylester	
	Rs	T_m (min)
diMe-βCD + *RS*-**1**	1.93	44.53
diMe-βCD	0.685	18.9
diMe-βCD + 18C6	0	22.5

[CD] = 28.6 mM; [RS-**1**] = 47.0 mM; [buffer] = 44.7 mM; [18C6] = 47.0 mM.

3.4. Optimization of the separation of TyMe with the dual system diMe-βCD + RS-1

As for the separation of TrMe, the change in cyclodextrin altered the complex-formation in the background electrolyte, which can be observed from the response surfaces (Fig. 8 versus Fig. 4). The best Rs values are lower than those obtained with Me-βCD and no minimum was observed in the response surface. The efficiency of the selector system increases largely when the concentrations of *RS*-**1** and mainly diMe-βCD are increased, while an increase in the buffer concentration has a small but negative impact on the Rs. The electropherogram measured in the optimum region (high CD and *RS*-**1** concentrations and low buffer concentration) and resulting in Rs of 1.05 is shown in Fig. 9.

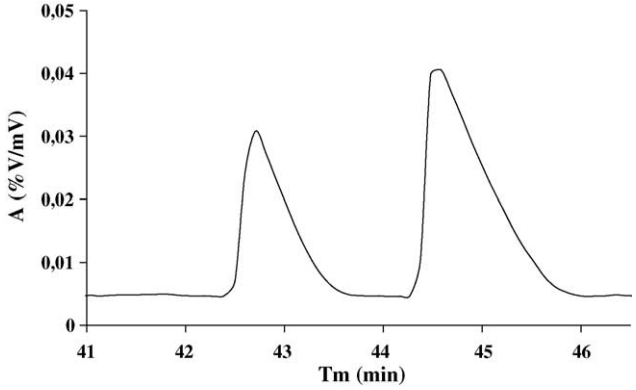

Fig. 7. Separation of the TrMe enantiomers in a dual diMe-βCD and *RS*-**1** system. [CD] = 28.6 mM, [RS-**1**] = 47 mM and [buffer] = 44.7 mM. Other experimental values: see text.

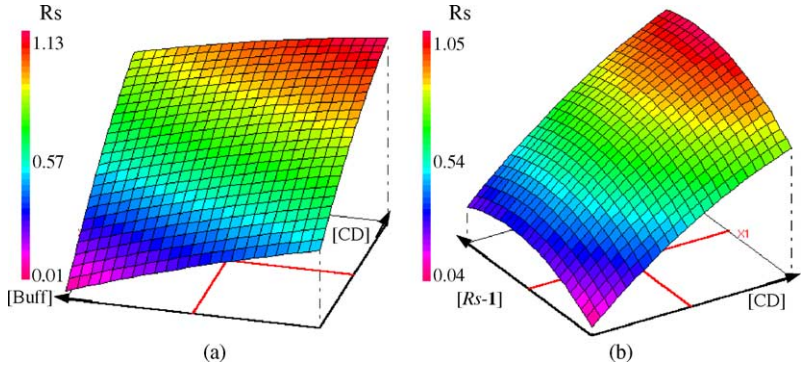

Fig. 8. Resolution of TyMe enantiomers as a function of diMe-βCD and buffer concentrations (a), and of CD and *RS*-**1** concentrations (b) at constant *RS*-**1** and buffer concentration (30 mM and 50 mM), respectively.

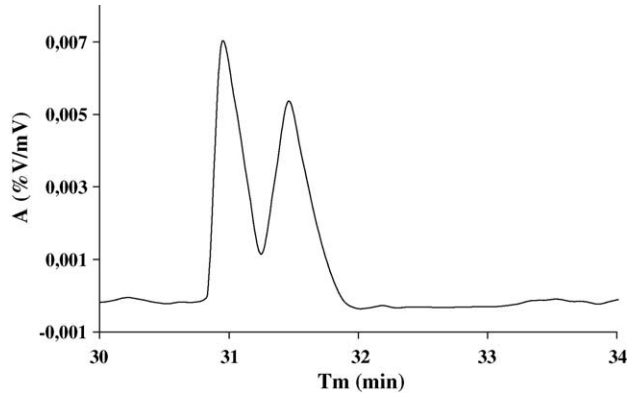

Fig. 9. Separation of TyMe enantiomers in presence of diMe-βCD and *RS*-**1** selectors. [CD] = 40 mM, [RS-**1**] = 40 mM and [buffer] = 30 mM.

4. Conclusion

In this study, different dual selector systems containing cyclodextrin derivatives (methyl-β-cyclodextrin and dimethyl-β-cyclodextrin) and a new diaza-crown-ether derivative were studied in the enantioselective separation of tryptophan-methylester and tyrosine-methylester enantiomers by means of capillary electrophoresis. The dual system results were compared with CD + 18C6 containing systems, and the single CD system. The response surfaces showed that, in general, *RS*-**1** has a positive influence on the selectivity. However, migration times also increased using the dual system. At optimal conditions baseline separation was not always obtained. However, the use of *RS*-**1** + CD dual selector systems resulted always in better resolutions than the other systems tested and than the starting point of the development (centre point of the design). The use of methyl-β-cyclodextrin + *RS*-**1** proved to be more successful in the separation of tryptophan-methylester isomers, while the use of dimethyl-β-cyclodextrin + *RS*-**1** gave better results in the separation of tyrosine-methylester isomers. Our results demonstrated that the new diaza-crown-ether derivative might be a helpful tool to improve the separation of amino acids and their derivatives, when used in dual systems with a cyclodextrin derivative as second selector.

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References

[1] S.C. Stinson, Chem. Eng. News 78 (2000) 55–78.
[2] H. Caner, E. Groner, L. Levy, I. Agranat, Drug Discov. Today 9 (2004) 105–110.
[3] G. Gubitz, M.G. Schmid, Biopharm. Drug Dispos. 22 (2001) 291–336.
[4] J. Haginaka, J. Pharm. Biomed. Anal. 27 (2002) 357–372.
[5] A. Amini, Electrophoresis 22 (2001) 3107–3130.
[6] N.W. Smith, J. Chromatogr. A 652 (1993) 259–262.
[7] C.A. Lucy, R. Brown, K.K.C. Yeung, J. Chromatogr. A 745 (1996) 9–15.
[8] K.D. Altria, M.A. Kelly, B.J. Clark, TRAC 17 (1998) 214–226.
[9] N.T. Nguyen, R.W. Siegler, J. Chromatogr. A 735 (1996) 123–150.
[10] B. Chankvetadze, Capillary Electrophoresis in Chiral Analysis, Wiley, Chichester, 1997.
[11] N. Matthijs, C. Perrin, M. Maftouh, D.L. Massart, Y. Vander Heyden, J. Pharm. Biomed. Anal. 27 (2002) 515–529.
[12] C. Perrin, Y. Vander Heyden, M. Maftouh, D.L. Massart, Electrophoresis 22 (2001) 3203–3215.
[13] K.H. Gahm, L.W. Chang, D.W. Armstrong, J. Chromatogr. A 759 (1997) 149–155.
[14] D.W. Armstrong, L.W. Chang, S.S.C. Chang, J. Chromatogr. A 793 (1998) 115–134.
[15] M. Newcomb, J. Toner, R. Helgeson, D.J. Cram, J. Am. Chem. Soc. 101 (1979) 4941–4947.
[16] M. Hilton, D.W. Armstrong, J. Liq. Chromatogr. 14 (1991) 9–28.
[17] R. Kuhn, F. Erni, T. Bereuter, J. Hausler, Anal. Chem. 64 (1992) 2815–2820.
[18] R. Kuhn, F. Stoecklin, F. Erni, Chromatographia 33 (1992) 32–36.
[19] R. Kuhn, V. Wagner, V. Walbroehl, V. Bereuter, Electrophoresis 15 (1994) 828–834.
[20] R. Kuhn, C. Steinmetz, T. Bereuter, P. Haas, F. Erni, J. Chromatogr. A 666 (1994) 367–373.

[21] T. Iványi, K. Pál, I. Lázár, D.L. Massart, Y. Vander Heyden, J. Chromatogr. A 1028 (2004) 325–332.
[22] Y. Vander Heyden, C. Perrin, D.L. Massart, in: K. Valkó (Ed.), Handbook of analytical separations: separation methods in drug synthesis and purification, Elsevier, Amsterdam, 2000, pp. 163–211.
[23] S.A.C. Wren, R.C. Rowe, J. Chromatogr. 603 (1992) 235–241.
[24] P. Dehouck, Uncertainty determination in pharmaceutical analysis, Ph.D. Thesis, Catholic University Leuven, Belgium, 2004 [Chapter 6–8].