ELSEVIER

Journal of Chromatography A, 945 (2002) 249–255

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Chiral separation of underivatized amino acids by ligand-exchange capillary electrophoresis using a copper(II)–L-lysine complex as selector

Xiaoning Lu, Yi Chen*, Lei Guo, Yifang Yang

Group 205, Center for Molecular Sciences, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100080, China

Received 6 June 2001; received in revised form 6 November 2001; accepted 6 November 2001

Abstract

A ligand-exchange capillary electrophoretic method was explored, with L-lysine as the ligand and copper(II) as the central ion. Its applicability was demonstrated with underivatized aromatic amino acids, namely D,L-phenylalanine, D,L-tryptophan, D,L-tyrosine and D,L-β-phenylserine. Optical resolutions of a single pair of amino acid enantiomers, and of mixed amino acids were obtained with a running buffer of 10 m*M* NH_4Ac, 6.67 m*M* Cu(II) and 13.33 m*M* L-lysine, pH 7.0. Sodium dodecylsulfate (SDS) was shown to be necessary for simultaneous separation of the mixed amino acids. The resolution was found to increase with the concentration of the copper(II) complex at a copper(II)-to-lysine ratio of 1:2. If the total concentration of copper(II) and lysine was kept at 20 m*M*, decreasing the ratio of copper(II) to lysine caused a resolution loss of tryptophan, but a slight resolution improvement of the other three amino acids. The pH of buffer is another important factor controlling the separations. For all the studied amino acids, the optimum pH was 6.0. An interesting phenomenon was observed in this study. SDS induces precipitation at a concentration below 32 m*M* at room temperature (16±2 °C), possibly due to the formation of neutral substance from the SDS monomer and the copper(II)–lysine complex.

Keywords: Enantiomer separation; Ligand-exchange capillary electrophoresis; Complexation; Lysine; Amino acids

1. Introduction

Ligand-exchange chromatography has become an important method of resolving the enantiomers of amino acids by HPLC, since 1971 when Davankov and Rogozhin first reported their work [1]. Based on this principle, amino acids can also be optically resolved by capillary electrophoresis (CE). In 1985, Gassman et al. [2] reported the first application of ligand-exchange capillary electrophoresis (LECE) to the resolution of dansylated amino acids, using copper, Cu(II)–L-histidine complex as a selector. They also employed Cu(II)–aspartame to improve the optical resolution [3]. Since then, other ligands have been introduced, such as *N,N*-didecyl-L-alanine [4,5] and L-arginine [6], with the central ions mainly being Cu(II) [4–10]. Schmid and Gübitz [7] found that when L-proline– or L-hydroxyproline–Cu(II) was used, high concentrations of complex were needed to yield an effective chiral separation. They then used *N*-alkyl derivatives of L-hydroxyproline to improve the enantioselectivity [8,9]. A L-hydroxy-

*Corresponding author. Tel.: +86-10-6261-8240; fax: +86-10-6255-9373.

E-mail address: chenyi@public.east.net.cn (Y. Chen).

0021-9673/02/$ – see front matter
PII: S0021-9673(01)01494-7

proline–Cu(II) complex was also employed as additive by Chen et al. to resolve the optical and positional isomers of tyrosine and flurophenylalanine [10], as well as tryptophan derivatives [11], using micellar electrokinetic chromatography (MEKC). The authors observed that the presence of sodium dodecylsulfate (SDS) in the running buffer greatly enhanced the separation, but its concentration should be kept below 15 m*M* to maintain a stable baseline.

These works show that ligand exchange is a versatile procedure to perform chiral separations. There are at least two crucial factors that can be varied to introduce new separation mechanisms and yield a better resolution. The first is the chiral ligand and the second an achiral additive like SDS. This opens a new window to explore more convenient approaches to chiral separation by CE.

The purpose of this paper is to investigate the potential of L-lysine (Lys) as a chiral ligand for the chiral separation of aromatic amino acids, based on the ligand exchange principle. Lys has a positively charged side chain which, we think, will interact with the electron-full aromatic ring of the studied amino acids and play an important role in the enantiorecognition. In the following, we will show that L-Lys is effective in the enantiorecognition of aromatic amino acids when Cu(II) is used as the central ion. Moreover, SDS was found to be able to enhance the enantioseparation dramatically.

2. Experimental

2.1. Chemicals and solutions

All amino acids were biochemicals except Lys which was of analytical grade. D,L-Phenylalanine (Phe), D,L-tryptophan (Trp), D,L-tyrosine (Tyr) and L-Lys were purchased from Sigma (St. Louis, MO, USA) and D,L-β-phenylserine (pSer) from the Institute of Microbiology, Chinese Academy of Sciences. L-Phe, L-Trp and L-Tyr and other reagents were all of analytical grade obtained from Beijing Chemical Work (Beijing, China).

Stock solutions of samples were prepared by dissolving 2–4 mg of amino acids in 1 ml double-distilled water. They were stored at 4 °C. Injected samples were prepared by diluting the stock solutions with water by a factor of 5–10. Running buffer was prepared by dissolving copper(II) sulfate and L-Lys in 10 m*M* ammonia acetic acid, adjusted to a desired pH with 1 *M* ammonia or 1 *M* acetic acid. All solutions were prepared in double-distilled water and filtered through a 0.45-μm membrane (Nihon Millipore, Japan) prior to use.

2.2. Instrumentation and separation conditions

A P/ACE 2050 capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) equipped with an UV detector was used. Sample detection was performed at 214 nm. Uncoated fused-silica capillaries of 57 cm (effective length 50 cm)× 50 μm I.D. were used (Yongnian Optic Fiber Work, Heibei, China). The capillary temperature was maintained at 20 °C and the room temperature at 16±2 °C. Dimethyl sulfoxide (DMSO) was used to mark the electroosmotic flow (EOF).

Samples were injected with pressure at 0.5 p.s.i. for 2 s and separated at 20 kV, unless noted otherwise (1 p.s.i.=6894.76 Pa). Prior to use, the capillaries were rinsed successively with water, 0.1 *M* NaOH, and water for 5 min each. It was observed that Lys adsorbed onto the capillary through electrostatic interactions. In order to ensure the reproducibility of migration time, capillaries were sequentially washed between two successive runs with: (i) water, (ii) 1% HNO_3, (iii) water, (iv) 0.1 *M* NaOH, and (v) water for 2.0 min each.

Peaks were identified by adding an amount of a known solute to the sample solutions.

3. Results and discussion

3.1. Basic consideration

Lys is a basic amino acid with an isoelectric point (p*I*) at 9.4, possessing a side chain of an amino butyl group; all the tested amino acids are neutral with their p*I* values at around 6.0. Lys was selected so that it can (or possibly can) interact with negatively charged additive such as SDS. Thus, a concerted effect may be generated between the chiral complex and SDS to enhance the resolution.

3.2. Chiral separation of a single amino acid

When Lys is used as a chiral ligand and Cu(II) as a central ion, the enantiomers of an amino acid can easily be separated by either capillary zone electrophoresis (CZE) or MEKC. CZE is easier to carry out than MEKC, but its separation efficiency and resolution are poorer than MEKC (comparing Fig. 1A and B). The EOF under the pH of the buffer 6.0–8.0 in CZE is so small that it is not measurable. The EOF suppression may be due to the adsorption of the positively charged side chain of Lys onto the capillary wall. Such adsorption also leads to band broadening. This negative effect is prevented by the addition of SDS to compensate for the positive charge.

3.3. Chiral separation of mixed amino acids

Mixed amino acids cannot be well resolved by CZE no matter how the separation is optimized. The reason is that the mobilities of these amino acids are too close (Fig. 2). This problem has been solved by addition of SDS into the CZE running buffer, namely by using MEKC (Fig. 3A).

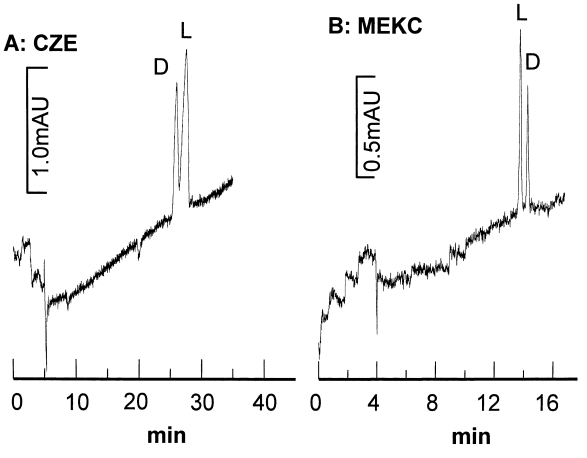

Fig. 1. Elution order of the enantiomers of Trp as SDS is absent (A) and present (B) in the buffer. The ratio of L-Trp to D-Trp in the sample is 2:1. Conditions for (A) buffer: 10 m*M* NH_4Ac, 6.67 m*M* Cu(II) and 13.33 m*M* L-Lys at pH 7.0; separation voltage: 23 kV. (B) Buffer: 10 m*M* NH_4Ac, 1.33 m*M* Cu(II), 2.67 m*M* L-Lys and 50 m*M* SDS at pH 6.2. For other conditions, see Experimental.

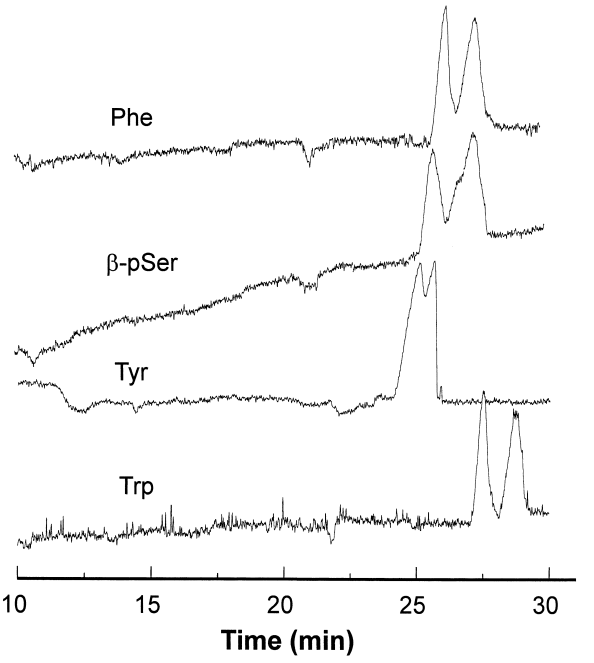

Fig. 2. Typical electropherograms of the chiral separations of the four amino acids by CZE. For all analytes, the D-form elutes before the L-form. Buffer: 10 m*M* NH_4Ac, 6.67 m*M* Cu(II) and 13.33 m*M* L-Lys, at pH 7.0; for other conditions see Experimental.

3.4. Migration order

Fig. 2 shows that the elution order of the enantiomers is reversed when SDS is added. In CZE, the D-form elutes before the L-form, while the opposite occurs in MEKC. In CZE, a Cu(II) ternary complex may form in the presence of an aromatic amino acid in addition to Lys. Under neutral pH, such complexes are positively charged and will move faster than the free amino acids. In addition, in such a ternary complex, electrostatic interaction can take place between the positively charged side chain of Lys and the electron-full aromatic ring of the amino acids. According to Zare and co-workers [2,3], the interaction groups are closer in the configuration of L-Cu–D complex than L-Cu–L complex. Thus, the D enantiomer forms a more stable complex and migrates faster than the L enantiomer. In MEKC the SDS micelles, each having a negatively charged exterior and hydrophobic interior, can interact with the positively charged amino group of L-Lys through electrostatic interaction and the residue of the aro-

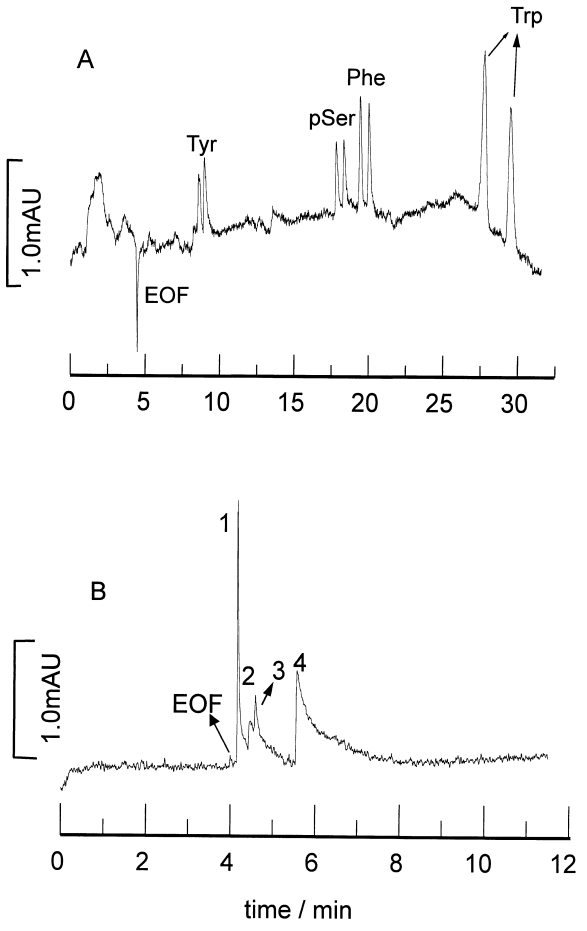

Fig. 3. Electropherogram of separation of the mixture of the four amino acids with (A) or without (B) Cu(II)–L-Lys complex. Peaks: 1=Tyr; 2=β-pSer; 3=Phe; 4=Trp. Conditions: (A) buffer: 10 m*M* NH_4Ac, 50 m*M* SDS, 3.33 m*M* Cu(II) and 6.67 m*M* L-Lys, at pH 6.0; (B) buffer: 10 m*M* NH_4Ac and 50 m*M* SDS, at pH 6.0; for other conditions, see Experimental.

matic amino acids through hydrophobic interaction, respectively. Again, the L-Lys–Cu(II)–D-amino acid complexes are more stable than the corresponding L-Lys–Cu(II)–L-amino acid complexes, since the residues of L-Lys and the aromatic amino acids in the former complex are closer and can interact simultaneously with SDS micelles more conveniently. However, because of the high density of the negative charges that a SDS micelle possesses, the interactions between the SDS micelles and Cu(II) complexes make the Cu(II) complexes migrate like anions. This is evident in the electropherogram where the aromatic amino acids pass the detection window after the EOF (see Fig. 3A). Consequently, the migration order is reversed.

3.5. Critical conditions

For an ideal separation, all factors have to be considered, such as buffers, temperature, voltage, and so forth. But usually, we just optimized some critical conditions namely (1) the concentration of the Cu(II)–Lys complex which in turn affects the ratio of Lys to Cu(II); (2) the buffer pH and (3) the SDS concentration.

3.5.1. Cu(II)–L-Lys complex

Achiral separation of Tyr, pSer, Phe, and Trp by MEKC was performed but the resolution and peak shape were poor (Fig. 3B). This poor separation was greatly improved when the Cu(II)–L-Lys complex was present in the running buffer, although the migration times are prolonged (Fig. 3A). This indicates that the chiral complex greatly influences the migration behavior of the amino acids. Further studies demonstrated that the optical resolution, and also the elution time, increases steadily with the concentration of Cu(II)–L-Lys complex (Table 1), while the molar ratio of Cu(II) to L-Lys was kept at 1:2.

If the molar ratio of Cu(II) to L-Lys is changed, a more complicated impact on resolution can be observed. Fig. 4 shows that the resolution of Trp is much more sensitive to the ratio than the others: its resolution decreases rapidly as the ratio decreases; on the contrary, the resolutions of the other amino acids slightly increases. In addition, pSer and Phe tend to overlap at the low ratio range. Thus, no optimum ratio exists in the tested range and we have to compromise between the resolution of Trp and of the other amino acids. A ratio at 1:2 was shown to be suitable for our separations.

Generally, increasing the ratio of L-Lys, while the total concentration of Cu(II) and L-Lys is kept constant, reduces the competitive opportunity for the solutes to form complex with Cu(II). Consequently, the optical resolution of the solutes should decrease. This is true for the resolution of Trp. As for the other amino acids, the increasing resolution with the ratio of L-Lys may be because of their different molecular

Table 1
Influence of concentration (C) of Cu(II) and L-Lys on separation by MEKC

C (mM)	Tyr			β-pSer			Phe			Trp		
	t_L (min)	t_D (min)	R_s	t_L (min)	t_D (min)	R_s	t_L (min)	t_D (min)	R_s	t_L (min)	t_D (min)	R_s
0	4.17	4.17	0.00	4.45	4.45	0.00	4.59	4.59	0.00	5.57	5.57	0.00
4	6.28	6.73	0.75	11.73	11.93	0.60	12.27	12.46	0.73	15.66	16.12	1.10
8	7.21	7.42	0.80	12.48	12.73	0.90	13.23	13.48	0.96	16.53	17.13	1.50
12	7.81	8.11	0.90	13.74	14.03	0.90	14.78	15.10	0.96	18.47	19.22	1.80
16	7.90	8.25	1.21	14.13	14.50	1.21	15.10	15.45	1.21	19.08	20.10	2.11
20	8.15	8.57	1.70	15.59	16.12	1.45	18.48	18.70	1.53	24.49	26.10	3.10

Conditions: buffer: 10 mM NH_4Ac, 50 mM SDS and different amounts of Cu(II) and L-Lys at ratio 1:2, pH 6.0; for other conditions, see Experimental; t_L and t_D are the migration times of the L- and D-enantiomers, respectively.

structures from that of Trp. Each of these three amino acids contains a phenyl group while Trp has a indolyl ring. Additionally, the migration times of all the amino acids decrease with the ratio of Cu(II) to L-Lys, because more free amino acids which move faster than the corresponding Cu(II) complex appear in the buffer as the concentration of L-Lys increases.

3.5.2. pH

Considering biological applications, it is preferable to perform separations around a neutral pH. The influence of pH on the optical resolution was thus investigated from 5.0 to 8.0 at an increment of 0.5, with a running buffer containing 10 mM NH_4Ac, 50 mM SDS, 3.33 mM $CuSO_4$ and 6.67 mM L-Lys. Distinct maxima of the chiral separations could be observed at pH 6.0 (Fig. 5). The existence of a maximum pH is explained as follows. As pH decreases from 8.0 to 5.0, the free amino acids are changed from negative ions to the neutral form (at their pI around 6.0) or even positive ions, while the Cu(II) complexes remain to migrate as anions in the presence of SDS micelles. As a result, the difference in mobilities between the free amino acids and the corresponding Cu(II) complexes is enhanced. Thus, the chiral resolutions should be improved. But decreasing pH may also reduce the extent of complexation of the Cu(II) ternary complex because H^+

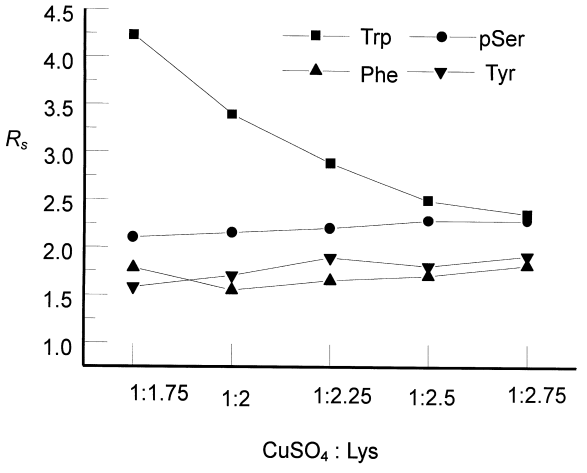

Fig. 4. Dependence of resolution on the ratio of Cu(II) to L-Lys. Buffer: 10 mM NH_4Ac, 20 mM of Cu(II) and L-Lys at different ratio, and 50 mM SDS, pH 6.0; for other conditions, see Experimental.

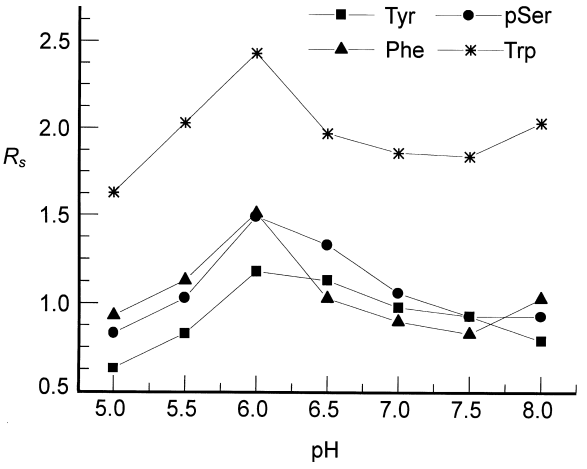

Fig. 5. Influence of pH on the optical resolutions of the four amino acids by MEKC. Conditions: buffer: 10 mM NH_4Ac, 3.33 mM Cu(II) and 6.67 mM L-Lys, and 50 mM SDS, at different pH values; for other conditions, see Experimental.

will complete with Cu(II) ions for the amino groups of the amino acids. This untoward effect is more considerable when pH is in the acidic range. The compromise of these two effects results in a maximum pH for the chiral separation. The pH point at 8.0 is a bit abnormal, where optical resolutions of Phe and Trp are better than those at pH 7.5. As expected, the migration time increased with pH. Thus, running buffer at pH 6.0–6.5, was adopted to yield better resolution and shorter separation time.

3.5.3. SDS

Different concentrations of SDS were added to the buffer containing 10 m*M* NH_4Ac, 6.67 m*M* Cu(II) and 13.33 m*M* L-Lys at pH 6.0. As we hoped, the presence of SDS greatly improved enantioseparation of mixed amino acids (Fig. 3A).

An interesting phenomenon was observed: precipitation appeared when SDS was added at below a certain concentration; the precipitation disappeared when more SDS was added. The critical concentration was around 32 m*M* at room temperature (16±2 °C). This phenomenon has not been mentioned in the literature to date. Further investigation revealed that the mixing SDS with 13.33 m*M* L-Lys or with 6.67 m*M* $CuSO_4$ did not lead to precipitation, as the concentrations of SDS varied from 0 to 60 m*M*. The phenomenon may be caused by interactions between the Cu(II) complex and SDS monomer or SDS micelle. At low SDS concentrations (<32 m*M*), the interaction between SDS monomer and the Cu(II) complex should dominate, which will result in the formation of neutral and insoluble substance. At higher SDS concentrations, the interaction between SDS micelles and Cu(II) complex should dominate, leading to the formation of negatively charged and soluble complex of SDS, Cu(II), Lys and an amino acid.

As expected, both chiral and achiral resolutions of amino acids increased with SDS concentration above the critical concentration of 32 m*M*. Too high a concentration of SDS prolongs the separation time and also increases the running current (see Table 2). Very concentrated SDS is not required. Simultaneous baseline chiral resolution of the four amino acids could be achieved at 50 m*M* SDS when the running buffer containing Cu(II) complex above 10 m*M* (Fig. 3A).

4. Conclusion

The chiral Cu(II)–L-Lys complex shows effective enantiorecognition toward the studied amino acids, namely Phe, Trp, Tyr, and pSer, both in CZE and MEKC. Moreover, the presence of SDS in the buffer can greatly improve the chiral separation. A simple method for simultaneous separation of the enantiomers, based on the exchange ligand principle, has been thus developed. Further efforts will be made to extent the applications of this method.

Acknowledgements

The authors would like to thank the National Natural Sciences Foundation of China (no. 29825112) and the Chinese Academy of Sciences for the financial support of this work.

Table 2
Influence of SDS concentration on the chiral separation by MEKC

	40 m*M*			50 m*M*			60 m*M*			70 m*M*		
	t_L (min)	t_D (min)	R_s	t_L (min)	t_D (min)	R_s	t_L (min)	t_D (min)	R_s	t_L (min)	t_D (min)	R_s
Tyr	7.39	7.71	0.90	7.49	7.84	1.70	8.15	8.57	1.70	8.74	9.20	1.10
pSer	14.33	14.87	1.01	15.59	16.12	2.00	16.14	16.86	1.56	18.45	19.17	1.60
Phe	16.25	16.74	0.96	18.18	18.70	1.53	19.94	20.86	1.35	21.89	22.68	1.40
Trp	22.37	24.08	2.10	24.49	26.10	3.00	27.12	29.29	3.50	30.08	32.38	3.80

Conditions: buffer, 10 m*M* NH_4Ac, 6.67 m*M* Cu(II) and 13.33 m*M* L-Lys, with different amounts of SDS, pH 6.2; for other conditions, see Experimental.

References

[1] V.A. Davankov, S.V. Rogozhin, J. Chromatogr. 60 (1971) 280.
[2] E. Gassman, J. Kuo, R. Zare, Science 230 (1985) 813.
[3] P. Gozel, E. Gassman, H. Michelsen, R. Zare, Anal. Chem. 59 (1987) 44.
[4] A.S. Cohen, A. Paulus, B.L. Karger, Chromatographia 24 (1987) 15.
[5] N.G. Sundin, T.M. Dowling, N. Grindberg, G. Bicker, J. Microcol. Sep. 8 (1996) 323.
[6] Z. Yuan, L. Yang, S. Zhang, Electrophoresis 20 (1999) 1842.
[7] M.G. Schmid, G. Gübitz, Enantiomer 1 (1996) 23.
[8] Á. Végári, M.G. Schmid, F. Kilár, G. Gübitz, Electrophoresis 19 (1998) 2109.
[9] M.G. Schmid, R. Rinaldi, D. Dreveny, G. Gübitz, J. Chromatogr. A 846 (1999) 157.
[10] Z. Chen, J.M. Lin, K. Uchiyama, T. Hobo, J. Chromatogr. A 813 (1998) 369.
[11] Z. Chen, J.M. Lin, K. Uchiyama, T. Hobo, Chromatographia 49 (1999) 436.