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# Enantioselective capillary electrophoresis of amino acid derivatives on cyclodextrin Evaluation of structure–resolution relationships

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**Abstract**

In this work a strategy for enantioselective separations of α-amino acid derivatives in cyclodextrin (CD)-modified capillary zone electrophoresis (CZE) is presented and the effects of various experimental parameters such as temperature, pH, applied voltage and the use of organic modifiers (methanol, *tert.*-butyl methyl ether, carnitin) have been studied in detail. For the modification of α-amino acids, also helpful in order to improve sensitivity by UV or fluorescence detection, well known derivatives such as dinitrobenzoyl, dinitrophenyl, dimethylaminonaphthylsulfonyl, carboxybenzyl and 9-fluorenylmethoxycarbonyl and the new 6-aminoquinolyl-N-hydroxysuccinimidylcarbamoyl derivatives have been prepared, and the chiral derivatives have been tested with respect to their resolvability in CZE using different cyclodextrins (α-, β-, γ-CDs and CD derivatives) as chiral additives to the electrophoretic buffer system. In general the selector–selectand interactions could be improved by using amino acid derivatives containing nitro- or dimethylamino groups in combination with extended (methylated or hydroxypropylated) CDs. Further enhancement of enantiomeric resolution was achieved by the addition of organic modifiers and/or lowering the temperature down to 5°C. At temperatures above 40°C a non-linear relationship of the decrease of resolution as function of $1/T$ was noticed.

## 1. Introduction

Capillary zone electrophoresis (CZE), introduced in the early 1980s [1], is one of the most rapidly developing separation techniques and stands for a high-efficiency non-stereoselective analytical separation method in free solution. By the addition of chiral additives to the electrophoretic buffer system it is possible to create diastereomeric associates, which should be resolvable due to their different conformation and thus electrophoretic mobilities [2]. Various additives have been used as chiral selectors in CE such as D-campher-10-sulfonate, L-menthoxyacetic acid [3], chiral micelle-forming detergents [4–6], chiral metal complexes [7–9], chiral crown ether [10,11], proteins [12], maltoheptaose [13], oligosaccharides [14] and most frequently cyclodextrins (CDs) [15–26] alone, and in combination with others [3,27,28]. CDs are non-ionic cyclic oligosaccharides consisting of six (α), seven (β) or eight (γ) α-(1,4)-linked D-(+)-glucopyranose units. The resulting geometrical structure has been characterized as a hollow truncated cone with a relatively hydrophobic cavity and a hydrophilic external surface. Chiral

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recognition seems predominantly be based on the inclusion of an aromatic or alkyl functionality into the cavity and additional electrostatic hydrogen bonding interactions between secondary hydroxyl groups around the cone opening and substituents of the guest molecule. Consequently, the formation of the inclusion complex depends on spatial factors, hydrophobic interactions, hydrogen bonding and solvation effects. The chiral discrimination mechanism using CDs is based on the differential electrophoretic mobilities as a result of the different conformation and stability of inclusion-type complexes between CD and enantiomer moieties [29–31].

In this paper a general strategy for the direct enantiomeric separation of selected α-amino acid derivatives is presented and an attempt is made to elucidate the complexity of structure–resolution relationships. Derivatization of amino acids is of general use in terms of improved sensitivity by UV or fluorescence detection and to enhance chiral discrimination mechanisms. The utility for CE application of recently developed amino acid derivatization methods, e.g. 6-aminoquinolyl-N-hydroxysuccinimidylcarbamoyl (AQC) [32], and several amino acid derivatization reagents [e.g. carboxybenzyl (CBZ), dinitrobenzoyl (DNB), dinitrophenyl (DNP), 5-dimethylaminonaphthylsulfonyl (Dns), 9-fluorenylmethoxycarbonyl (FMOC); for chemical formulas see Fig. 1], which have already been widely used for enantiomeric separations with alternative separation techniques, was studied.

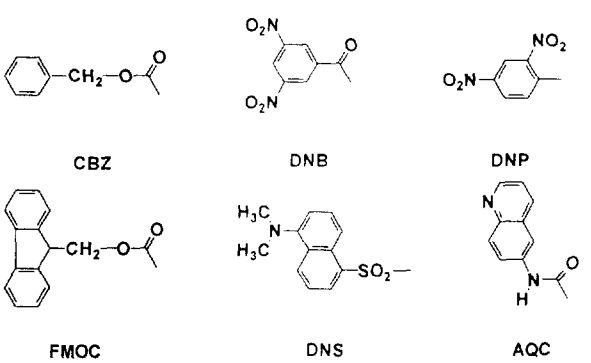


Fig. 1. Molecular structures of amino acid N-derivatives tested for their potential of amino acid enantiomeric separations by CD-modified CZE.

The chemical/spatial structure–inclusion/interaction relationship is discussed and evaluated with respect to size of the aromatic group, substitution, electronic density properties, distance from aromatic moiety to center of chirality, additional hydrogen-bonding facilities etc.

The influence of other experimental CE parameters such as pH, temperature, applied voltage, concentration and type of electrolyte buffer and CD has been extensively studied and will be presented. The effect of organic buffer modifiers (e.g. methanol) was found to be a crucial parameter in terms of lowering electroosmotic flow (EOF) and hence facilitating enantiomeric separations.

## 2. Experimental

### 2.1. Reagents and materials

All chemicals were of analytical-reagent grade unless stated otherwise. All cyclodextrins: α-CD, β-CD, γ-CD, 2-hydroxypropyl (HP)-β-CD (substitution degree 0.9), 2,3,6-methyl (Me)-β-CD (substitution degree 1.8), HP-γ-CD, Me-γ-CD were purchased from Wacker Chemie (Salzburg, Austria), sodium hydroxide and all electrolyte buffer solutions (sodium phosphate, sodium tetraborate decahydrate, sodium citrate, 20 m*M* each, pH range 3–9) were obtained from Fluka (Buchs, Switzerland). L-Carnitin hydrochloride [$HOOC-CHOH-(CH_2)_2-N^+(CH_3)_3 \cdot HCl$], mesityloxide, the derivatizing agents 2,4-dinitrofluorobenzene (DNFB, Sanger's reagent), 3,5-DNB chloride (DNB-Cl), benzyloxycarbonylchloride (CBZ, Z), fluorenylmethylchloroformate (FMOC-Cl) and the final DNP-, DNB-, CBZ-, Dns and FMOC derivatives of different α-amino acids were purchased from Aldrich (Steinheim, Germany). The new AQC, trade name AccQ · Fluor) reagent was provided by Millipore, Waters Chromatography (Milford, MA, USA). Deionized water was prepared with a Milli-Q system (Millipore, Vienna, Austria). Water of 18 MΩ was used for the preparation of

all the solutions, electrolyte buffer and standards.

### 2.2. Apparatus

All CE separations were performed using a HP$^{3D}$ CE system [Hewlett-Packard (HP), Vienna, Austria], equipped with 50–70 cm × 50 μm I.D. uncoated fused-silica capillary with "bubble cell" (3 times extended optical path length). Hydrodynamical injection was achieved by applying a controlled pressure profile at 100 mbar for 3 s. A Peltier element allowed forced-air (10 m/s air velocity) temperature controlling of the capillary cassette down to 5°C (±0.1°C). Peak detection was accomplished by diode-array detection (DAD, 190–600 nm). Data processing was performed on a HP Vectra personal computer (486/66) with a HPCE $^{3D}$ChemStation.

### 2.3. Methods

The capillary was prewashed via maximum pressure application using a standardized washing procedure with 1.0 *M* NaOH for 5 min, 0.1 *M* NaOH for 3 min, water for 5 min and running buffer for 10 min at the beginning of each working day, and further with 0.1 *M* NaOH for 1 min and running buffer for 7 min prior to each analysis. Sample injections were followed by a 1.0-s injection of water in order to wash the electrode and the outside of the fused-silica capillary. After the final analysis of a sequence running overnight the capillary was washed with 0.1 *M* NaOH for 1 min, water for 15 min and air for 1 min.

## 3. Results and discussion

### 3.1. Effect of inclusion interaction between different amino acid derivatives and CD types

There is a direct relation between spatial extension (aromatic size) of the amino acid N-protection group and the inner diameter of the CD cavity (Table 1). Voluminous fluorescent derivatives (Dns, FMOC) showed best resolution with γ-CDs, whereas the smaller chromophores of DNP, DNB, CBZ and AQC could be efficiently separated with β-CD derivatives.

In addition, aromatic substitution with dinitro or dimethylamino functionalities increased chiral discrimination, presumably by the reduced or changed immersion into the CD cavity, thus facilitating additional hydrogen bonding of amino acid moieties (amino and carboxyl groups) towards the secondary hydroxyl groups of the CD rim. The importance of this effect besides hydrophobic inclusion is also made evident by the improved resolution using HP-CDs (Fig. 2) and by the general decrease in separation per-

Table 1
Possible host–guest inclusion interactions between various CDs and α-amino acid derivatives depending on the relationship of the spatial extension of the amino acid N-protection group and the inner diameter of the CD cavity

| CD type (cavity diameter at the CD rim in Å) | Amino acid derivative (min. × max. spatial extension in Å) | | | | | |
|---|---|---|---|---|---|---|
| | DNB (5.9 × 6.8) | DNP (5.9 × 6.8) | AQC (4.3 × 7.1) | FMOC (5.0 × 8.9) | Dns (7.0 × 8.0) | CBZ (5.0 × 6.7) |
| α-CD (5.7 nm) | | | | | | |
| β-CD (7.8 nm) | + | + | | | + | |
| Me-β-CD (7.8 nm) | + | + | | + | | |
| HP-β-CD (7.8 nm) | + + | + + | + + | | | + |
| γ-CD (9.5 nm) | | | + | + + | + + | + |
| Me-γ-CD (9.5 nm) | | | | | | |
| HP-γ-CD (9.5 nm) | | | | | + | |

+ = Separation possible; + + = baseline separation achieved.

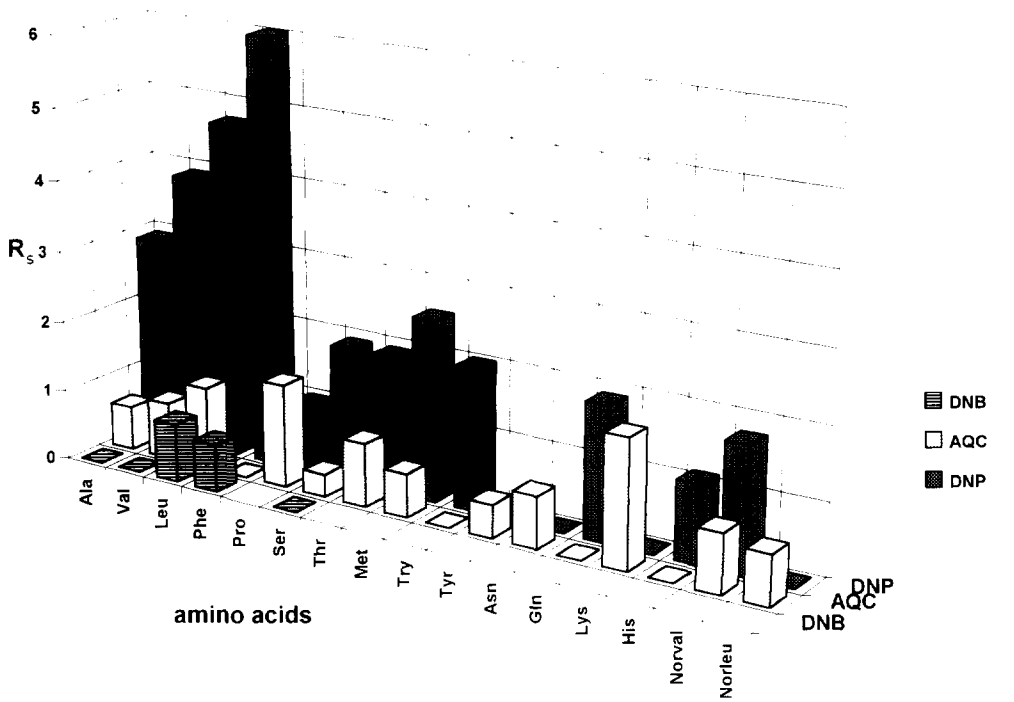


Fig. 2. Resolution ($R_s$) in CD CZE enantiomeric separation of several amino acid derivatives (100 ppm solutions in methanol) using HP-$\beta$-CD (30 m$M$) as a chiral selector. The background electrolyte was 20 m$M$ sodium tetraborate pH 9.2 for DNB- and DNP-amino acid separations and 70 m$M$ sodium phosphate pH 7 for AQC-amino acids with a methanol content of 10% as an organic modifier. CE separations were performed at 20 kV and 10°C capillary temperature for DNP- and AQC-amino acids and 25°C for DNB-amino acids. Blank cells have not been tested, yet.

formance when using Me-CDs. These facts also prove the recently postulated chiral recognition model of CD CZE [33] assuming a three-point interaction mechanism (aromatic inclusion plus two hydrogen bonding interactions), which was first proposed in HPLC on $\beta$-CD bonded stationary phase [34]. However, the restriction of this general approach, when separating DNB, DNP and AQC derivatives of various amino acids using HP-$\beta$-CD is also demonstrated in Fig. 2.

### 3.2. Effect of CD concentration on separation

The effect of CD concentration on the separation, reported as an essential parameter in CD-modified CZE by several authors [12–16], was investigated for DNP- and DNB-amino acids over the concentration range 15–100 m$M$ of HP-$\beta$-CD (Fig. 3). Optimum separations for the model substances DNP-Pro and DNB-Phe were achieved at a concentration of 50 m$M$ HP-$\beta$-CD, whereas DNP-Leu separation required 100 m$M$ of the selector. However, when rising the CD concentration up to an optimum level, the increase in separation factor $\alpha$ and in resolution was insignificant (mean increase of $\alpha$ ca. 2%), but it has to be paid for by prolonged separation time of about 35%. According to the theoretical model, first introduced by Wren and Rowe [29,30], we deduced that the investigated amino acid derivatives show very high affinity to the CD. The difference in apparent mobility between the two enantiomers ($\Delta\mu$) can be derived from the equation

$$\Delta\mu = \frac{C(\mu_1 - \mu_2)(K_B - K_A)}{1 + C(K_A + K_B) + K_A K_B C^2}$$

(where $\mu_1$ = mobility of the enantiomers, $\mu_2$ = mobility of the complexes, $K_A$ and $K_B$ =

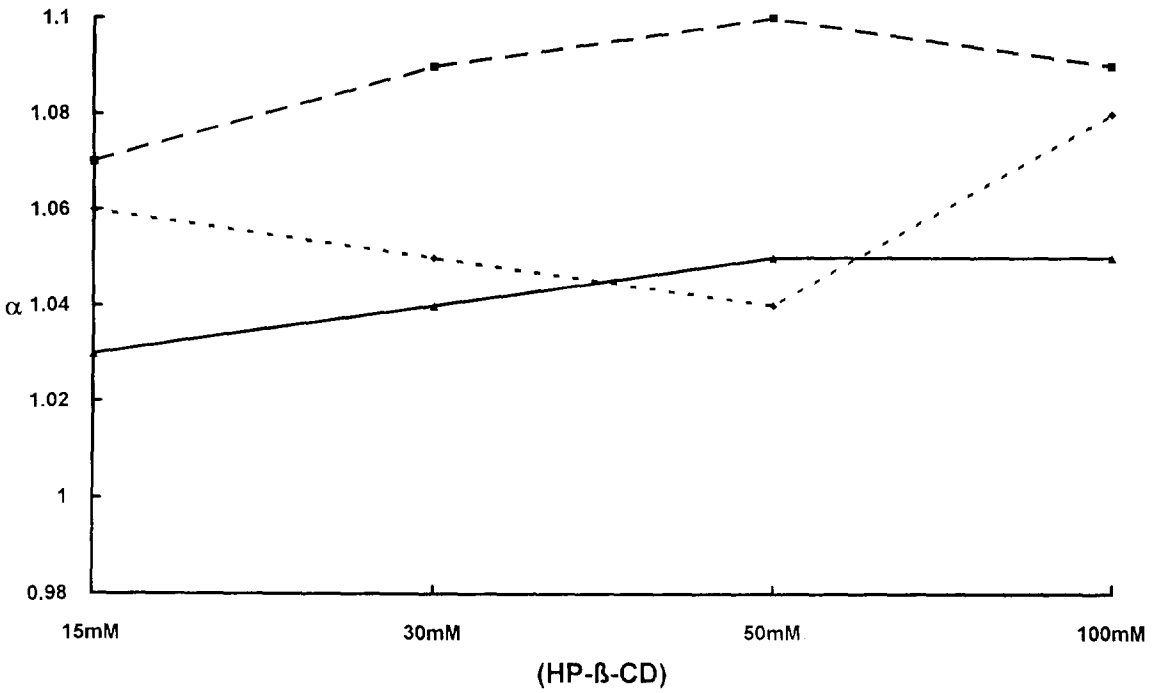


Fig. 3. Influence of HP-β-CD concentration on the separation factor α of selected α-amino acid derivatives. CE parameters: 10 kV electrode voltage, 15°C capillary temperature, electrolyte buffer was sodium tetraborate 20 m*M*, pH 9.2, analyte concentration 100 ppm in methanol. ◆ = DNP-Leu; ▲ = DNP-Pro; ■ = DNB-Phe.

formation constants of the enantiomer–CD complex and $C$ = CD concentration), whereby a maximum mobility difference is obtained at

$$C = \frac{1}{\sqrt{(K_A K_B)}}$$

However, the influence of this term is diminished for great values of the equilibrium constants $K_A$ and $K_B$, which has been proved by the CD CZE separation results of amino acid derivatives shown in Fig. 3. Consequently the optimum selector concentration can be very low, when the affinity of the enantiomers to the CD is high.

### 3.3. Effect of pH on separation

pH Effects were studied over the range of pH 5–9.2 for DNB-, DNP- and AQC-amino acids using sodium phosphate 70 m*M* as background electrolyte and HP-β-CD 30 m*M* as chiral selector (Fig. 4). As a result, all separations of DNB, DNP, Dns and FMOC derivatives showed enhanced resolution at pH values 7–9, since the resulting anionic analytes run counter to the EOF and the "pseudo-stationary" CD phase. AQC derivatives required a low pH setting (5–7) resulting in a reduction of the EOF, presumedly due to a slow kinetics of complex formation, otherwise the EOF may be too rapid, resulting in elution of solute before separation has occurred. However, Fig. 4 also demonstrates the exception of the rule with DNP-Phe showing best separation efficiency at pH 6.

### 3.4. Effect of the type and concentration of the running buffer

The use of sodium phosphate buffer showed slightly better resolution in CD CZE enantiomeric separation of amino acid derivatives as compared with borate buffer. This can be attributed to a possible complexation of borate anions

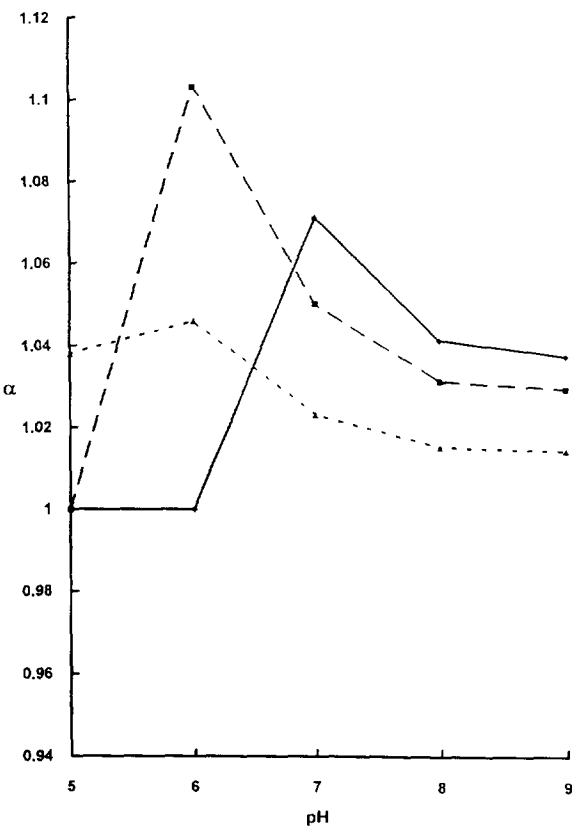


Fig. 4. Effect of electrolyte buffer pH on the separation factor $\alpha$ of selected amino acid derivatives using HP-$\beta$-CD (30 m$M$) as a chiral selector. CE parameters: 20 kV electrode voltage, 10°C capillary temperature, electrolyte buffer was sodium phosphate 70 m$M$ with a modifier content of 20% methanol. The analyte concentration was 100 ppm in methanol. ▲ = AQC-Leu; ■ = DNP-Phe; ◆ = DNB-Phe.

to the vicinal hydroxyl groups in positions 2 and 3 of the CD rim [35]. The optimum ionic strength, studied in a range from 10 to 100 m$M$, was found to be 20 m$M$ for borate buffer and 70 m$M$ for phosphate buffer.

### 3.5. Effect of applied voltage

The effect of the applied voltage in the range of 5–30 kV on the EOF and consequently on migration time as well as on separation was studied at a phosphate buffer system (70 m$M$) with HP-$\beta$-CD (30 m$M$) as chiral selector and DNB-Phe, DNP-Phe and AQC-Leu as model substances. Good enantiomeric separations were achieved by setting the voltage at 30 kV, which is also the limit of most CE devices. But, high analysis speed under elevated voltage setting had to be paid for by a slight decrease in the separation factor $\alpha$ of about 3%, due to the increased Joule heating of the capillary, revealing higher diffusion velocities and peak broadening. However, this effect could be kept to a minimum as a result of the highly sufficient forced-air cooling within the capillary cartridge. Even though, a voltage setting of 15–20 kV seems to be a good compromise of sufficient resolution with acceptable speed of analysis.

### 3.6. Effect of temperature

Since the host–guest complexation mechanism is a kinetically driven process and there is potential Joule heating within the capillary under usual voltage settings of 5–30 kV, temperature effects are supposed to be a crucial parameter for CD-modified CZE. Joule heating mainly depending on the power, capillary dimensions, conductivity of the running buffer and applied voltage was limited by using an active temperature control (a Peltier element allowing forced-air temperature controlling of the capillary cassette), moderate voltage of 20 kV as well as moderate running buffer concentration of 20 m$M$ and by using capillaries with a narrow inner radius and a large outer radius.

Accordingly our temperature study ranging from 5 to 60°C on enantiomeric separations of DNB-Phe, DNP-Phe and AQC-Leu revealed a significant but not Van 't Hoff-type (linear relation of ln $\mu$ or ln $\alpha$ versus $1/T$) relationship of increased migration and resolution versus decreasing temperature down to 5°C (Figs. 5 and 6A and B).The electropherograms of the separation of the DNB-Phe enantiomers as shown in Fig. 5 can be regarded as very useful, because especially DNB-derivatized amino acids showed good resolution results without any organic modifier addition (see Fig. 8). Therefore the possible additional effect of modifier on interaction with capillary wall, with the selector and/or the analyte could be neglected.

Once again, referring to the potential Joule

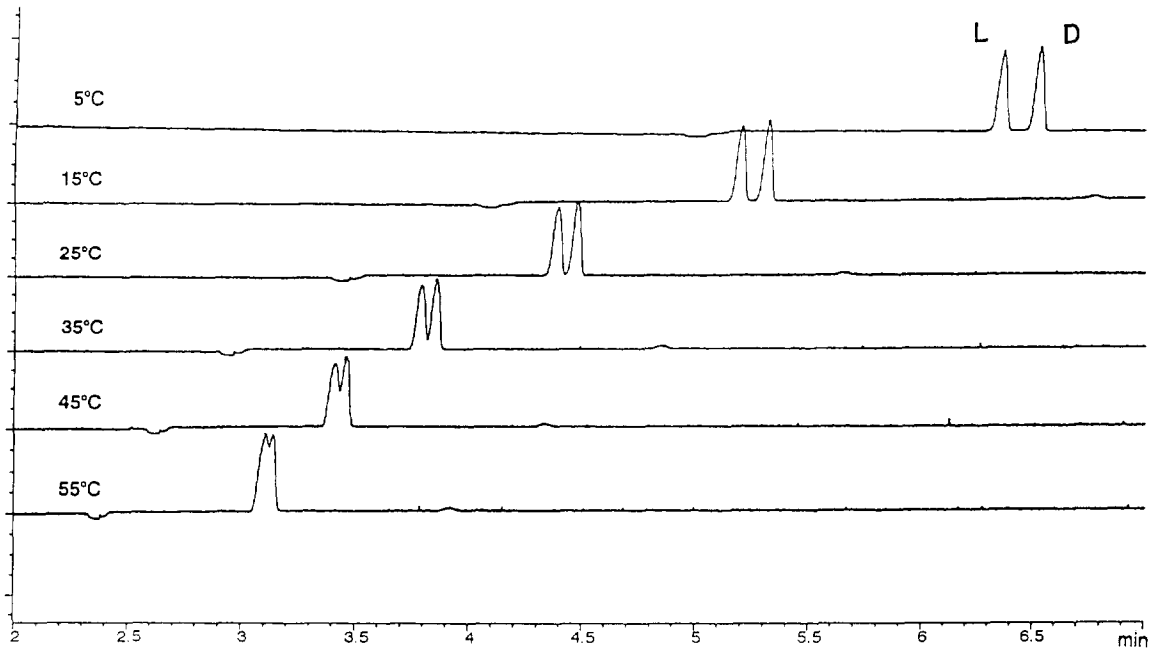


Fig. 5. Electropherogram of the CD CZE separation of DNB-Phe enantiomers (100 ppm racemate in methanol) using HP-β-CD (30 m*M*) as a chiral selector. CE parameters: fused-silica capillary 70 cm × 50 μm I.D., 20 kV electrode voltage, electrolyte buffer was sodium tetraborate 20 m*M*, pH 9.2, UV detection at 214 nm.

heating, it has been shown by Knox and McCormack [36] that the temperature rise $\Delta T$ in the capillary during electrophoresis, the so-called Joule heating, is proportional to a good approximation to the power dissipated, that is

$$\Delta T = aEI$$

where $E$ = field strength (i.e. the applied voltage per unit length, $V/L$), $I$ = current and $a$ = power temperature coefficient depending on the cooling and the outer diameter.

Concerning this direct proportionality, the non-linear relationship of ln $k'$ (capacity factor) versus reciprocal of absolute temperature cannot be attributed to the so-called "unstable and undefined" temperatures in the separation system, because whenever there exists Joule heating, it would not result in such sudden non-linearity.

The relationship of enantiomeric separation, complex formation and temperature is given by the Gibbs–Helmholz equation:

$$-\Delta\Delta G = RT \ln \alpha$$

where $R$ is the general gas constant (8.31 J $K^{-1}$ $M^{-1}$), $T$ is the temperature in K and $\Delta\Delta G$ is the difference in the molar Gibbs energy of the two diastereomeric complexes. The separation factor $\alpha$ was calculated by the quotient of the migration times $[\alpha(\mu) = \mu_B/\mu_A]$ or alternatively by the quotient of capacity factors $[\alpha(k') = k'_B/k'_A)$ in order to compensate the influence of EOF. $k'$ was calculated as $(\mu_{analyte} - \mu_{EOF})/\mu_{EOF}$, where $\mu_{EOF}$ is the migration time of mesityloxide used as a neutral marker.

Usually the Gibbs energy is known to be mainly dependent on the enthalpy of the complex formation revealing a linear relationship of ln $\mu$ or ln $\alpha$ versus $1/T$. But the formation of inclusion complexes with CDs is also influenced by entropy-controlled factors, e.g. the disarrangement of the solvation status of the CD and the loss of translational and rotational degrees of freedom during complex formation as has been postulated by Kuhn et al. [37] for a chiral 18-crown-6 ether. Consequently $\alpha$ is dependent on the enthalpy difference ($\Delta\Delta H$) and the entropy

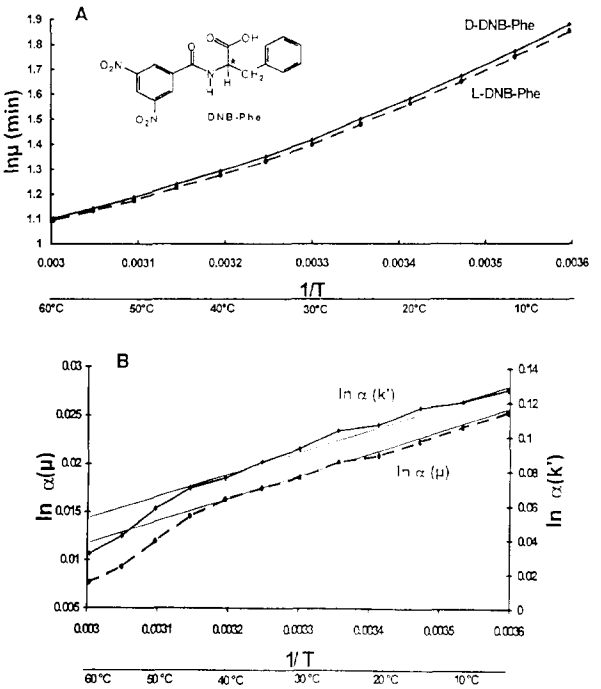


Fig. 6. Van 't Hoff plots showing the non-linear behavior of temperature on the separation of DNB-Phe enantiomers (100 ppm racemate in methanol) using HP-β-CD (30 m*M*) as a chiral selector. CE parameters: fused-silica capillary 70 cm × 50 μm I.D., 20 kV electrode voltage, electrolyte buffer was sodium tetraborate 20 m*M*, pH 9.2. (A) Relationship of migration behavior as ln $\mu$ versus $1/T$. (B) Relationship of discrimination behavior as ln $\alpha$ versus $1/T$. The separation factor $\alpha$ was calculated as quotient of the two migration times or alternatively to compensate for the EOF as quotient of $k'$ values. The linearity regions are presented by the parallel linear lines.

difference ($\Delta\Delta S$) of the inclusion interaction following the equation:

$$\ln \alpha = -\frac{\Delta\Delta H}{RT} + \frac{\Delta\Delta S}{R}$$

From the non-linear relationship of ln $\mu$ versus $1/T$ of our results (Fig. 6A) one might conclude that the migration behavior of the diastereomeric complexes is also significantly influenced by the contribution of entropy to the CD-complex formation with our model amino acid derivatives. A Van 't Hoff plot of ln $\alpha$ versus $1/T$ (Fig. 6B) shows an approximately linear relation in the temperature range from 5 to 40°C. Exceeding this temperature range to higher temperatures resulted in a significant change in the migration behavior and consequently separation of the diastereomeric complexes as a result of additional, predominantly entropy-controlled, effects.

The unusual temperature dependency of these entropy factors might mainly be attributed to temperature-controlled changes in the bulk liquid structure and viscosity thus affecting the rotational and translational degrees of freedom of the host–guest associates and/or to a significantly changed mechanism of interaction (e.g. inclusion-type complex formation is replaced by electrostatic hydrogen bonding at the exterior CD surface). Calculation or prediction of these effects, however, remains difficult. Consequently, the calculation of thermodynamic parameters ($\Delta\Delta H$, $\Delta\Delta S$), as reported for HPLC-type separation using covalently bonded β-CD chiral stationary phase [38], via extrapolation of the linear part of the curve appears to be questionable.

Recently, Lamparczyk and co-workers [39–41] found a non-linear relationship of capacity factor (ln $k'$) versus reciprocal of absolute temperature using β-CD modified mobile phase for the separation of estradiol and other stereoisomers by HPLC. According to this work the mentioned phenomenon seems to be more complex but without any doubt relevant.

Undoubtedly, this non-linear relationship of ln $k'$ versus $1/T$ is the result of many temperature-dependent parameters i.e. expansion of the liquid, resistance due to the change in the conductivity of the running buffer, which itself arises from the change in the viscosity of the buffer, the electroosmotic and the electrophoretic mobilities and the complexation constants of the reversible diastereomerical enantiomer–CD complex.

Further, recent computational studies on the dynamical features of CDs brought into light that CDs can no longer be seen as a rigid cone, but more likely as a flexible, twisting basket [42], which also allows an induced fit of the guest molecule. Obviously, these dynamical features are strongly temperature controlled and may also enhance the contribution of the complexation

entropy to the molar Gibbs energy and consequently to the separation factor $\alpha$.

However, dynamical features of CD and changes of the interaction from host–guest type to electrostatic hydrogen bonding of the exterior CD surface have been thoroughly discussed before on the basis of molecular modelling (MM) calculations [43] and results from MM have to be applied precautiously in CE, regarding the aqueous system of CE.

The influence of temperature on chiral separation in modified capillaries using CD or CD derivatives as chiral selectors could be studied in more detail, concerning the fact that the performance of chiral separations is strongly influenced by alteration of surface coating [44,45] referring not only the chiral separation of bases but also of organic acids.

However, these results stand in contrast to the experiments by Nielen [23] where hardly any differences and certainly no improvements with a $C_{18}$-coated capillary could be achieved by separating racemic drugs by CD and CZE.

### 3.7. Effect of modifiers on separation

Organic modifiers added to the electrolyte buffer system are in many cases reported to facilitate a "finetuning" of enantiomeric separations. We have studied this effect on the enantiomeric separations of DNP-, DNB- and AQC-amino acids adding 20% (v/v) methanol alone and in combination with 1% (v/v) *tert.*-butyl methyl ether or alternatively 5 m*M* carnitin to a sodium tetraborate (20 m*M*, pH 7–9) background electrolyte system with 30 m*M* HP-$\beta$-CD as chiral selector at a capillary temperature of 10°C.

The influence of methanol on the enantiomeric separation of DNP, DNB and AQC derivatives of the model amino acid phenylalanine is depicted in Figs. 7 and 8. There was a significant increase in migration time of DNB- and DNP-amino acids of ca. 40%, which could be attributed to the decreased EOF, probably via interaction of the modifier with the capillary wall thus altering charge and hydrophobicity, and consequently changing $\zeta$ potential as a driving force of EOF.

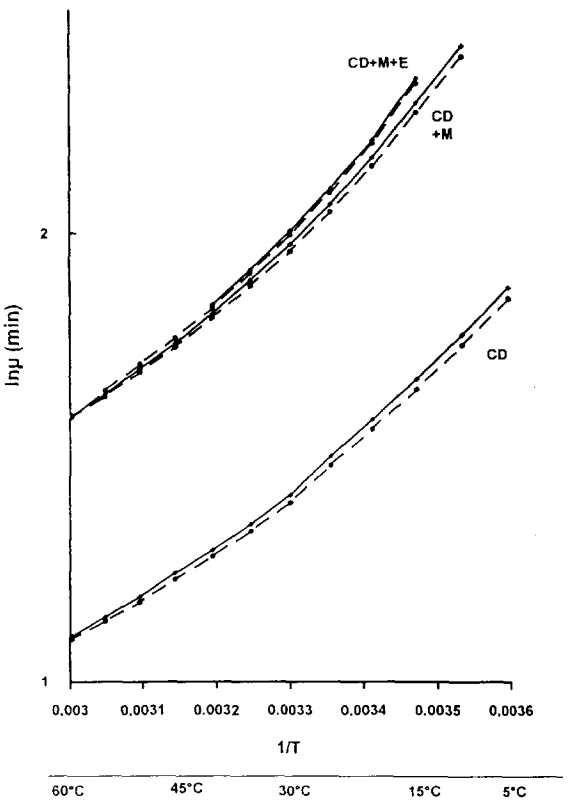


Fig. 7. Van 't Hoff plots showing the influence of different modifiers at variable temperatures on migration and on the separation of DNB-Phe enantiomers (100 ppm racemate in methanol) using HP-$\beta$-CD (30 m*M*) as a chiral selector. CE parameters: fused-silica capillary 70 cm × 50 $\mu$m I.D., 20 kV electrode voltage, electrolyte buffer was sodium tetraborate 20 m*M*, pH 9.2, without modifier (CD), or alternatively with addition of 20% (v/v) methanol (CD + M) or alternatively with addition of 20% (v/v) methanol and 1% (v/v) *tert.*-butyl methyl ether in combination (CD + M + E).

Moreover the addition of methanol enabled enantiomeric separations of DNP-Asp, DNP-Phe, DNP-Pro, DNP-His and DNP-Try, which could not be accomplished without the modifier. Accordingly, the modifier effect cannot be simply reduced to the influence on EOF. An additional effect on chiral recognition mechanism via interaction with the selector and/or the analyte can be assumed. Also, the change in bulk liquid viscosity and consequently diffusion velocity

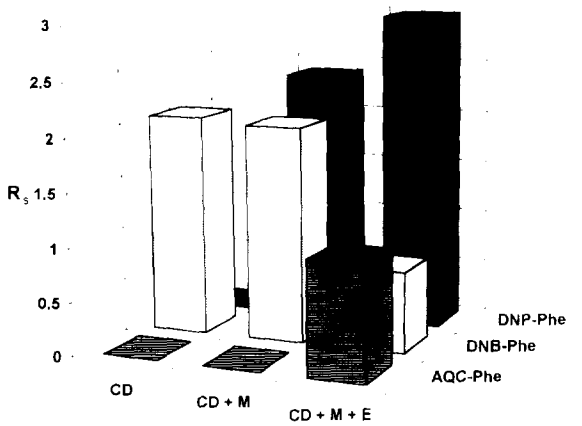


Fig. 8. Modifier effects on the CZE separation of different (*R*)- and (*S*)-Phe derivatives (100 ppm racemate in methanol) using HP-β-CD (30 m*M*) as a chiral selector. CE parameters: fused-silica capillary 70 cm × 50 μm I.D., 20 kV electrode voltage, 10°C capillary temperature, electrolyte buffer was sodium tetraborate 20 m*M*, pH 9.2 for DNB-Phe and DNP-Phe and pH 7 for AQC-Phe. The additives used were 20% (v/v) methanol (M) or 1% (v/v) *tert.*-butyl methyl ether (E).

and/or interaction kinetics should not be neglected.

The additive *tert.*-butyl methyl ether did not significantly improve resolution of DNP- and DNB-amino acids, with DNP-Phe as a single exception of the rule (Fig. 8).

As to the enantiomeric separation of AQC-amino acids the methanol effect on EOF was negligible, due to the lower pH revealing a priori low EOF conditions. But, as shown in this contribution, the combination of methanol and *tert.*-butyl methyl ether rendered possible enantiomeric separations of AQC-Phe, AQC-His, AQC-Cit and AQC-Ser, which have not been accomplished with methanol or without any modifier.

As a result, organic additives proved to be a useful tool to increase resolution and sometimes even to make a separation possible, which always has to be paid for by prolonged analysis time due to the lower EOF.

The use of 5 m*M* L-carnitin as an organic modifier improved resolution of DNP-Phe and AQC-Leu, whereas resolution of DNB-Phe was decreased. The assumed interaction of the zwitterionic and quaternary amine type compound L-carnitin with the silanol groups of the fused-silica capillary, consequently altering the direction of EOF, was obviously overlaid by other interaction equilibria between the CD selector, the electrolyte system, the modifier and the analyte. Further studies are planned to elucidate the complex mechanism of interactions with this unusual modifier.

In general, the prediction of modifier effects seems to be hampered by the high complexity of the various equilibria involved. Consequently, the successful application of organic modifiers for resolution enhancement in CD CE relies still on "trial and error".

## 4. Conclusions

CDs of different cavity size (α-, β-, γ-CD) have proved to be effective chiral selectors for the enantiomeric separation of various α-amino acid derivatives by CZE. Their potential for the separation of a wide range of chemical different structures was even enhanced by the use of chemical modified (HP and Me) CDs showing improved solubility in aqueous electrolyte buffer systems and facilitating chiral recognition mechanism. This work also demonstrates the influence of several experimental conditions on enantiomeric separations, such as type of buffer, electrolyte pH, CD concentration, organic modifiers, applied voltage and capillary temperature. Table 2 is an attempt to give a general guideline for sufficient enantiomeric separation of various α-amino acids by CE using suitable derivatization methods, appropriate types of CD, different modifiers and optimized CE parameters. However, the prediction of optimum conditions based on the chemical structures of the analytes remains difficult.

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Table 2
General guideline for sufficient enantiomer separation of α-amino acids by CD CZE using suitable derivatization methods, appropriate types of CD, different modifiers and optimized CZE parameters

| Amino acid | AQC | DNP | DNB | Dns | CBZ | FMOC |
|---|---|---|---|---|---|---|
| Ala | HP-β-CD + M + E | HP-β-CD + M | – | | γ-CD | Me-β-CD |
| Val | HP-β-CD + M + E | HP-β-CD + M | | γ-CD | | γ-CD + M |
| Nva | HP-β-CD + M + E | HP-β-CD + M + E | | | | |
| Leu | HP-β-CD + M + E | HP-β-CD + M | HP-β-CD + M | HP-γ-CD, β-CD | HP-β-CD | γ-CD + M |
| Ile | – | HP-β-CD + M | | | | |
| Nle | HP-β-CD + M + E | – | | | | |
| Phe | HP-β-CD + M + E | HP-β-CD + M | HP-β-CD, β-CD | | | γ-CD + M |
| Pro | HP-β-CD + M | HP-β-CD + M | – | | | |
| Ser | HP-β-CD + M + E | HP-β-CD + M | – | | | |
| Thr | HP-β-CD + M | HP-β-CD + M | | | | |
| Cys | – | – | | | | |
| Met | HP-β-CD + M | HP-β-CD + M | | γ-CD | | |
| Trp | – | HP-β-CD + M | | | | |
| Tyr | HP-β-CD + M + E | | | | | |
| Asn | HP-β-CD + M + E | | | | | |
| Gln | – | HP-β-CD + M | | | | |
| Asp | – | | | | | |
| Glu | – | | | | | |
| Lys | HP-β-CD + M + E | | | | | |
| Arg | – | | | | | |
| His | HP-β-CD + M + E | HP-β-CD + M | | | | |
| GABA | – | – | | γ-CD | | |

CE parameters: uncoated fused-silica capillary 70 cm × 50 μm I.D., 20 kV electrode voltage, 10°C capillary temperature, electrolyte buffer was sodium tetraborate 20 m*M*, pH 9.2, and for DNP and AQC derivatives sodium phosphate 70 m*M*, pH 7, the concentration of the chiral selector (CD) was 30 m*M* except for β-CD (max. solubility 15 m*M*), analyte concentration 100 ppm in methanol, UV detection at 214 nm and at 280 nm for AQC. GABA = γ-aminobutyric acid; HP-CD = hydroxypropylated cyclodextrin; Me-CD = methylated cyclodextrin; + M = addition of 20% (v/v) methanol; + E = addition of 1% (v/v) *tert.*-butyl methyl ether; – = no results due to derivatization, solubility or separation problems; blank cells not tested, yet.

HP[3D] CE system in our laboratories for a limited time.

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## References

[1] J.W. Jorgenson and K.D. Lukacs, *Anal. Chem.*, (1981) 1298–1302.
[2] S. Terabe, K. Otsuka and H. Nishi, *J. Chromatogr. A*, 666 (1994) 295–319.
[3] H. Nishi, T. Fukuyama and S. Terabe, *J. Chromatogr.*, 553 (1991) 503–516.
[4] S. Terabe, M. Shibata and Y. Miyashita, *J. Chromatogr.*, 480 (1989) 403–411.
[5] K. Otsuka and S. Terabe, *J. Chromatogr.*, 515 (1990) 221–226.
[6] A. Dobashi, T. Ono and S. Hara, *Anal. Chem.*, 61 (1989) 1986–1988.
[7] P. Gozel, E. Gassmann, H. Michelsen and R.N. Zare, *Anal. Chem.*, 59 (1987) 44–49.
[8] E. Gassmann, J.E. Kuo and R.N. Zare, *Science*, 230 (1985) 813–814.
[9] S. Fanali, L. Ossicini, F. Foret and P. Boček, *J. Microcol. Sep.*, 1 (1989) 190–194.
[10] R. Kuhn, F. Stoecklin and F. Erni, *Chromatographia*, 33 (1992) 32–36.
[11] J. Snopek, H. Soini, M. Novotny, E. Smolkova-Keulemansova and I. Jelínek, *J. Chromatogr.*, 559 (1991) 215–222.
[12] S. Birnbaum and S. Nilsson, *Anal. Chem.*, 64 (1992) 2872–2874.

[13] S. Terabe, Y. Miyashita, Y. Ishihama and O. Shibata, *J. Chromatogr.*, 636 (1993) 47–55.
[14] A.D. Hulst and N. Verbeke, *J. Chromatogr.*, 608 (1992) 275–287.
[15] S. Terabe, H. Ozaki, K. Otsuka and T. Ando, *J. Chromatogr.*, 332 (1985) 211–217.
[16] S. Fanali, *J. Chromatogr.*, 545 (1991) 437–444.
[17] S. Fanali, *J. Chromatogr.*, 474 (1989) 441–446.
[18] S. Fanali and P. Boček, *Electrophoresis*, 11 (1990) 757–760.
[19] A. Pluym, W. Van Ael and M. de Smet, *Trends Anal. Chem.*, 11 (1992) 27–32.
[20] T.L. Bereuter, *LC · GC*, 12 (1984) 748–766.
[21] T.E. Peterson and D. Towbridge, *J. Chromatogr.*, 603 (1992) 298–301.
[22] M.E. Swartz, *J. Liq. Chromatogr.*, 14 (1991) 923–938.
[23] M.W.F. Nielen, *Anal. Chem.*, 65 (1993) 885–893.
[24] H. Nishi, Y. Kokusenya, T. Miyamoto and T. Sato, *J. Chromatogr. A*, 659 (1994) 449–457.
[25] T. Schmitt and H. Engelhardt, *Chromatographia*, 37 (1993) 475–481.
[26] T.E. Peterson, *J. Chromatogr.*, 630 (1993) 353–361.
[27] J. Prufionosa, R. Obach, A. Diez-Cascón and L. Gouesclou, *J. Chromatogr.*, 574 (1992) 127–133.
[28] T. Ueda, F. Kitamura, R. Mitchell, T. Metcalf, T. Kuwana and A. Nakamoto, *Anal. Chem.*, 339 (1991) 63–64.
[29] S.A.C. Wren and R.C. Rowe, *J. Chromatogr.*, 603 (1992) 235–241.
[30] S.A.C. Wren and R.C. Rowe, *J. Chromatogr.*, 609 (1992) 363–367.
[31] S.A.C. Wren, *J. Chromatogr.*, 636 (1993) 57–62.
[32] S.A. Cohen and D.P. Michaud, *Anal. Biochem.*, (1993) in press.
[33] C. Quang and M. Khaledi, *Anal. Chem.*, 65 (1993) 3354–3358.
[34] D.W. Armstrong, T.J. Ward, R.D. Armstrong and T.E. Beesley, *Science*, 232 (1986) 232–235.
[35] P.J. Oefner, A.E. Vorndran, E. Grill, C. Huber and G.K. Bonn, *Chromatographia*, 34 (1992) 308–316.
[36] J.H. Knox and K.A. McCormack, *Chromatographia*, 38 (1994) 279–282.
[37] R. Kuhn, F. Erni, T. Bereuter and J. Häusler, *Anal. Chem.*, 64 (1992) 2815–2820.
[37] K. Cabrera and D. Lubda, *J. Chromatogr. A*, 666 (1994) 433–438.
[38] H. Lamparczyk and P.K. Zarzycki, presented at the *5th International Symposium on Chiral Discrimination, Stockholm, 25–28 September 1994.*
[40] D. Sybilska, M. Asztemborska, A. Bielejewska, J. Kowalczyk, H. Dodziuk, K. Duszczyk, H. Lamparczyk and P. Zarzycki, *Chromatographia*, 35 (1993) 637–642.
[41] H. Lamparczyk, P.K. Zarzycki and J. Nowakowska, *J. Chromatogr. A*, 668 (1994) 413–417.
[42] K.B. Lipkowitz, *J. Chromatogr. A*, 694 (1995) 15.
[43] D.G. Durham and H. Liang, *Chirality*, 6 (1994) 239–244.
[44] D. Belder and G. Schomburg, *J.Chromatogr. A*, 666 (1994) 351–365.
[45] D. Belder and G. Schomburg, *J. High Resolut. Chromatogr.*, 15 (1992) 686.