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Application of tetraoxadiaza-crown ether derivatives as chiral selector modifiers in capillary electrophoresis

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Abstract

Two new diaza-crown ether derivatives (*R*-**1**, *RS*-**1**) have been synthesized from 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane and tested as potential chiral selectors in capillary electrophoresis (CE) for the chiral separation of five amino acid derivatives. The individual use of the selectors did not lead to chiral differentiation. However, they enhanced the enantioselective effect of different cyclodextrins in dual selector systems. In this paper, we report the effect of different substituted diaza-crown ether derivatives on the separation results obtained in dual systems with cyclodextrins.

Keywords: Enantiomer separation; Derivatization, electrophoresis; Chiral selectors; Crown ethers; Diaza-crown ethers; Cyclodextrins; Amino acids

1. Introduction

Capillary electrophoresis (CE) is a widely used analytical tool, which provide fast and reproducible separations of different nature. It is often considered as a first choice technique for chiral separations. The most frequently used chiral selectors in CE are the different cyclodextrins [1–7], other "macrocycle-type" molecules, like calixarenes [8,9] and different types of crown ethers [10,11]. However, the crown ethers also might be added for other purposes, e.g. separation of metal ions, of different amines, and of positional isomers. The most commonly used crown ethers in capillary electrophoretic separations are the "18-crown-6"-type molecules, discovered by Pederson [12,13]. One important property is their selective complex-formation ability with metal ions or protonated organic cations, based on electrostatic interactions between the cation and the unshared pair of electrons from oxygen donor atoms. Alkali, alkaline earth, transition metal and lanthanide cations have been separated using the neutral 18-crown-6 (18C6), modifying their electrophoretic mobility by in situ complex formation [14–17].

CE is also extensively used for the investigation of organic amines, which are commonly applied as chemicals in pharmaceutical synthesis. They are protonated under acidic conditions and if they contain a primary amino group they form inclusion complexes with crown ethers via three hydrogen bonds between the protonated amino group and the ring-oxygen atoms [10]. For secondary and tertiary amines the host–guest interaction does not appear due to steric hindrance [11]. Primary, secondary and tertiary amines and positional isomers of aromatic amines were separated with 18C6, based on this theory [11,18,19].

However, the neutral crown ethers can only be applied for the separation of cations. Mori et al. [20] showed the possibility to separate negatively charged positional isomers using a twice positively charged nitrogen-atoms-containing crown ether derivative.

As already mentioned, the most frequent application of CE is the chiral separation of pharmaceutically important organic compounds including the optical-purity testing of chiral drugs. Since the enantiomer differentiation of primary amines requires chiral center containing substituents, the non-chiral 18C6 can not achieve chiral separation individually, but it can induce or enhance resolution when used simultaneously with a cyclodextrin in a dual chiral selector system. The crown ether supports the interaction between

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the analyte and the cyclodextrin [21–24]. In these dual selector systems, the crown ether forms selective host–guest complexes with the different enantiomers via their protonated amino group and the hydrophobic substituent of the analytes are incorporated into the cavity of the cyclodextrin. The possible interaction between the analytes and selectors depends on their chemical structure, but the exact mechanism is not solved yet. The 18C6 might also have no or a negative effect on the separation.

The crown ether's positive effect on the cyclodextrin–amine interaction was proved when β-cyclodextrin was functionalised with diaza-crown ether [25]. Higher binding constants for alkali metal or ammonium salts of aromatic compounds were measured than when using the unmodified cyclodextrin, as it was expected.

The only chiral crown ether, which has already been studied as chiral selector is the 18-crown-6-tetracarboxylic acid ($18C6H_4$), introduced by Kuhn et al. [10]. The primary electrostatic interaction between protonated amines and the polyether ring in itself is not sufficient for the separation, but the four carboxylic groups form additional (ionic and hydrogen bonding) interactions with the substituents of the enantiomers. Depending on the molecular structure of the analytes the electrophoretic mobility of the different enantiomers is modified by the host–guest complexation and separation is observed. Under acidic conditions, positively charged primary amines and the negatively charged $18C6H_4$ migrate into opposite directions, causing enhanced resolution. Several underivatized and esterified amino acids and peptides, containing the stereogenic centre located mainly in the α-position to the primary amino group, have been successfully separated in this way [10,26–29]. When neither $18C6H_4$ nor cyclodextrins individually achieved enantiomer separation, dual selector systems, containing both $18C6H_4$ and cyclodextrin, were already successfully applied [10].

In our laboratory, different tetraoxadiaza-crown ether derivatives have been synthesised recently. Development of molecules *R*-**1** and *RS*-**1** was a continuation of our efforts to synthesize new macrocyclic derivatives for multipurpose use, mainly as metal ion complexing agents and host molecules for chiral and positional isomer separations. The technique of choice for this application was capillary electrophoresis. The two diaza-crown ether derivatives were synthesised from 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane (Kryptofix 22) and studied for the chiral separation of five amino acid derivatives, namely tryptophan (Try), tryptophan methyl ester (TryMe), tryptophan ethyl ester (TryEt), tryptophan butyl ester (TryBu) and tyrosine methyl ester (TyrMe). The crown ether derivatives examined were (i) an optically pure *R*-enantiomer *N*-[(2*R*)-2-(1,4,10,13-tetraoxa-7,16-diazacyclooctadecan-7-yl)propanoyl]glycine: compound *R*-**1**; and (ii) a racemic *N*-[2-(1,4,10,13-tetraoxa-7,16-diazacyclooctadecan-7-yl)propanoyl]glycine:compound *RS*-**1** (Fig. 1).

Both new molecules have a propanoylglycine pendant arm with a free carboxylic end that is required for efficient metal ion coordination. The presence of the carboxyl group might show less advantageous effect on the dual selector systems, but its magnitude can not be preestimated. The virtually inadvantageous effect is overcompensated on the other hand by the multifunctionality of *R*-**1** and *RS*-**1** molecules. The macrocycle ring still bears a free secondary amino group which is intended to be used for attaching additional functional groups to them in order to improve coordination/host–guest behaviours and to serve as a connecting point for anchoring macrocycles to a solid support. The place of the chiral centre in the pendant arm was tailored to provide maximum interaction with the chiral amino compound when it is coordinating to the macrocycle. That interaction, depending on the absolute configurations of the chiral centres, leads to differences in bonding energies, which may result in enantiomer separation.

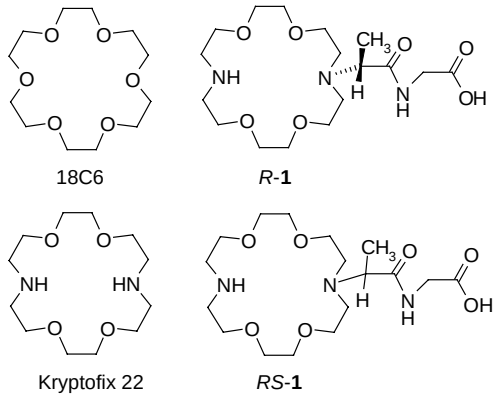


Fig. 1. Structures of 18-crown-6, Kryptofix 22 and the newly synthesized *R*-**1** and *RS*-**1** molecules.

In this paper, we report how the two nitrogen atoms replacing two oxygens in the crown-ether ring affect the separation results in dual systems with cyclodextrins and how the chiral pendant arm in the diaza-crown ethers does. The optically pure *R*-**1** derivative was also tested individually for its enantioselective properties.

2. Experimental

2.1. Chemicals and instrumentation

D,L-Tryptophan hydrochloride, D-tryptophan and D-tryptophan methyl ester were obtained from Aldrich (Steinheim, Germany), D,L-tryptophan methyl ester hydrochloride; D,L-tryptophan ethyl ester hydrochloride; D,L-tryptophan butyl ester hydrochloride; D,L-tyrosine methyl ester hydrochloride, α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), methyl-β-cyclodextrin (Me-β-CD), heptakis(2,3-di-*O*-methyl)-β-cyclodextrin (DM-β-CD1, Sigma) and heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD) were purchased from Sigma (St. Louis, MO, USA), γ-cyclodextrin (γ-CD), hydroxypropyl-β-cyclodextrin (HP-

β-CD) and dimethyl-β-cyclodextrin (DM-β-CD2, Beckman) were gifts from Beckman (Fullerton, CA, USA), 18-crown-6 and mesityl oxide was obtained from Fluka Chemie (Bornem, Belgium), and 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane (Kryptofix 22) from Merck. Water for solution preparation was produced in-house by the Milli-Q System (Millipore, Milford, MA, USA). Compounds *R*-**1** and *RS*-**1** were synthesized in our laboratory.

A SpectraPhoresis Ultra capillary electrophoresis instrument (Thermo Separation Products, San Jose, CA, USA) equipped with a fast scanning UV-Vis detector was used for the experiments. Separations were executed in an uncoated fused-silica capillary (Composite Metal Services, The Chase, Hallow, UK) with 56.5 cm total length (50.3 cm to the detector) and 75 μm in diameter. Samples were injected hydrostatically using a positive pressure of 0.8 (psi = 6894.76 Pa) for 2 s (injected volume 6.7 nl). The separations were carried out at 25 °C applying 15 kV voltage and all analytes were monitored at 218, 236 and 254 nm. Between two different electrolyte systems, the capillary was rinsed with 0.1 M NaOH and Milli-Q water, each for 2 min, and with the running electrolyte solution for 5 min, respectively. Before each separation the capillary was conditioned with the running electrolytes for 2 min.

Solvents of reagent grade for the synthesis were purchased from Spektrum-3D (Debrecen, Hungary) and used without further purification. Other reagents were purchased from Aldrich and Merck (Darmstadt, Germany). Mass spectrometry analyses were carried out with a Bruker BIFLEX III matrix-assisted desorption ionization (MALDI) time-of-flight (TOF) mass spectrometer equipped with a TOF analyser (Leipzig, Germany). ^{1}H spectra were recorded on a Bruker AM 360 spectrometer (Bruker, Germany), and processed with the Mestre-C 2.3 (Santiago, Spain) software.

AM1 semiempirical quantum chemical calculations were performed by the computer program HyperChem 7.1 (Hypercube, Gainesville, FL, USA) on a dual processor 2 × 2.4 GHz Compaq Evo 6000 computer using 1 GB RAM.

2.2. Synthesis of R-1 and RS-1

Compounds *R*-**1** and *RS*-**1** have been synthesized from 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane in a two-step reaction. First the appropriate (*S*)- or (*RS*)-(2-bromo-propionylamino)-acetic acid benzyl ester (6.32 g, 21.05 mmol) was added to the diaza-crown ether (11.04 g, 42.08 mmol), in an anhydrous dipolar aprotic solvent, preferably acetonitrile (50 ml). 1,4,10,13-Tetraoxa-7,16-diazacyclooctadecane was used in excess in order to achieve regioselectivity and to bind hydrogen bromide formed in the reaction. Dry column vacuum chromatography was applied in order to purify *R*-**1** or *RS*-**1** from the possible byproducts. The benzyl protecting group was then removed by catalytic reduction with hydrogen using palladium on activated carbon (10%, 940 mg) in a water/methanol (4/1, v/v) mixture (40 ml). The obtained *RS*-**1** is a light yellowish powder (3.58 g). Reaction scheme for *RS*-**1** is given in Fig. 2. More detailed description of the synthesis will be published elsewhere. Additional information obtained about the reaction product is: ^{1}H NMR (360 MHz): δ_H (D_2O, pD = 9.45): 1.15 (d, J = 6.84 Hz, 3H, CH–CH_3), 2.62–2.68 (m, 2H, ring N–CH_2), 2.82–2.89 (m, 2H, ring N–CH_2), 3.00–3.03 (m, 4H, ring N–CH_2), 3.66–3.73 (m, ring OCH_2, CH, CO–NH–CH_2), 3.90 (d, CO–NH–CH_2 J = 17.4 Hz), 8.32 (CO–NH–CH_2), and MALDI–TOF–MS data: $[M + H^+]_{calc.}$ = 392.24, $[M + H^+]_{meas.}$ = 392.32; $[M + Na^+]_{calc.}$ = 414.22, $[M + Na^+]_{meas.}$ = 414.30; $[M + K^+]_{calc.}$ = 430.20, $[M + K^+]_{meas.}$ = 428.29.

2.3. Electrolytes

A 50 mM phosphate buffer, pH 2.27 was prepared by adjusting the pH of a 50 mM sodium dihydrogen phosphate (Merck) solution with 50 mM orthophosphoric acid (Fluka). A 10 mM Tris-citrate buffer was prepared by dissolving Tris (LKB-Produkter, Bromma, Sweden) in MQ-water and adjusting to the desired pH with citric acid (Merck) before completing the volume. The chiral selector-containing electrolyte solutions were prepared by dissolving the required amount of cyclodextrin (30 mM of α-CD, Me-β-CD, DM-β-CD1, DM-β-CD2, TM-β-CD and HP-β-CD; 15 mM of β-CD and γ-CD) with or without a crown-ether derivative (30 mM) in the buffer solution filtering through a 0.45 μm nylon syringe filter (Chromafil) and degassing in an ultrasonic bath. A volume of 1.3 ml buffer solution was used for three injections.

2.4. Sample preparation

Sample stock solutions were prepared by dissolving the racemic compounds in Milli-Q water in a concentration of 1 mg/ml with a small excess of pure D-enantiomers added when available and were stored in refrigerator. The stock solutions were diluted five times with Milli-Q water

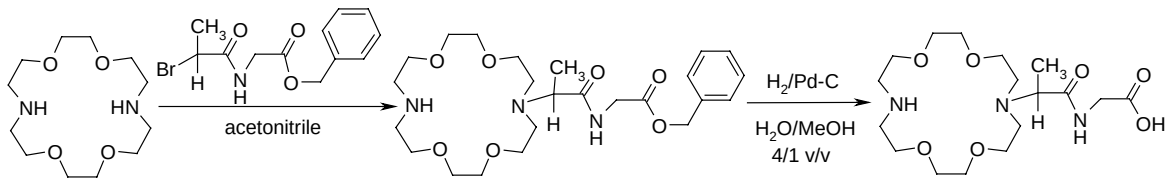
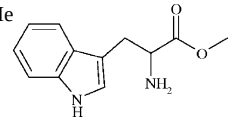
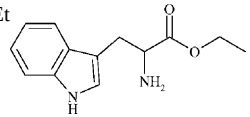
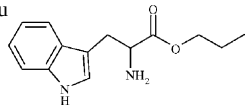
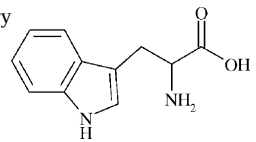
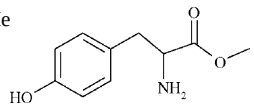


Fig. 2. Synthesis of macrocyclic derivatives *R*-**1** and *RS*-**1**.

Table 1
Chiral separation data of chiral amino acid derivatives

Amino acid derivative	Cyclodextrin	CD alone		CD + 18C6		CD + Kryptofix 22		CD + *R*-**1**		CD + *RS*-**1**	
		t_1/t_2 (min)	R_s	t_1/t_2 (min)	R_s	t_1/t_2 (min)	R_s	t_1/t_2 (min)	R_s	t_1/t_2 (min)	R_s
TryMe 	α-CD	11.58 (14.0/14.1)	0 (0.6)	17.62 (17.6/17.7)	0 (0.6)	40.91	0	28.98/29.23	0.60	37.31/37.79	0.76
	β-CD	12.85/12.94 (15.0/15.2)	0.58 (0.9)	15.57/15.71 (17.6/17.8)	0.58 (0.9)	25.91/26.19	0.73	32.17/32.77	1.07	44.93/46.18	1.32
	γ-CD	12.75 (14.2/14.3)	0 (0.7)	17.02 (18.0)	0 (0)	17.91/17.99	0.40	25.41/25.69	0.71	25.23/25.52	0.79
	Me-β-CD	16.14/16.37	0.59	16.37	0	59.23/60.23	1.04	37.26/37.94	1.03	39.25/40.09	1.01
	DM-β-CD1	11.45/12.08 (21.5/21.8)	0.59 (0.9)	46.71 (26.9)	0 (0)	n.p.	–	37.99/39.13	1.11	40.98/42.58	1.79
	DM-β-CD2	17.95 (21.5/21.8)	0 (0.9)	17.47 (26.9)	0 (0)	53.10/54.02	0.86	35.19/35.50	0.56	38.10/38.52	0.68
	TM-β-CD	14.96	0	20.02	0	40.65/41.08	0.67	37.13/37.34	0.47	30.72/30.95	0.63
	HP-β-CD	15.33/15.47 (17.5/17.8)	0.58 (1.4)	18.69/18.89 (20.2/20.5)	0.62 (1.4)	30.77	0	43.29/44.59	1.55	42.97/44.32	1.50
TryEt 	α-CD	13.19	0	17.99	0	45.35	0	39.78	0	56.85	0
	β-CD	13.67/13.92 (17.4/17.8)	0.98 (1.5)	17.24/17.55 (19.7/20.1)	0.82 (1.5)	23.23/23.55	1.14	42.34/44.26	2.31	59.97/63.88	2.76
	γ-CD	13.71/13.79 (15.4/15.6)	0.48 (0.9)	18.55/18.83 (20.7/21.1)	0.90 (1.5)	19.31/19.52	0.95	29.96/30.45	1.04	56.46/54.56	1.18
	Me-β-CD	19.86/20.27	1.19	18.47/18.76	0.74	55.15/55.76	0.57	50.80/53.86	2.91	56.72/60.75	2.95
	DM-β-CD1	25.55/26.76 (24.4/25.5)	1.72 (1.5)	42.02/42.64 (30.4/31.0)	0.71 (1.4)	n.p.	–	49.05/51.94	2.78	51.10/54.11	2.47
	DM-β-CD2	19.52/19.73 (24.4/25.5)	0.68 (1.5)	20.75/20.86 (30.4/31.0)	0.41 (1.4)	61.97/63.42	1.21	44.90/46.15	1.47	54.86/56.03	1.35
	TM-β-CD	16.28/16.39 (16.7/16.9)	0.6 (0.8)	22.58/22.97 (18.4/18.6)	0.84 (0.9)	44.01	0	29.79/30.29	1.10	33.59/34.22	1.22
	HP-β-CD	17.00/17.38 (19.6/20.2)	1.31 (1.5)	20.96/21.29 (22.8/23.4)	0.99 (1.5)	41.81/43.04	1.81	52.87/56.86	3.55	57.57/62.55	3.66
TryBu 	α-CD	16.91	0	25.71	0	33.35/34.06	1.43	n.p.	–	n.p.	–
	β-CD	16.22	0	24.78	0	32.42/32.78	0.72	n.p.	–	n.p.	–
	γ-CD	17.38/17.50 (21.0/21.2)	0.42 (0.7)	25.09/25.68 (28.3/28.9)	0.98 (1.5)	38.13/38.67	0.87	57.54/59.12	1.12	n.p.	–
	Me-β-CD	27.30/28.04	1.17	22.98/23.41	0.82	75.02	0	n.p.	–	126.88/140.64	3.19
	DM-β-CD1	33.28/33.89 (30.1/30.6)	0.70 (1.3)	60.36 (37.4/37.9)	0 (1.0)	n.p.	–	62.79/64.26	0.99	63.15/64.58	0.86
	DM-β-CD2	26.23/27.03 (30.1/30.6)	1.35 (1.3)	26.34/27.01 (37.4/37.9)	1.08 (1.0)	58.33	0	88.42/97.37	3.41	92.95/102.48	3.62
	TM-β-CD	19.85/20.29 (16.0)	1.02 (0)	30.51 (23.5/23.7)	0 (0.7)	68.81/70.82	1.57	40.80/42.21	1.96	47.61/49.49	2.14
	HP-β-CD	23.16/23.91 (26.2/26.6)	1.39 (1.0)	29.05/30.03 (32.1/32.8)	1.46 (1.3)	57.93/60.64	2.99	n.p.	–	n.p.	–
Try 	α-CD	16.36/16.70 (19.6/19.9)	1.33 (1.8)	24.01/24.69 (25.2/25.8)	1.56 (2.0)	n.p.	–	n.p.	–	n.p.	–
	β-CD	18.43	0	23.30	0	n.p.	–	n.p.	–	n.p.	–
	γ-CD	18.13	0	20.09	0	n.p.	–	73.98	0	n.p.	–
	Me-β-CD	25.65	0	23.14	0	n.p.	–	93.90	–	155.55/159.41	1.03
	DM-β-CD1	22.73/22.90	0.56	42.19	0	n.p.	–	48.38/49.49	1.36	65.31/67.65	2.01
	DM-β-CD2	24.74	0	25.80	0	n.p.	–	80.61	0	n.p.	–
	TM-β-CD	23.43/23.59	0.55	n.p.	–	n.p.	–	62.17	0	n.p.	–
	HP-β-CD	22.30	0	27.55	0	n.p.	–	n.p.	–	n.p.	–
TyrMe 	α-CD	11.41	0	14.75	0	22.37	0	27.70	0	36.73	0
	β-CD	11.92 (15.5/15.6)	0 (0.6)	17.32 (19.3/19.4)	0 (0.7)	29.29/29.70	1.00	35.01/35.51	0.79	46.85/47.63	0.72
	γ-CD	12.49	0	18.81	0	19.19	0	25.48	0	45.92	0
	Me-β-CD	16.11/16.32	0.57	19.28/19.75	1.08	73.51	0	34.63/35.32	1.17	36.09/36.90	1.17
	DM-β-CD1	16.97 (19.7)	0 (0)	37.26/38.02 (28.1/28.3)	0.59 (0.7)	n.p.	-	30.31/30.76	0.92	31.60/32.11	0.81
	DM-β-CD2	14.89/15.02 (19.7)	0.56 (0)	19.64/20.19 (28.1/28.3)	1.22 (0.7)	73.34	0	30.50/31.06	1.10	31.54/32.10	1.06
	TM-β-CD	14.18 (15.1)	0 (0)	18.70/18.86 (16.4/16.5)	0.54 (0.6)	44.82	0	23.36	0	27.09	0
	HP-β-CD	15.46 (16.9)	0 (0)	19.88 (21.0/21.1)	0 (0.5)	53.08	0	36.52	0	39.79	0

Effect of the diaza-crown ether and its derivatives as chiral selector additives on the enantiomer separation. Data between brackets obtained by Armstrong et al. [23]; abbreviations and experimental conditions are explained in the text. n.p.: no peak was observed within 100 min.

(~200 μg/ml), filtered through a 0.45 μm nylon syringe filter and degassed in an ultrasonic bath. The diluted solutions were stored in a refrigerator and could be used for 2 weeks.

3. Results and discussion

In this paper, we report the test results of the applicability of two newly synthesized diaza-crown ether derivatives *R*-**1** and *RS*-**1** (Fig. 1) in the chiral separation of five primary amino group containing amino acid derivatives (Table 1), which are commonly used as building blocks in the synthesis of new chiral drugs. The experiments were carried out under acidic conditions, at which the primary amines are positively charged and the analyte–selector interaction might occur. Under acidic conditions, the diaza-crown ether derivatives are also positively charged—the protonation constants for *R*-**1**, *RS*-**1** were found to be $\log K_1$ 9.51, $\log K_2$ 5.52 and $\log K_3$ 2.74—thus both the analyte and the selector migrate towards the cathode. The separation of the amino-acid enantiomers was evaluated using eight different cyclodextrins. These cyclodextrins were tested alone as chiral selectors and in dual systems combined with 18C6, Kryptofix 22, *R*-**1** and *RS*-**1** (Fig. 1), respectively. The experimental conditions applied by Kuhn et al. [10] and Armstrong et al. [23] were maintained throughout the study to allow comparison with the literature values. Thus, the effect of *R*-**1** and *RS*-**1** when replacing the 18C6 was evaluated.

The separation results are summarized in Table 1, in which the resolution values were calculated by the equation $R_s = 2(t_2 - t_1)/(w_2 + w_1)$, with t_1, t_2 the migration times and w_1, w_2 the peak widths at baseline of the first and second enantiomer, respectively. The Parabolic–Lorentzian variance-modified Gaussian (PLMG) deconvolution model [30] was applied for highly skewed overlapped peaks in order to determine the individual migration times and peak widths. Mathematical and computational treatment of several chromatographic peak-fitting procedures using Microsoft Excel has been published by Nikitas and co-workers [31] and was used as a guideline for performing calculations with the PLMG model. The R_s values were then calculated with the parameters of the peaks obtained with the deconvolution model.

The electroosmotic flow was found very low at the pH applied, it could not be measured in any applied selector system.

3.1. Single selector system

3.1.1. Diaza-crown ether R-*1*

In the first part of this study individual applicability of compounds *R*-**1**, *RS*-**1** was considered. In lack of any enantiomer excess, the racemic compound *RS*-**1** is obviously not suitable for enantiomer recognition in se, thus only the compound *R*-**1** was individually tested. Experimental conditions used were taken from the paper of Kuhn et al. [10], in which the individual effect of the chiral $18C6H_4$ for chiral recognition of tryptophan enantiomers was examined. In our work $18C6H_4$ was replaced with compound *R*-**1**. The influences of the buffer composition, the pH (between 2.5 and 6.0) and the concentration of the chiral selector were studied. Tris-containing citrate buffer and phosphate buffer were tested and the concentration of *R*-**1** was varied between 5 and 100 mM. We observed that the migration times of the analytes increased with the concentration of compound *R*-**1**, which could be the consequence of either the increasing viscosity of the electrolyte system or more likely the increasing size of the analyte–selector association complex plus the increasing ratio of the analyte–selector complex in the equilibrium system. Enantiomer separation, however, could not be found for any analyte, indicating that the structure of the diaza-crown ring plus amino acid molecule is too flexible to form stable diastereomers with significantly different physical properties for chiral recognition.

3.1.2. Results with cyclodextrins

The five amino acid derivatives were analyzed using several cyclodextrins. Eight cyclodextrins were tested. Six of them (α-CD, β-CD, γ-CD, DM-β-CD1, TM-β-CD, HP-β-CD) were also used by Armstrong et al. [23], further Me-β-CD and DM-β-CD2 were added. The DM-β-CDs were purchased from different suppliers, which might make their separation properties different. The DM-β-CD2 (Beckman) was of pure CE grade, while DM-β-CD1 (Sigma) contained only 58% of pure dimethyl-β-CD and 42% of over-methylated homologues, primarily hexakis(2,6-di-*O*-methyl) and (2,3,6-tri-*O*-methyl)-β-cyclodextrin. This explains why the results obtained with the two DM-β-CDs are significantly different (see Table 1). Although the magnitude of the obtained migration times was similar to the results obtained by Armstrong et al. [23], much lower R_s values were observed and in the majority of runs, separations were considered non-acceptable. In a few cases however, depending on the type and molecular structure of the applied selector and analyte pairs, baseline separations were achieved. Nevertheless, none of the cyclodextrins could be considered as a superior selector for all five chiral amino-acid derivatives studied.

3.2. Dual selector systems

Since neither diaza-crown ether *R*-**1** nor cyclodextrins provided sufficient chiral separation individually, their interactions with tryptophan- and tyrosine esters were tested in dual selector systems containing compound *R*-**1** and a cyclodextrin simultaneously. Initial experimental conditions were taken from Armstrong et al. [23], who studied the influence of the non-chiral 18-crown-6 on six different cyclodextrins for the separation of over fifty racemic compounds. In our work, five amino acid derivatives and the six cyclodextrins used by Armstrong et al., plus two others (Me-β-CD and DM-β-CD2), were cross-tested. 18-Crown-6, Kryptofix 22, *R*-**1** and *RS*-**1** were used as a co-selector in dual systems with

the cyclodextrins. In this experimental arrangement, effects of cyclodextrin ring size, number and nature of substituents on the cyclodextrins, nature of the donor atoms in the macrocycle ring and presence or absence of the chiral pendant arms attached to the macrocycle ring could be compared.

3.2.1. Effect of adding 18-crown-6

The effect of 18C6 on the different cyclodextrin systems was investigated by adding 18C6 to the different cyclodextrin containing electrolytes. For the studied tryptophan derivatives, 18C6 had mainly no or a negative effect on the separation. In a small number of cases synergistic effect was observed when 18C6 was used with γ-CD or TM-β-CD for TryEt, with γ-CD or HP-β-CD for TryBu and with α-CD for tryptophan. Tyrosine methyl ester enantiomers were for some cyclodextrins better separated with 18C6 than without (Table 1).

In general, in these dual selector systems the migration times of the analytes were moderately increased compared to the single cyclodextrin selector system. This can be explained by either the increased size of the proposed three-body association complexes formed between the CD + analyte + 18C6 relative to the two-body association complexes between the CD + analyte, or the increased electrolyte viscosity caused by adding the crown-ether additive.

3.2.2. Effect of adding tetraoxadiaza-crown ether (Kryptofix 22)

The effect of Kryptofix 22, the parent ring of compounds *R*-**1** and *RS*-**1**, on the enantiomer separation was investigated by replacing 18C6 with that diaza-crown ring in the experimental setup described above. The general observation was that migration times of the analytes increased significantly compared to the effect of the 18C6 additive. It was assumed that three-body complexes consisting of the analyte + CD + Kryptofix 22 had been formed with relatively stronger intermolecular interactions than those in the analyte + CD + 18C6 association. It has also to be taken into consideration, that Kryptofix 22, in contrast with 18C6, is protonated under the applied pH and capable of forming extra hydrogen bonds. The effect of Kryptofix 22 on the R_s values depends on the molecular structure of the samples and cyclodextrins applied. For tryptophan esters Kryptofix 22 had generally a positive effect with cyclodextrins, but there were cases when it had no or negative effect compared to 18C6.

The tryptophan peak did not appear within a reasonable period of time (100 min) with any cyclodextrin–Kryptofix 22 selector system. For TyrMe ester the diaza-crown ether had a nearly general negative effect on the separation with only one exception: β-cyclodextrin's separation power was enhanced.

*3.2.3. Effect of adding R-**1** and RS-**1***

This replacement allowed us to evaluate the influence of the pendant arm of macrocycles *R*-**1** and *RS*-**1** relative to their simple unsubtituted ring. The alanyl–glycine pendant arm containing macrocycles, *R*-**1** and *RS*-**1**, induced a significant increase in the enantiomer resolution and made the analytes baseline separated in several cases. In the inclusion complexes an additional hydrogen bonding interaction may occur between the diaza-crown-ether derivatives' carboxylic group and the hydrophilic part (e.g. hydroxyl, hydroxypropyl groups) of the cyclodextrin inducing a remarkably increased host-guest interaction between the analyte + diaza-crown-ether derivative + CD, thus enhancing the chiral resolution.

For TryMe ester the *R*-**1**/*RS*-**1** induced or enhanced separation, compared to cyclodextrins alone or to CD +18C6 or CD + Kryptofix 22 selector systems (Table 1). The only exception was DM-β-CD2 + *R*-**1**/*RS*-**1** systems, which showed worse separation as compared to DM-β-CD2 + Kryptofix 22 selector system.

Significantly higher resolution values were obtained for TryEt in almost every dual selector system compared to the separations for TryMe. For example, using HP-β-CD + *R*-**1**/*RS*-**1** dual selector system the R_s for TryMe were 1.55/1.50, while for TryEt 3.55/3.66 was obtained (Table 1, Fig. 3). As only exception, the α-CD + *R*-**1**/*RS*-**1** system did not show any ability for chiral separation, probably partly due to the α-cyclodextrin's small cavity size for complexation.

TryBu ester and Try did not always elute within a reasonable period of time. In some cases the experiments were run for 3 h without detecting any peaks. In other cases, migration times longer than 120 min were recorded.

Nevertheless, the results found within a practically applicable time frame obviously prove the positive synergistic effect of *R*-**1**/*RS*-**1** on the cyclodextrins as compared to any other selector system tested (Table 1). In order to find an explanation for the very long migration times, semi-empirical quantum chemical calculations were performed for the structure and electric charge of the three-body associate. It has been taken into consideration that tryptophan was in approximately 50% deprotonated state on its carboxylic group under the experimental conditions (pH 2.27, pK_1 = 2.35) and the macrocyclic nitrogens are also fully protonated (see later in detail). As it has been pointed out by AM1//AM1 semi-empirical calculations, in the trimolecular complex the protonated tryptophan lost its last proton, leading to a one-positively charged three-body species. As electrophoretic mobility is linearly proportional with the electric charge of the analyte, that low specific charge resulted in very long migration times. A question emerged whether long migration times were a general feature of the dual selector system with free carboxylic group-containing analytes, or would it be possible to decrease migration times significantly. In theory, decreasing the pH would gradually increase the overall electric charge of the complex and thus decrease the migration time. However, as the pH can not be reduced unlimitedly and at the actual stage of development the number of experimental data is very limited, it is

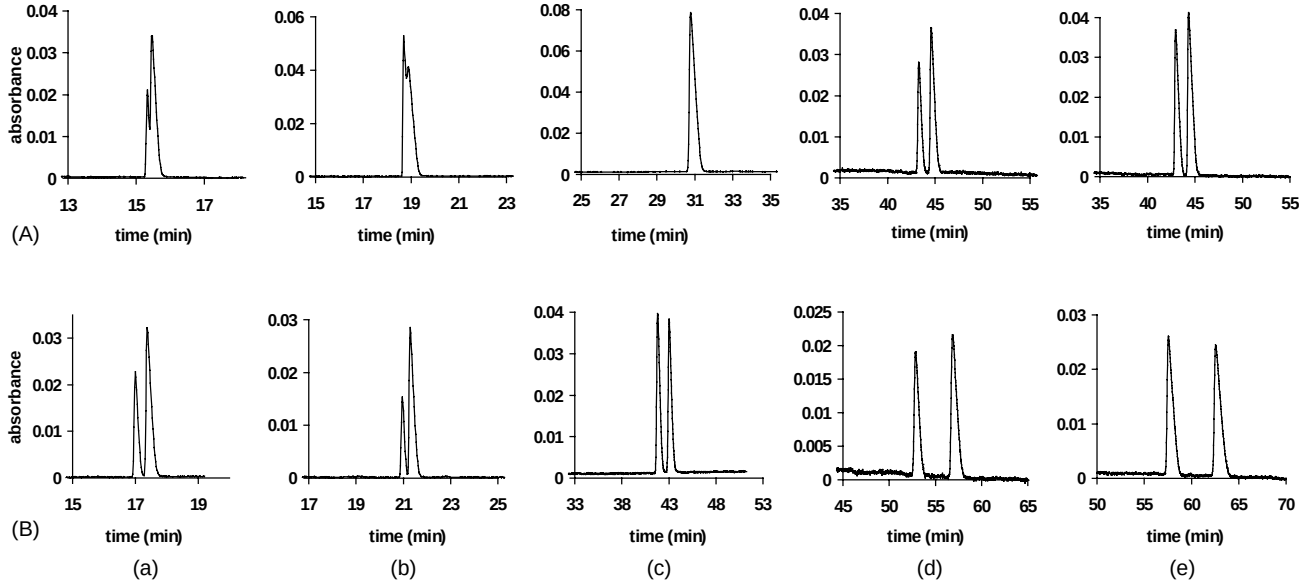


Fig. 3. Electropherograms of (A) tryptophan methyl ester and (B) tryptophan ethyl ester applying (a) HP-β-CD, (b) HP-β-CD + 18C6, (c) HP-β-CD + Kryptofix 22, (d) HP-β-CD + *R*-**1** and (e) HP-β-CD + *RS*-**1** selector systems.

not possible to estimate the effect of the pH on dual selector systems for any other free carboxylic group-containing analytes.

The worst effect of *R*-**1**/*RS*-**1** on the enantioseparation was found for TyrMe ester. There are only four cyclodextrins from the eight applied which induced or enhanced enantioseparation when involved in those dual systems, compared to the selector system with cyclodextrin alone (Table 1). In case of DM-β-CD2 the resolution was even slightly better when 18C6 additive was used.

We also observed that in general racemic *RS*-**1** caused slower migration and a comparable or slightly better separation than the pure enantiomer *R*-**1**. Presumably, the *S*-enantiomer provided a somewhat more favourable interaction with the analytes than the *R*-enantiomer, thus using the racemic derivative stronger averaged interaction might occur. However, to predict which one of the interactions influences the complex formation most, thorough molecular modeling studies would be needed. Several molecular properties like the size of cyclodextrin ring, the nature and property of its substituents, the structures of the analytes and of the *R*-**1**, *RS*-**1** derivatives, the effect of the anions/cations present in the solution as well as the critical interactions with the water molecules should simultaneously be taken into consideration, as they all play important roles in the host-guest complex formation.

Coordination of *R*-**1** to the tryptophan ester + CD complex takes place mainly through formation of hydrogen bonds between the analyte, the hydroxyl groups of the CD and the pendant arm, as has been pointed out by quantum chemical calculations. Presence of the negatively charged dihydrogenphosphate anions in the outer sphere is also very important and promotes association of the positively charged species leading to a virtual plus three-charge within the complex. However, due to strong electric repulsion between the positively charged groups that are present within the complex in a limited space a hydrogen ion from the protonated amino group of the tryptophane esters is forced to migrate to the neighbouring dihydrogenphosphate ion resulting in a twice-positively charged three-body complex cation by the end of the association process, as calculated by the AM1//AM1 quantum chemical method. When tryptophan was used as the analyte an additional deprotonation of the carboxylic group already took place leading to a plus one-charged complex cation. Since the electrophoretic mobility is linearly proportional with the electric charge of the species, the peak of the tryptophan complex does not appear within 100 min while tryptophan methyl and ethyl esters can be observed within 40–65 min. Due to the time- and hardware-consuming nature of semiempirical calculations, several factors could have not been taken into consideration, like the role of solvent water molecules, the presence of other cations and anions, and the number and type of substituents of the cyclodextrin rings. As it can be seen in Table 1, migration times depend on the nature, charge, steric properties, etc. of the substituents, therefore a deeper understanding of the interactions between the analyte and the selectors would require extended calculations.

Both mono-substituted diaza-crown ether derivatives (*R*-**1**, *RS*-**1**) had a significant positive effect on the enantioseparation along with many cyclodextrins. However, the migration times might still be considered as quite high. Therefore, optimization of the experimental conditions for each individual dual selector systems would be required in order to reduce analysis times.

4. Conclusion

Two diaza-crown ether derivatives (*R*-**1**, *RS*-**1**) were synthesized for the first time and tested for their analytical potential as chiral selectors in the separation of five amino acid derivatives in capillary electrophoresis. Neither of them produced enantiomer separation individually, thus they were applied as electrolyte modifiers in eight different cyclodextrins-containing dual selector systems. Depending on the molecular structure of the analyte and on the applied cyclodextrin, *R*-**1** and *RS*-**1** derivatives induced or enhanced enantiomer separation compared to the cyclodextrin alone, to CD + 18C6 or to CD + Kryptofix 22 dual selector systems. The most significant effect was achieved on the resolution of tryptophan ethyl ester, while a deleterious effect of adding the diaza-crown-ether derivatives on the separation was found only in a few cases.

Our results have confirmed the beneficial use of diaza-crown ether type molecules in cyclodextrin-containing dual selector system for enantiomer separation of primary amino group containing amino acid derivatives. In order to ascertain the chemical and structural background of the mechanism of the enantiomer separation process and to identify essential factors that influence the resolution, further examinations are required to test new types of analytes and new derivatives of diaza-crown ether type additives. Systematic studies on finding shorter migration times and optimized experimental conditions are under development.

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