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Chiral separation of unmodified amino acids with non-aqueous capillary electrophoresis based on the ligand-exchange principle

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Abstract

A ligand exchange mechanism in non-aqueous capillary electrophoresis was employed for the separation of eight unmodified amino acids using chiral complexes of copper(II) with L-proline and L-isoleucine. The electrophoretic medium consisted of 25 m*M* ammonium acetate and 1 *M* acetic acid in methanol. We were able to completely separate the enantiomeric pairs of each of the investigated racemic amino acids. We also report the optimization of the separation parameters, such as pH*, composition of the complex, and concentration of the complexing agents. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Non-aqueous capillary electrophoresis; Ligand-exchange capillary electrophoresis; Enantiomer separation; Amino acids

1. Introduction

Since Davankov and Roghozin 1971 [1] first reported ligand-exchange chromatography, this separation principle has become a very useful method for the separation of amino acids by HPLC. Gassmann et al. 1985 [2] introduced this method for the separation of dansylated amino acids by using capillary electrophoresis (CE). This group used L-histidine as a ligand at a pH value between 7 and 8. Under the same pH conditions, Gozel et al. [3] used aspartame, an enantiopure dipeptide, which is commonly used as a sweetener, for the separation of dansylated amino acids. They also investigated the order in which the enantiomers of the analytes migrate; it was observed that the D-enantiomers migrated first. This observation suggests that the D-enantiomer is more strongly bound to the Cu(II)–selector complex than the L-enantiomer [3]. Further investigations with ligand-exchange capillary electrophoresis (LECE) were reported by Desiderio et al. in 1994 [4]. These authors used aspartame, L-proline, and L-hydroxyproline as selectors for the separation of α-hydroxy acids and studied the influence of capillary temperature, applied voltage, and pH of the background electrolyte on the extend of the separations. In 1996 Schmid and Gübitz [5] reported the chiral separation of unmodified amino acids with LECE with L-proline and L-hydroxyproline as ligands. Through use of these selectors, only amino acids containing aromatic moieties could be resolved. Improved selectivity was observed with *N*-

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(2-hydroxyoctyl)-L-4-hydroxyproline [6]; this selector as well as *N*-(2-hydroxypropyl)-L-4-hydroxyproline also showed enantioselectivity for aliphatic amino acids and dipeptides [7]. In the same year Sundin et al. [8] reported the chiral separation of dansyl amino acids by using micellar electrokinetic capillary chromatography and the ligand exchange principle. The separations were achieved with a copper complex of *N,N*-didecyl-alanine. This ligand has a high hydrophobicity and solubilisation was possible only in the presence of sodium dodecylsulfate at concentrations above the critical micelle concentration. This group was able to separate eleven dansyl amino acids into enantiomers with this separation system.

In the course of our studies with non-aqueous capillary electrophoresis (NACE) [9,10], we have investigated for the first time the combination of LECE with the advantages of NACE for the chiral separation of unmodified amino acids. In a medium of methanol containing ammonium acetate and acetic acid, it was possible to separate each of the eight amino acids into their enantiomers. As chiral selector we used a chiral complex of copper(II) with L-proline and L-isoleucine. We carried out investigations to optimize the separation parameters, such as pH* (apparent pH in non-aqueous media), the concentration of the complexing agents and the composition of the complex.

2. Experimental

2.1. Instrumentation and separation conditions

A P/ACE 2200 capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) equipped with a UV detector was used. Sample detection was performed at 214 nm. Uncoated fused-silica capillaries 37 cm (effective length 30 cm)×50 μm)] were used (Polymicro Technologies, Phoenix, USA). The pH* were measured with a Microprocessor pH meter, pH 537 (WTW, Weilheim, Germany) with the following electrode: pH-Elektrode SenTix 41; pH 0...14; 0...80°C (WTW). Analytes were dissolved in methanol containing formic acid for better solubility. The pH* values of the electrophoresis media were adjusted with NaOH and formic acid. Samples were injected hydrodynamically for 3 s. The applied voltage was 25 kV. The capillary temperature was set to 25°C. Prior to use, the capillaries were rinsed with doubly distilled water (10 min), 1 *M* NaOH (10 min) and methanol (10 min). Between the runs the capillaries were rinsed with methanol (2 min) and with the running electrophoresis medium (2 min).

2.2. Chemicals

Acetic acid, $CuCl_2 \cdot 2H_2O$, and formic acid (analytical-reagent grade) were purchased from Merck (Darmstadt, Germany). NaOH was supplied by Chemapol (Prague, Czech Republic). L-Proline, L-tryptophan, D,L-tryptophan, and D,L-kynurenine were obtained from Reanal (Budapest, Hungary). L-isoleucine and D,L-α-methyltyrosine were purchased from Serva (Heidelberg, Germany). D,L-Phenylalanine was from Bergakademie Freiberg (Freiberg, Germany), D,L-thyronine was purchased from Hoffmann-La Roche (Grenzach, Germany), D,L-tyrosine was from Bayer (Leverkusen, Germany), D,L-α-aminophenyl acetic acid was supplied by Janssen (Beerse, Belgium), and D,L-histidine was purchased from Schuchardt (Munich, Germany). In the following text, we will disregard the prefix D,L for the racemic analytes. Methanol (HPLC grade) was purchased from AppliChem (Darmstadt, Germany). Water was doubly distilled.

3. Results and discussion

For all investigations the electrophoresis medium was methanol and contained 25 m*M* ammonium acetate and 1 *M* acetic acid. Cu(II) was used as the complexing agent. When Cu(II) was replaced by Co(II), no resolution of the enantiomers could be observed.

3.1. Influence of the pH*

The electrophoretic runs were performed in the pH* range from 3.5 to 5.0 containing 5.86 m*M* $CuCl_2 \cdot 2H_2O$ and 16 m*M* L-proline. At pH*>5.0 the amino acids migrated with the electroosmotic flow. Distinct maxima of the separations could be ob-

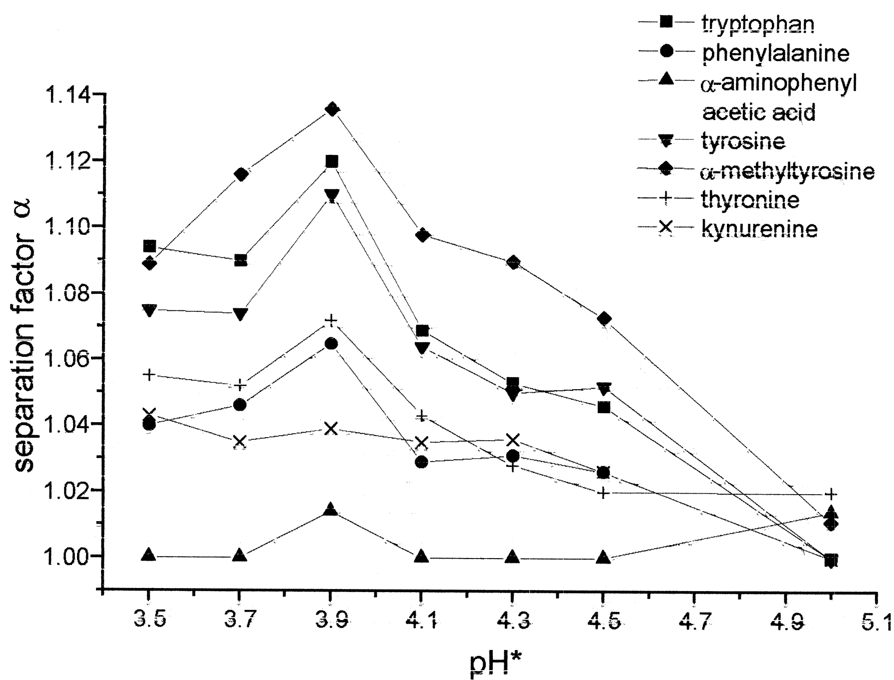

Fig. 1. Dependence of the separation factors on pH*. Conditions: methanol; 25 m*M* ammonium acetate; 1 *M* acetic acid; 5.86 m*M* $CuCl_2 \cdot 2H_2O$; 16 m*M* L-proline; uncoated capillary 37 cm (effective length 30 cm)×50 μm; 25 kV; 214 nm; 25°C.

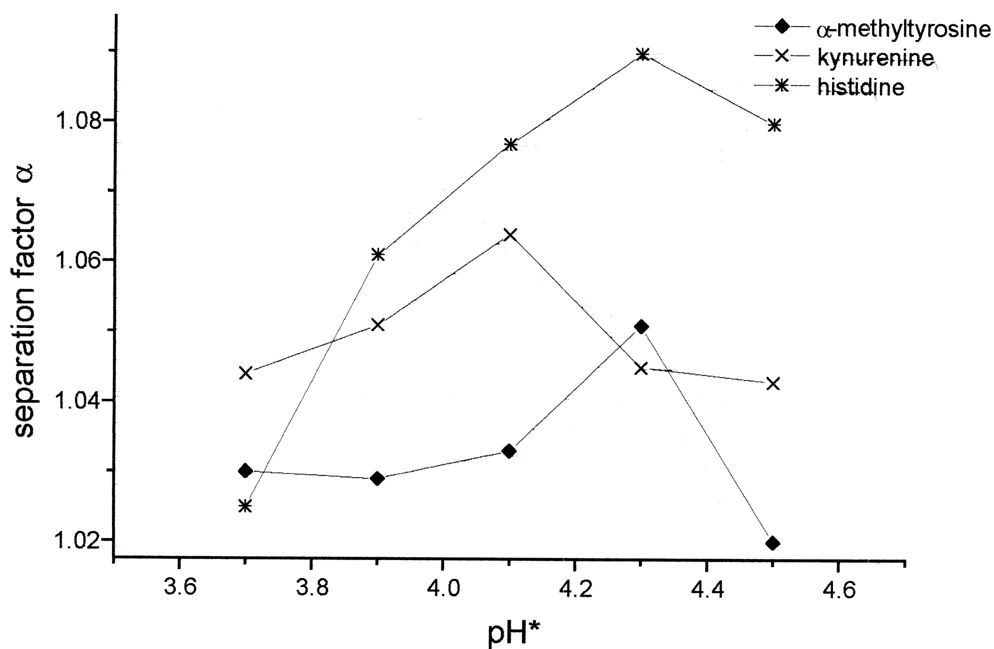

Fig. 2. Dependence of the separation factors on pH*. Conditions: methanol; 25 m*M* ammonium acetate; 1 *M* acetic acid; 5.86 m*M* $CuCl_2 \cdot 2H_2O$; 16 m*M* L-isoleucine; uncoated capillary 37 cm (effective length 30 cm)×50 μm; 25 kV; 214 nm; 25°C.

served at pH* 3.9 (Fig. 1) for all analytes except histidine, which could not be separated with this ligand. Only in the case of kynurenine was this maximum not so clear; this analyte was the only one with two basic moieties in the molecule. Under these conditions, all analytes (except histidine) could be separated at baseline resolution. In further investigations, we used L-isoleucine as a ligand (Fig. 2). With this selector it was possible to separate histidine. Better separation than with L-proline was also observed for kynurenine. Based on these results, it is obvious that those two analytes, which in contrast to the other investigated amino acids have two basic moieties, could be separated with L-isoleucine. Only α-methyltyrosine (the analyte for which the best results with L-proline were obtained) could also be separated with L-isoleucine, however, the results were not as good as with L-proline (compare Figs. 1 and 2).

3.2. Influence of the composition of the complex

The ratio of Cu(II) to ligand, which would be optimal for the chiral separations of the unmodified amino acids, was investigated. These investigations were carried out with tryptophan, phenylalanine, and thyronine (Figs. 3 and 4). Distinct maxima for the separations of the two investigated essential amino acids were observed at a ratio of 1:3 (Cu(II)–L-proline). The separation factors of thyronine did not increase further at a ratio of 1:2.75, but the separations were far better than at a ratio of 1:2. These results show clearly that the ratio of 1:2 for the use of LECE in non-aqueous media is not optimal, as it has been described for investigations in aqueous media [3,8].

3.3. Influence of the concentrations

3.3.1. Concentration of the complexing agents

The concentrations of Cu(II) and L-proline were increased at a constant ratio of 1:3 and the resulting changes recorded (Fig. 5). Only Histidine was separated at the lowest of the investigated concentrations. All the other represented analytes

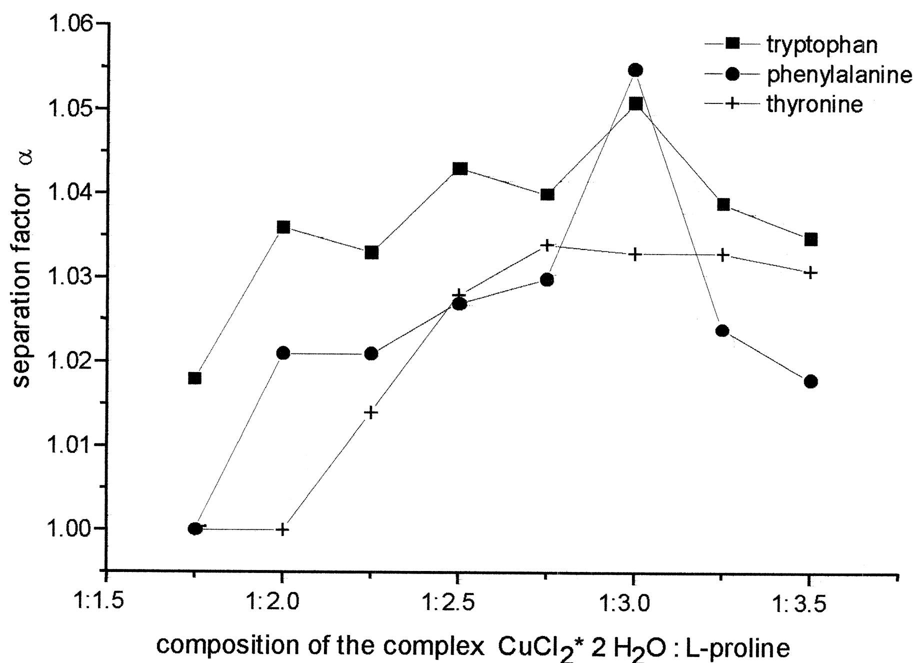

Fig. 3. Dependence of the separation factors on the composition of the complex. Conditions: methanol; 25 mM ammonium acetate; 1 M acetic acid; 16 mM L-proline; pH* 3.92; uncoated capillary 37 cm (effective length 30 cm)×50 μm; 25 kV; 214 nm; 25°C.

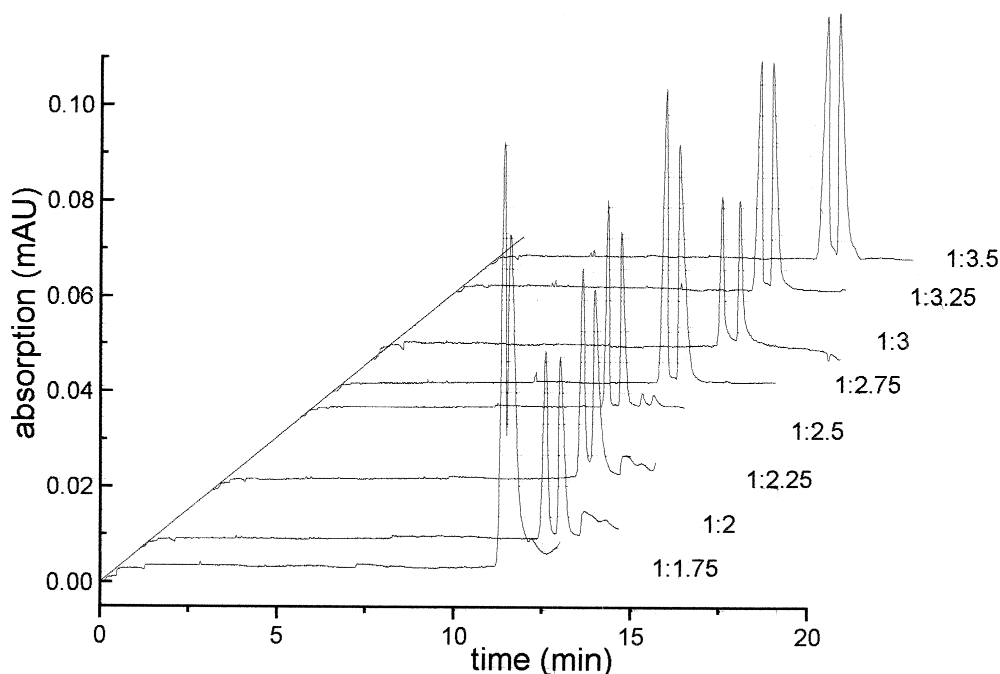

Fig. 4. Dependence of the separations of tryptophan on the composition of the complex. Conditions: methanol; 25 m*M* ammonium acetate; 1 *M* acetic acid; 16 m*M* L-proline; pH* 3.92; uncoated capillary 37 cm (effective length 30 cm)×50 μm; 25 kV; 214 nm; 25°C. 1:1.75, α=1.010; 1:2, α=1.030; 1:2.25, α=1.030; 1:2.5, α=1.040; 1:2.75, α=1.040; 1:3, α=1.050; 1:3.25, α=1.030; 1:3.5, α=1.035.

showed increased separations with increasing concentrations, however, a linear dependence could not be observed. The strongest improvement of the separations showed α-methyl-tyrosine, which has a maximum of the separation factors at 5 m*M* $CuCl_2 \cdot 2H_2O$ and 15 m*M* L-proline. Improvements in the separations at even higher concentrations could be obtained for all other investigated analytes. We established that concentrations of 5 m*M* $CuCl_2 \cdot 2H_2O$ and 15 m*M* L-proline under the given conditions were sufficient for the chiral separations of the analytes (except histidine). However, increasing these concentrations might increase the separation of some analytes (phenylalanine, thyronine).

3.3.2. Concentration of the analytes

In order to optimize the UV signal for the detection of the amino acid complexes, it was necessary to vary the concentrations of the analytes. The optimal concentrations were 1 mg/ml for phenylalanine, 0.25 mg/ml for thyronine and 0.5 mg/ml for tryptophan (Fig. 6). Better separations could be observed with lower concentrations, however, the signal-to-noise ratio of these separations was poor.

3.4. Influence of the composition of the ligand of L- and D-proline

Thus, we always used the L-enantiomer of the ligand amino acid for the chiral separations. Next the effect of an exchange against the opposite enantiomer was investigated. We used tryptophan spiked with the L-enantiomer as the analyte to observe the migration order (Fig. 7). L-Proline was replaced progressively by D-proline without changing the total concentration of the amino acid ligand. The separation of tryptophan decreased because of the mixing of the ligand enantiomers by increasing concentration of the amount of D-proline. No separation could be observed at the 1:1 ratio of the proline enantiomers. As expected, the separation increased

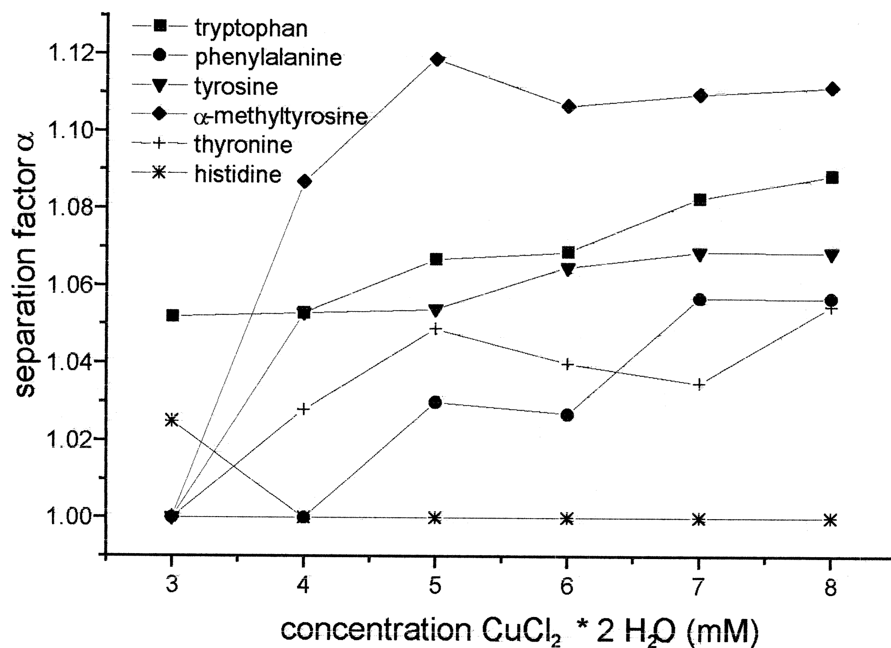

Fig. 5. Dependence of the separation on the concentration of the complexing agents. Conditions: methanol; 25 m*M* ammonium acetate; 1 *M* acetic acid; pH* 3.92; ratio $CuCl_2 \cdot 2H_2O$–L-proline=1:3; uncoated capillary 37 cm (effective length 30 cm)×50 μm; 25 kV; 214 nm; 25°C.

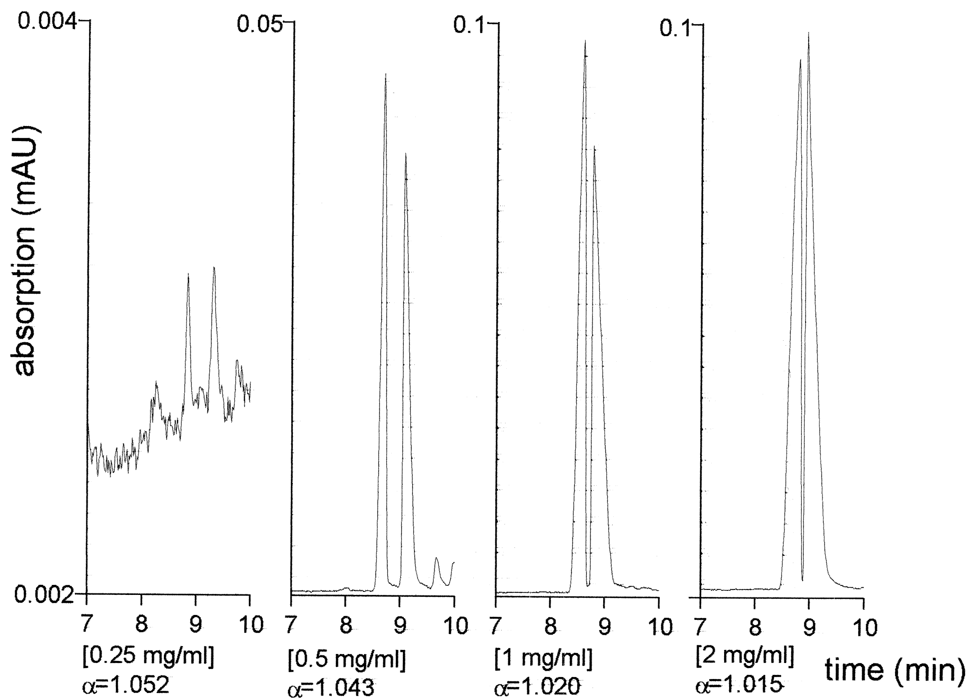

Fig. 6. Dependence of the separations on the concentration of the analytes using tryptophan as an example. Conditions: methanol; 25 m*M* ammonium acetate; 1 *M* acetic acid; pH* 3.92; 6.4 m*M* $CuCl_2 \cdot 2H_2O$; 16 m*M* L-proline; uncoated capillary 37 cm (effective length 30 cm)×50 μm; 25 kV; 214 nm; 25°C.

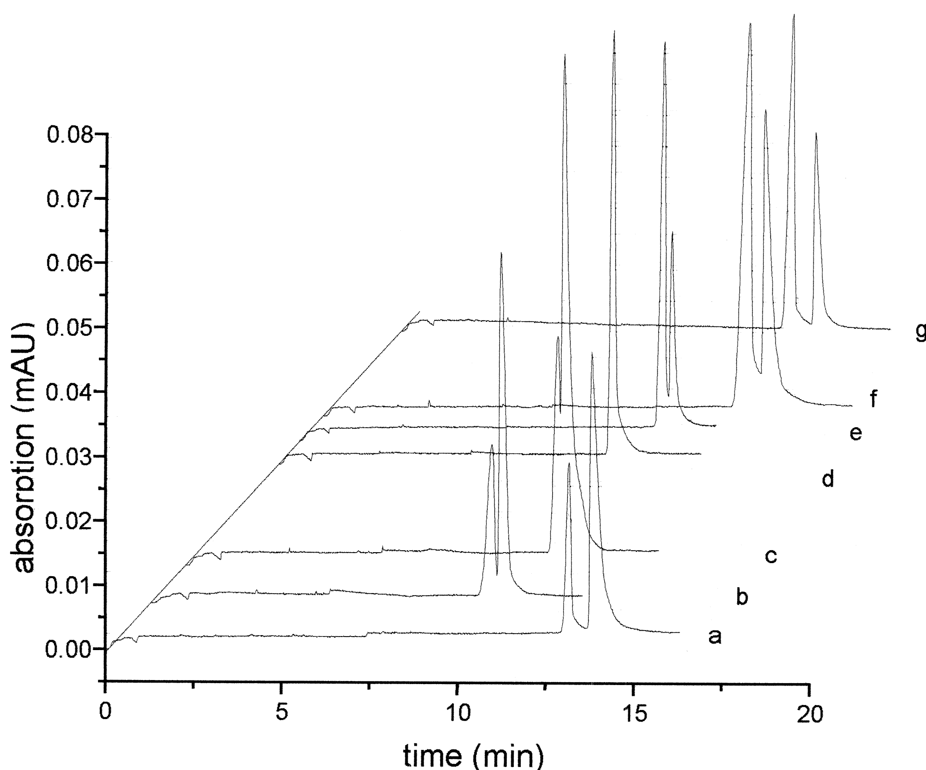

Fig. 7. Dependence of the separations on the composition of the ligand of L- and D-proline. Conditions: methanol; 25 m*M* ammonium acetate; 1 *M* acetic acid; pH* 3.92; 5.86 m*M* $CuCl_2 \cdot 2H_2O$; uncoated capillary 37 cm (effective length 30 cm)×50 μm; 25 kV; 214 nm; 25°C. a=16 m*M* L-proline, α=1.049; b=14 m*M* L-proline–2 m*M* D-proline. α=1.025; c=12 m*M* L-proline–4 m*M* D-proline, α=1.015; d=8 m*M* L-proline–8 m*M* D-proline, α=1.000; e=4 m*M* L-proline–12 m*M* D-proline, α=1.022; f=2 m*M* L-proline–14 m*M* D-proline, α=1.037; g=16 m*M* D-proline, α=1.059.

again after the ratios of L- and D-proline was reversed. With a majority of L-proline, the D-enantiomer migrated first. The reversal of the migration order of the analyte enantiomers could be observed very clearly by changing the ratio of the ligand enantiomers in a stepwise manner. The faster migration of the analyte D-enantiomer could also be observed for all other analytes; this is in agreement with the results of LECE in aqueous media [3].

4. Conclusion

This paper describes the high potential of NACE in the analysis of unmodified amino acids by using the ligand-exchange mechanism. It is the first report of non-aqueous media used successfully in LECE. We were able to completely separate the enantiomeric pairs of each of eight investigated racemic amino acids and to optimize the separation parameters. Further investigations of the separation of other analytes (α-hydroxy acids, β-amino alcohols) with the ligand-exchange mechanism in NACE are in progress.

Acknowledgements

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