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Journal of Chromatography A, 716 (1995) 371–379

JOURNAL OF CHROMATOGRAPHY A

Evaluation of an optically active crown ether for the chiral separation of di- and tripeptides

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Abstract

The direct optical resolution of a number of di- and tripeptides was achieved by capillary zone electrophoresis using an enantioselective crown ether as buffer additive. The protonated primary amines form inclusion complexes with the crown ether. Chiral resolution is based on different stability constants of the diastereomeric complexes thereby changing the electrophoretic mobilities of the enantiomers. Enantioselectivity is strongly affected by the distance between the amine functionality and the chiral carbon atom. This effect was studied using di- and tripeptides especially synthesized for this purpose. In general, baseline resolution was obtained for those peptides with the amine group located as far as four bonds from the stereogenic center. Additionally, tripeptides possessing two chiral centers were separated to investigate the potential of the chiral selector for the analysis of complex analytes with related structures. Experimental factors such as crown ether concentration, buffer pH and temperature also show a strong influence on the resolution. These factors can be successfully employed for method optimization.

1. Introduction

Due to the growing significance of peptide-based drugs, liquid- and solid-phase syntheses of biologically active peptides have become an important challenge in the pharmaceutical industry. Today, analytical characterization of these peptides comprises not only determination of the proportion of by-products from synthesis but also the enantiomeric purity of the drug. In routine analysis chromatographic techniques are established for this purpose since chiral stationary phases became commercially available. The resolution of dipeptides was described by Oi et al. [1] using gas chromatography. Lindner et al. [2] employed HPLC to separate peptides after derivatization and Pirkle and co-workers [3,4] resolved enantiomeric di- and tripeptides using Pirkle-type chiral stationary phases. Stationary phases based on optically active crown ethers were used by Cram and coworkers [5,6], Hilton and Armstrong [7] and Esquivel et al. [8]. Even though a huge number of stationary phases in liquid and gas chromatography are at hand, which virtually cover most separation problems, the results often show poor efficiency and method optimization may be time-consuming.

In 1985 Gassmann et al. [9] were the first to

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0021-9673/95/$09.50
SSDI 0021-9673(95)00371-1

describe the chiral resolution of enantiomers by capillary zone electrophoresis (CZE). The chiral selector was simply added to the buffer system. Enantio-separation was accomplished by the formation of diastereomeric complexes of the selector with the analytes leading to a change in electrophoretic mobilities. Subsequently, this basic principle has been extended by using other selectors. Today, enantio-separation in CZE is accomplished by utilizing ligand–exchange complexation [10], solubilization with optically active micelles [11,12], proteins [13] and host–guest complexation using cyclodextrins [14–17].

In previous papers we described the use of a chiral crown ether in CZE for the first time [18–21]. The focus of the present study is to evaluate the potential of 18-crown-6 tetracarboxylic acid ($18C6H_4$) for the enantio-separation of di- and tripeptides in CZE. The effect of the location of the interactive amine functionality in relation to the chiral center is discussed.

2. Experimental

2.1. Instrumentation

Experiments were carried out using an HP ^{3D}CE instrument (Hewlett-Packard, Waldbronn, Germany). Separations were performed in untreated open-tube fused-silica capillaries (58 cm × 75 μm I.D.) applying a potential of 15 kV. If not otherwise stated the capillary temperature was maintained constant at 25°C. A 10 m*M*

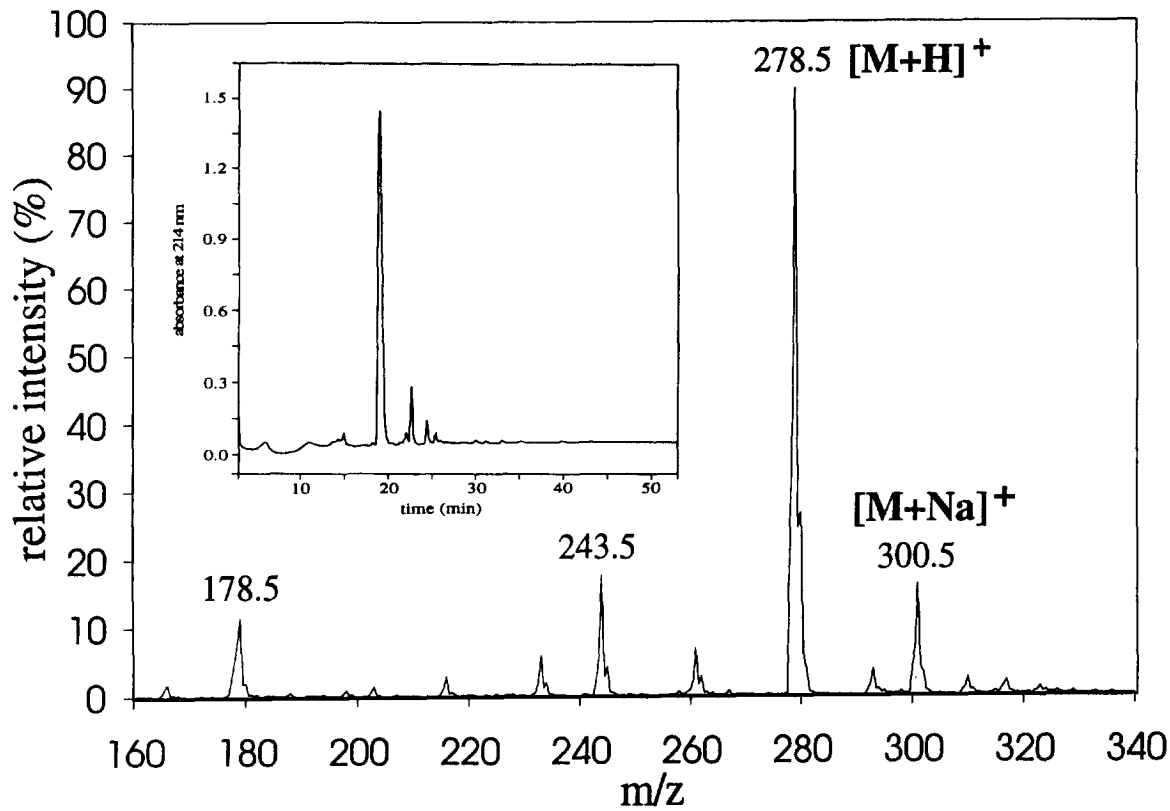


Fig. 1. HPLC profile and electrospray mass spectrum (main peak) of crude peptide D-Phe–Aca after cleavage from the resin. HPLC column: Nucleosil C_{18} (200 × 2 mm, 5 μm, Grom Herrenberg, Germany); gradient 0–100% (within 45 min) acetonitrile–water (containing 0.1% trifluoroacetic acid). The flow-rate was 0.3 ml/min. ES–MS: a triple quadrupole API III mass spectrometer equipped with a pneumatically assisted electrospray ion source (Sciex, Thornhill, Canada) was used for recording spectra in the positive mode. Solutions of 1 mg peptides in 1 ml *tert.*-butyl alcohol–water (4:1, v/v) were injected into the ion source at a flow-rate of 5 μl/min using an autosampler [23].

Tris–citrate buffer (pH 2.5) with 10 m*M* $18C6H_4$ in double-distilled water was used, unless stated otherwise. An appropriate amount of each sample (usually 0.05%, w/v) was dissolved in methanol–water (50:50, v/v) and injected by pressure for 2 s at 50 mbar and detected by UV absorbance at 210 nm. Whenever possible the elution order of the enantiomers was determined by spiking the mixture with the pure enantiomers.

2.2. Chemicals

All reagents were of analytical grade if not otherwise stated. Tris, citric acid, 18-crown-6 tetracarboxylic acid ($18C6H_4$, purum grade) and sodium hydroxide were from Merck (Darmstadt, Germany). Gly–DL-Trp and Gly–DL-Phe were purchased from Sigma (St. Louis, MO, USA). Gly–DL-Leu–DL-Ala was from Serva (Heidelberg, Germany). Acetic acid, dimethylformamide (DMF), dichloromethane, diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt), methanol and *tert.*-butyl alcohol were from Fluka (Buchs, Switzerland). All other di- and tripeptides were synthesized by solid-phase peptide synthesis according to the following procedure. Their structures were confirmed by HPLC and mass spectrometry. No further purification of the synthesis products was performed.

2.3. Peptide synthesis

The peptides were synthesized on a robot system (Tecan, Switzerland) equipped with software for multiple peptide synthesis (MultiSyn Tech, Bochum, Germany). Fmoc-L-amino acid-2-chlorotrityl resin [22] (30 mg, 0.6 μmol/mg) was distributed in separate small filter tubes. Fmoc deprotection was effected with 0.2 ml 50% piperidine in DMF for 8 min and repeated once. Couplings were carried out using 10-fold excess of Fmoc-amino acids and HOBt–DIC (1:1, v/v) activation in DMF within 1 h. After coupling and deprotection the tubes were washed with DMF (0.3 ml) three and seven times, respectively.

Removal of the peptides from the resin was achieved with acetic acid–dichloromethane–methanol (1:3:1, v/v, 2 ml) within 2 h. The filtrate was evaporated and the residue was dissolved in *tert.*-butyl alcohol–water (4:1, v/v) and lyophilized. The identity and purity of the free peptides was determined by HPLC and electrospray mass spectrometry (Fig. 1).

3. Results and discussion

Crown ethers are macrocyclic polyether ring systems consisting of a number of oxygens joined by ethylene bridges. Crown ethers of the 18-crown-6 type contain a cavity which is able to form inclusion complexes (host–guest complexes) with potassium, ammonium and protonated primary amines. Although complexation of the primary amine is indispensable for chiral separation, it is insufficient for the discrimination of the enantiomers. This can be obtained by additional interactions of the four substituents of $18C6H_4$ with the ligand (Fig. 2).

If $18C6H_4$ is simply added to the buffer electrolyte, diastereomeric complexes of the crown ether with the enantiomers are formed which result in a change of their electrophoretic mobilities. In a previous study [19] we proposed two recognition mechanisms. First, the carboxylic acid pairs on both sides of the ring system behave like chiral obstacles dividing the space available for the substituents of the asymmetric center into two cavities. The position and size of these substituents affect the binding energies of the host–guest complexes. The second mechanism is directly related to the carboxylic acids which may show electrostatic interactions with polar substituents of the enantiomers.

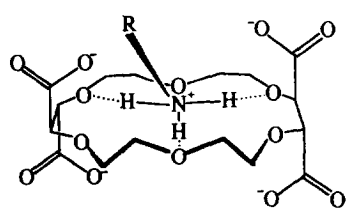
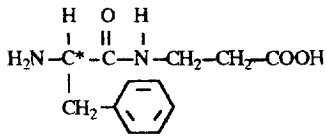
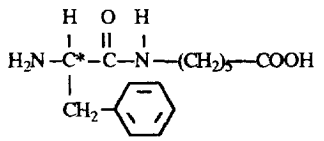
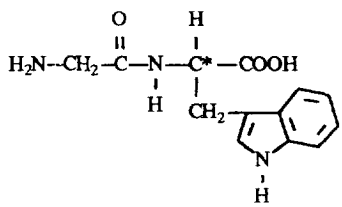
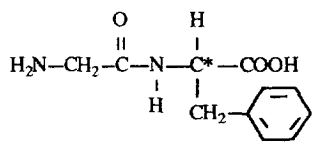
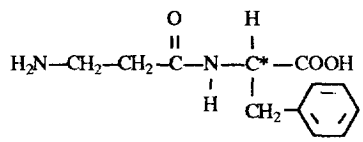
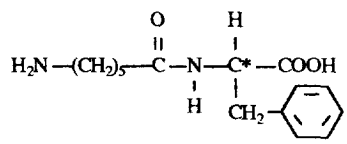


Fig. 2. Chemical structure of a host–guest complex of $18C6H_4$ with a protonated primary amine.

Table 1
Analytical data of the chiral resolution of dipeptides using 5 m*M* $18C6H_4$

Compound	Structure	t_1 (min)	α	R_s
DL-Phe–β-Ala		10.77	1.378	7.20
DL-Phe–Aca[a]		12.98	1.383	9.49
Gly–DL-Trp[b]		13.20	1.084	3.09
Gly–DL-Phe[b]		18.64	1.141	1.45
β-Ala–DL-Phe		14.10	1.000	–
Aca[a]–DL-Phe		13.90	1.000	–

[a] Aca: 6-aminocaproic acid.
[b] Separation with 10 m*M* $18C6H_4$ at 40°C.
α = separation factor, R_s = resolution.

In order to obtain enantiomeric separations with selectors of the "18-crown-6"-type a primary amine functionality is essential. Neither secondary nor tertiary nor other functional groups provide inclusion complexation required for chiral separation. As already demonstrated [20,21] the requisite interactions are dependent on charge and size. In addition, the distance of the interactive amine and the stereogenic center also influences the chiral recognition. In Table 1 the analytical data of 6 racemic dipeptides analyzed using 18C6H$_4$ are summarized. The dipeptides vary in their distances of the primary amine and the stereogenic center. While in DL-Phe–β-Ala and DL-Phe–Aca the chiral carbon is adjacent to the amine, four bonds separate the chiral carbon from the amine in the cases of Gly–DL-Trp and Gly–DL-Phe. Finally, β-Ala–DL-Phe and Aca–DL-Phe have their asymmetric center 5 and 8 bonds from the amine, respectively. The separation factor α is calculated by

$$\alpha = \frac{t_2}{t_1} \quad (1)$$

where t_1 is the migration time of the first eluting enantiomer and t_2 the migration time of the antipode. The resolution is calculated by

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2} \quad (2)$$

where w_1 and w_2 are the peak widths of both enantiomers. As one can readily see from Table 1 the separation factor and the resolution were the highest when the chiral center was adjacent to the amine function. Thus, both dipeptides DL-Phe–β-Ala and DL-Phe–Aca were excellently resolved using standard experimental conditions. The separation is shown in Fig. 3.

Hilton and Armstrong [7] investigated the "distance effect" on enantio-selectivity of dipeptides using a chiral crown ether in liquid chromatography. They found that dipeptides of the glycyl-amino acid type could not be baseline separated in HPLC. However, in capillary zone electrophoresis good separations were obtained for Gly–DL-Trp and Gly–DL-Phe (results not shown) with a resolution of 3.09 and 1.45, respectively. β-Ala–DL-Phe and Aca–DL-Phe could not be separated under these experimental conditions. It is obvious, that their stereogenic centers are located too far from the amine function and, consequently, from the crown ether substituents in the complex to show different interactions of the enantiomers.

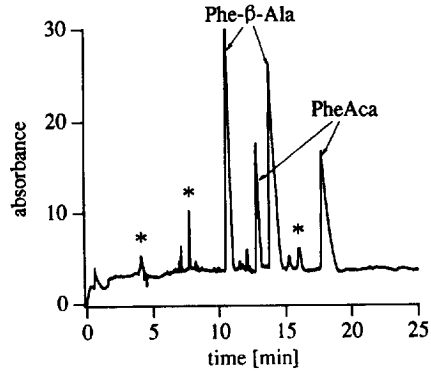
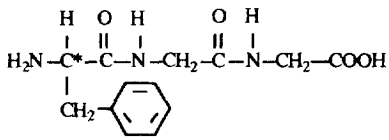
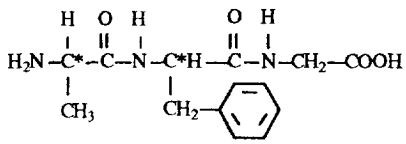
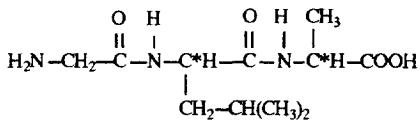
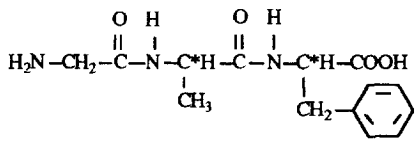
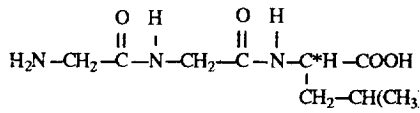


Fig. 3. Chiral separation of DL-Phe–β-Ala and DL-Phe–Aca by CZE using 5 m*M* 18C6H$_4$ as buffer additive. Peaks marked by an asterisk are impurities from synthesis.

In a second series of experiments chiral separation of tripeptides containing one or two asymmetric carbon atoms was studied. The analytical data are summarized in Table 2. Again, best separations were obtained if the stereogenic center is in close proximity to the protonated amine. For instance, DL-PheGlyGly and L-Ala–L-PheGly/D-Ala–D-PheGly showed resolutions of 4.92 and 6.55, respectively. The separation of the latter two enantiomers and one diastereomer is depicted in Fig. 4. While the diastereomer was separated from the enantiomers in an ordinary buffer system without any crown ether, chiral separation of the enantiomers succeeded in the same buffer containing 10 m*M* 18C6H$_4$. Fairly good results were also obtained for Gly–DL-Leu–DL-Ala. However, in order to resolve the four optical isomers the buffer pH had to be adjusted to pH 3.0. This minor change had some dramatic effects on the separation: (i) the complex formation of the isomers with the crown ether are strongly enhanced causing anionic migration of the peptides, (ii) migration times were approx. twice as long as those obtained at pH 2.5. Interestingly the enantiomers of GlyGly–DL-Leu were also recognized by the crown ether even

Table 2
Analytical data of the chiral resolution of tripeptides using 10 m*M* $18C6H_4$

Compound	Structure	t_1 (min)	α	R_s
DL-PheGlyGly		11.83	1.075	4.92
L-Ala–L-PheGly/ D-Ala–D-PheGly		12.43	1.158	6.55
Gly–DL-Leu–DL-Ala[a]		35.34/ 37.88	1.014/ 1.079	1.56/ 2.94
Gly–L-Ala–L-Phe/ Gly–D-Ala–D-Phe		17.69	1.054	1.01
GlyGly–DL-Leu		46.18	1.034	0.70

[a] Separation at pH 3.0 and 15 m*M* $18C6H_4$.
α = separation factor, R_s = resolution.

though resolution was poor under the experimental conditions.

3.1. *Influence of the experimental conditions*

Systematic investigations of the experimental factors revealed the method to be highly sensitive with respect to crown ether concentration, buffer pH and temperature.

The influence of the crown ether concentration on the resolution of DL-Phe-Aca and DL-Phe-β-Ala is shown in Fig. 5. According to the law of mass action an increase of the crown ether concentration enhances the complex formation and should improve the resolution. This holds for low crown ether concentrations. However, by increasing the concentration to approx. 5–10 m*M* the curves level off. Higher concentrations than 10 m*M* $18C6H_4$ did not significantly improve the resolution but led to marked longer

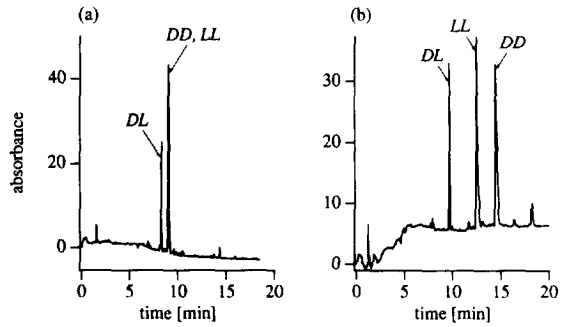


Fig. 4. Separation of the enantiomers L-Ala–L-PheGly and D-Ala–D-PheGly and the diastereomer D-Ala–L-PheGly in (a) 10 m*M* Tris–citrate (pH 2.5) and (b) 10 m*M* Tris–10 m*M* $18C6H_4$–citrate (pH 2.5).

Table 3
Influence of the buffer pH on the migration time of the first eluted isomer of Gly–DL-Leu–DL-Ala

pH	Migration time (min)	Remark
2.2	16.55	1 peak detected
2.5	16.13	3 peaks detected
3.0	35.34	complete separation
3.5, 4.0, 5.0, 6.0	>180	no peaks detected

Experimental conditions: temperature 20°C; pH 2.2 and 2.5: 15 m*M* Tris–15 m*M* $18C6H_4$ adjusted to the given pH with citric acid; pH 3.0 to 6.0: 15 m*M* $18C6H_4$ adjusted to the given pH with Tris; detection wavelength, 200 nm. Other conditions are given in the Experimental section

migration times. Similar effects of crown ether concentration on the resolution were found in earlier investigations [17,18].

The pH of the buffer system is the most powerful and most sensitive factor for method optimization. The pH effects the dissociation of the crown ether's carboxylic acids and consequently the electrophoretic mobility of the selector. In addition, the association of a ligand to $18C6H_4$ is known to depend on the dissociation degree of the crown ether because its carboxylate groups stabilize the complex stronger than the non-dissociated acids. The influence of the pH was investigated using Gly–DL-Leu–DL-Ala. Only a small pH range (pH 2.2–3.0) could be used for method optimization. Table 3 shows the influence of the pH on the migration time of the first isomer. Obviously, pH values higher than 3.0 are not applicable because no peaks could be detected within 180 min. It can be assumed that the increasing net charge of the crown ether at higher pH values and the stronger complexation of the enantiomers were responsible for this results.

Finally, the temperature also has an influence on the separation. In general, increasing the temperature causes a decrease in the separation factor and resolution. This was demonstrated in a previous study with DL-tryptophan, DL-phenylalanine and (±)-naphthylethylamine [19]. However, the dipeptide Gly–DL-Phe showed an oppo-

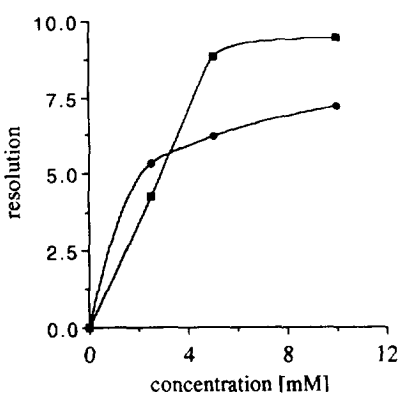


Fig. 5. Influence of the concentration of $18C6H_4$ on the resolution of DL-Phe–β-Ala (●) and DL-Phe–Aca (■). Experimental conditions are given in the Experimental section.

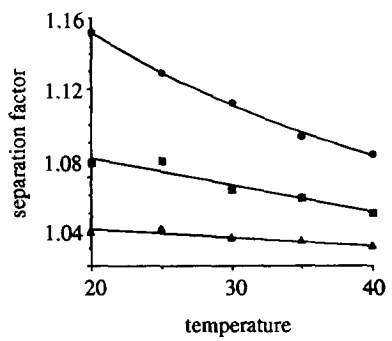


Fig. 6. Plot of the capillary temperature versus the separation factor for Gly–DL-Trp (●) and both enantiomeric pairs of Gly–DL-Leu–DL-Ala (■,▲). The buffer composition for the separation of the tripeptides was 15 m*M* $18C6H_4$ adjusted to pH 3.0 with Tris. Detection wavelength was 200 nm. Other conditions are given in the Experimental section.

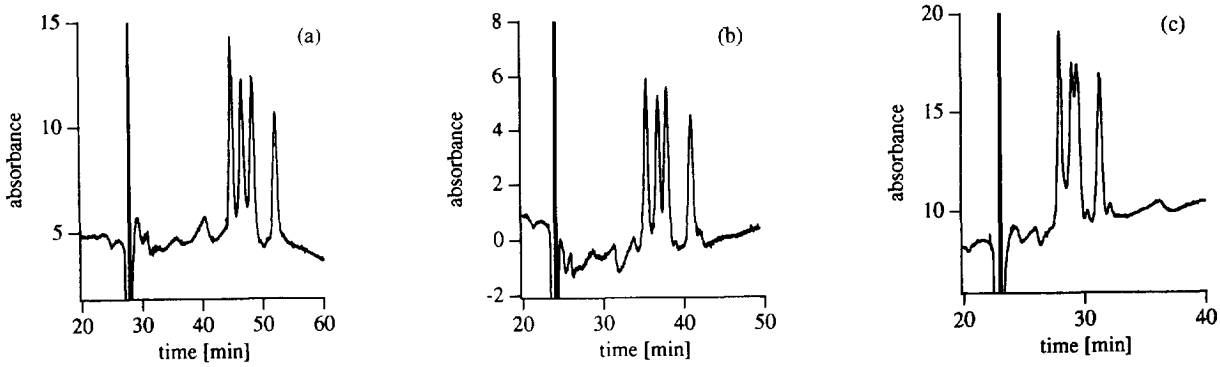


Fig. 7. Influence of the capillary temperature on the chiral separation of Gly–DL-Leu–DL-Ala: (a) 20°C, (b) 25°C, (c) 30°C. Other experimental conditions are described in Fig. 6.

site behavior in that investigation, which was explained by a different separation mechanism based on electrostatic interactions. In the present study we investigated the effects of the temperature on the separation factor of Gly–DL-Trp and Gly–DL-Leu–DL-Ala. The results are shown in Fig. 6. While the separation factor strongly decreases with increasing temperature in the case of Gly–DL-Trp, this effect is much less pronounced for both enantiomeric pairs of Gly–DL-Leu–DL-Ala. However, increasing the temperature decreased the resolution of all four components owing to the co-migration of two diastereomers (see Fig. 7a–c).

4. Concluding remarks

The present paper proves the suitability of $18C6H_4$ for enantio-separation of di- and tripeptides possessing one or two stereogenic centers. Most separation problems could be solved using a simple screening method. Nevertheless parameters such as crown ether concentration, buffer pH and temperature are effective for method optimization. However, even small changes of these parameters occasionally can have a dramatic effect on the separation power. Thus, careful adjustment of the experimental conditions is essential for obtaining reproducible results.

Acknowledgement

The authors thank Miss Beate Bachhuber for technical assistance.

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