Journal of Chromatography A, 1217 (2010) 1425–1428

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Short communication

Enantioseparation of α-amino acids on an 18-crown-6-tetracarboxylic acid-bonded silica by capillary electrochromatography

Taeho Lee [a], Wonjae Lee [b], Myung Ho Hyun [c], Jung Hag Park [a,*]

[a] Department of Chemistry, Yeungnam University, 214-1 Daedong, Gyeongsan 712-749, South Korea
[b] College of Pharmacy, Chosun University, Gwangju 501-759, South Korea
[c] Department of Chemistry, Pusan National University, Busan 609-735, South Korea

ARTICLE INFO

Article history:
Received 19 October 2009
Received in revised form
16 December 2009
Accepted 22 December 2009
Available online 4 January 2010

Keywords:
Enantioseparation
α-Amino acids
18-Crown-6-tetracarboxylic acid-bonded silica
CEC

ABSTRACT

(−)-(18-Crown-6)-2,3,11,12-tetracarboxylic acid-bonded silica was used as the chiral stationary phase in capillary electrochromatography (CEC) for enantioseparation of some α-amino acids. Separation data in CEC were measured in mobile phases of varying pH, and composition of methanol and buffer, and compared with those in capillary liquid chromatography (CLC). In CEC better enantioseparation was generally obtained in the eluent of lower pH, higher buffer concentration and intermediate MeOH content, usually at the expense of analysis time. CEC showed generally better enantioselectivity and resolutions than CLC for the amino acids investigated.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

18-Crown-6-2,3,11,12-tetracarboxylic acid (18C6H4) has been widely utilized as a chiral selector for the resolution of racemic amino compounds in capillary electrophoresis (CE) [1–4] and its silica-based stationary phase more widely as the chiral stationary phase (CSP) in high performance liquid chromatography (HPLC) [5,6]. Application of 18C6H4-bonded silica CSP has been reviewed recently [6]. Chiral recognition mechanism for the resolution of primary amino compounds on this CSP is believed to involve the tripodal complexation of the protonated primary amino group ($R\text{-}NH_3^+$) inside the cavity of the 18-crown-ether ring via three $^+N\text{–}H\cdots O$ hydrogen bonds and additional roles played by the two free carboxylic acid groups acting as chiral barriers, enantioselective hydrogen bonding sites, or ionic interaction sites [7–9].

Capillary electrochromatography (CEC) is a hybrid technique that combines the useful features of CE and HPLC, and its high efficiency and fast analysis make the technique an attractive tool for chiral separation [10,11]. Although there have been reported a couple of works in which crown ether-capped β-cyclodextrin-bonded silica [12,13] and crown ether-bonded polyacrylamide gels [14] were used as CSPs in CEC, the 18C6H4-based silica CSPs have not been utilized in enantioseparation of primary amino compounds by CEC. In this work we report enantiomer separation of four typical α-amino acids on a (−)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-bonded silica (CSP **1**) (Fig. 1) by reversed-phase CEC in the MeOH/Bis–Tris buffer mobile phases. The CSP **1** was originally developed for HPLC [15,16] and was used in this work without further optimization for CEC application. The effects of pH, and composition of methanol and buffer in the mobile phase were examined, and the CEC resolution data were compared with the capillary LC (CLC) data obtained in the same mobile phases.

2. Experimental

CSP **1** was prepared by bonding (−)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (from Aldrich, Milwaukee, USA) to aminopropyl silica (Kromasil, 5 μm, 100 Å, 320 m^2/g, surface coverage = 4.5 μmol/m^2) as previously reported [15]. Surface coverage of the chiral selector, 18C6H4, on CSP **1** based on the percent carbon from microanalysis was found to be 0.49 μmol/m^2. CSP **1** was slurry-packed in methanol by using Alltech slurry packer (Deerfield, USA) at *ca.* 60 MPa into a 100 μm I.D. × 363 μm O.D. fused silica capillary (Polymicro Technologies, Phoenix, USA) with packed bed length of 200 mm and total length of 360 mm during which the slurry reservoir was continuously sonicated. The end frits were manufactured from 5-μm silica and the mid-frit from sintering of the packing material while water flowed through the

* Corresponding author. Tel.: +82 53 810 2360; fax: +82 53 810 4613.
E-mail address: jhpark@ynu.ac.kr (J.H. Park).

0021-9673/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2009.12.064

column under a pressure of *ca*. 6.9 MPa, and the detection window was made immediately after the outlet frit by burning a section of polyimide coating with a heating coil. No attempt was made to optimize the packing procedure. HPLC-grade methanol was obtained from J.T. Baker (Phillipsburg, USA). Water was processed with an Elgastat UHQ water purification system (Bucks, UK). Bis(2-hydroxyethyl)iminotris(hydroxymethyl) methane (Bis–Tris), uracil, D- and L-phenylalanine (Phe), D- and L-phenylglycin (PhG), D- and L-tyrosine (Tyr) and D- and L-tryptophan (Trp) were obtained from Aldrich (Milwaukee, USA).

CEC measurements were carried out at 25 °C on an Agilent HP^{3D} CE system (Palo Alto, USA) with HP^{3D} CE ChemStation software. An external pressure of 1 MPa was applied to both buffer reservoirs. The mobile phases were mixtures of methanol with Bis–Tris buffer of varying composition. Bis–Tris buffers were prepared by titrating aqueous Bis–Tris solutions with citric acid to the desired pH. They were filtered through a nylon membrane filter of 0.2-μm pore size and degassed prior to use. The column was preconditioned for at least 12 h using the mobile phase before the chromatographic measurements. Approximately equal amounts of enantiomers of each amino acid dissolved in the mobile phase were injected electro-kinetically at 15 kV for 5 s and detected at 210 and 254 nm. Retention times of three consecutive injections were in agreement within 3%. Dead time was measured by injecting uracil. CLC measurements were carried out using the same column as used in CEC on an LC system composed of an Eldex MicroPro Series 1000 syringe pump (Napa, USA) set at a constant flow rate of 1 μL/min, a Valco Model 7620 injector with internal loop volume of 60 nL (Houston, USA), a Linear Model 200 UV–vis detector (San Jose, USA) for on-column detection at 210 nm, and chromatograms were recorded with Autochro-2000 chromatography data system from Young Lin Instruments Co. (Anyang, Korea).

3. Results and discussion

3.1. Influence of pH

On CSP **1** prepared with aminopropylsilica gel are there both residual aminopropyl groups with pK_a value of around 10 [17], residual silanol groups with pK_a values between 2 and 4 on the silica [18] and two residual carboxyl groups on the crown ether ring with pK_a values of 2.1 and 2.8 [19] after chemical modification. The

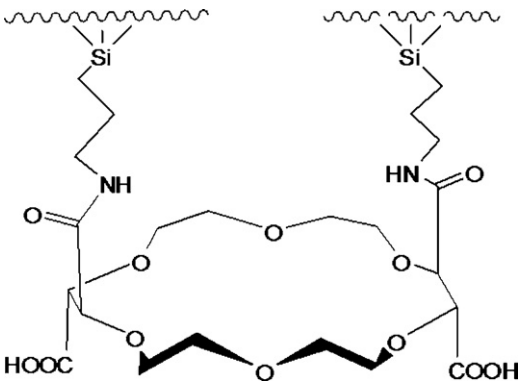

Fig. 1. Structure of CSP **1**.

magnitude and direction of electroosmotic flow (EOF) is dependent on the net charge of the surface at the pH of the mobile phase. In mobile phases with strongly acidic modifiers, which are generally incorporated in RPLC to effect protonation of the amino group of the analyte, basic residual aminopropyl groups are completely protonated to give positive charges while carboxyl and silanol groups are partially ionized to give negative charges. These two oppositely charged groups compensate each other and resultant net charge on the surface contributes to effective EOF. Instead of aqueous organic buffers with acid modifier such as sulfuric acid, which is usually employed in chiral separation by RPLC [15,16], we used MeOH/Bis–Tris buffers as the mobile phase. Bis–Tris was chosen as the buffer material as it does not bind to 18C6H4 and hence gave better enantioseparation of primary amines than other buffers in CE [20]. Fig. 2 shows exemplary chromatograms for the enantioseparation of Phe, along with theoretical numbers for the first-eluting enantiomer (N_1) and EOF measured by uracil on CSP **1** in 20:80 (v/v%) MeOH/Bis–Tris buffer at different pH, along with CLC chromatograms for Phe in the same mobile phases. Cathodic EOFs were observed at all times in the pH range investigated (3.0–4.5), indicating the net charge of CSP **1** is negative. Electrophoretic mobilities of protonated solutes are also cathodic, contributing to the transport of the solutes. Even at pH 3.0 reasonably fast EOF was observed to have the second enantiomer eluted in less than 30 min. Upon increasing pH from 3.0 to 3.5 the magnitude of EOF almost doubles and retention time decreases to one half. EOF increases monotonically with pH as more surface silanol groups dissociate and less

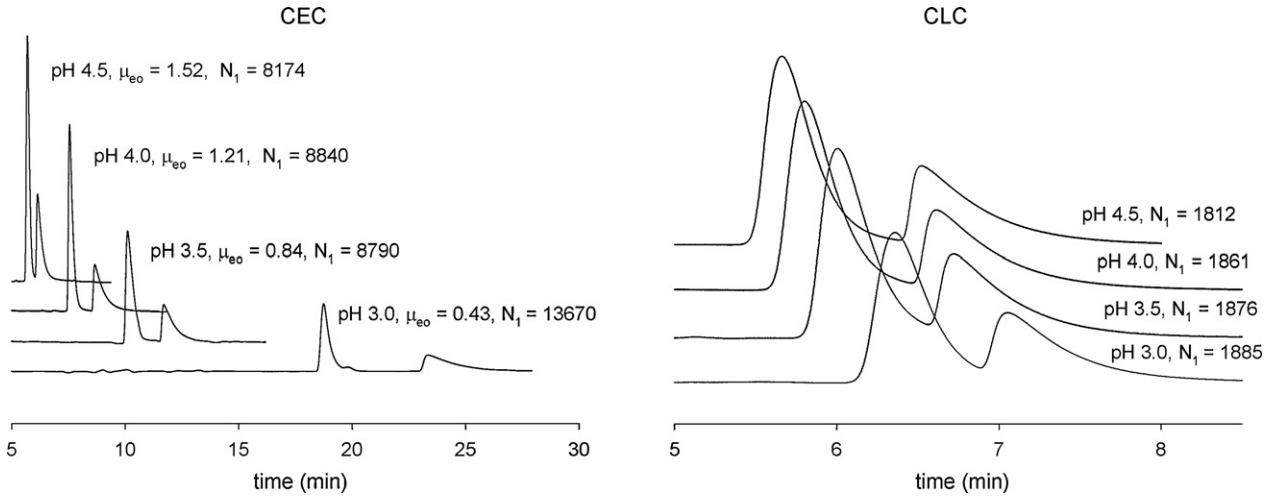

Fig. 2. Chromatograms for the enantioseparation of Phe on CSP **1** at different pH. Electroosmotic mobilities (μ_{eo} [10^{-4} cm^2 s^{-1} V^{-1}]) measured by uracil and theoretical numbers for the first-eluting enantiomers (N_1) are also shown. CEC conditions: mobile phase, 20:80 (v/v%) MeOH/Bis–Tris buffer (20 mM). Other conditions are as described in the footnote of Table 1. CLC conditions: flow rate = 1 μL/min; injection volume = 60 nL. Mobile phases are the same as in CEC.

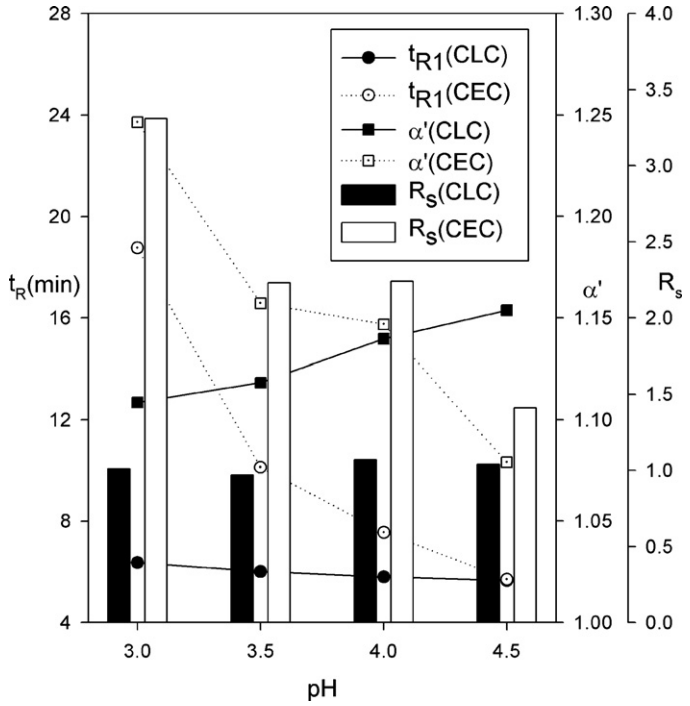

Fig. 3. Influence of pH on t_{R1}, α', R_s of Phe on CSP **1**. Conditions are as specified in Fig. 2.

amino groups in the tether are protonated to leave a greater net negative charge on the surface.

Enantioseparation data for the amino acids by CEC and CLC are listed in Table 1. Fig. 3 shows variations of retention time of the first-eluting enantiomer (t_{R1}), enantioselectivity (α'), resolution (R_s) of Phe with pH on CSP **1** as an example. In CEC three processes are generally involved in the enantioseparation of chiral cationic solutes on CSP **1** in eluents of acidic aqueous organic buffer: EOF, electrophoretic migration of the cationic solutes which is co-electroosmotic, and chiral discriminative interactions of the solute with the CSP. Chiral discrimination is based on the difference in binding strength of the analyte with CSP. Retention time decreases with pH as EOF increases with pH. Enantioselectivity for Phe decreases with pH in CEC. Tripodal complexation of the protonated primary amino group of the analyte inside the cavity of the crown ether ring via three $^{+}$N–H···O hydrogen bonds is essential for chiral discrimination [7]. The pK_{a1} values of the amino acids are all smaller than 3 and pK_{a2} values are greater than 9 [17]. In mobile phase of pH 3–4.5, the amino groups of the amino acids are completely protonated while the carboxylic acid groups are partially deprotonated. As pH increases the extent of protonation of the amino groups of the analytes decreases, which in turn will lead to weaker tripodal complexation, resulting in diminished enantioselectivity. Upon an increase of pH, ionization of the carboxylic groups of the amino acids increases. As a result, non-stereoselective ionic interaction of negatively charged carboxylic groups of the analyte with the positively charged amino groups of silica surface increases and in turn acts to hinder the desired tripodal complexation of the primary amino groups of the analyte with the crown ether ring, thereby causing enantioselectivity to diminish. Resolution also decreases with pH. Decreasing α values and plate numbers causes resolution to decrease according to the relationship, $R_s = 1/4 \cdot N^{1/2} \cdot (\alpha - 1)$ [21]. Similar trends in behaviors of retention, enantioselectivity and resolution are also observed for remaining amino acids. While the first-eluting peak for the weakly interacting enantiomer is not broad the second eluting peak is much broader the first one. This is likely due to the

Table 1
Chiral separation of amino acids in MeOH/Bis–Tris buffer with different compositions by CEC and CLC[a].

	Mobile phase[b]	Phe			Trp			PhG			Tyr		
		t_{R1}	α'	R_s	t_{R1}	α'	R_s	t_{R1}	α'	R_s	t_{R1}	α'	R_s
A	3.0	18.75 (6.36)	1.25 (1.11)	3.31 (1.01)	25.61 (8.66)	1.29 (1.17)	3.95 (1.39)	22.85 (6.41)	1.27 (1.16)	3.04 (1.03)	19.39 (6.68)	1.14 (1.08)	2.91 (0.73)
	3.5	10.12 (6.01)	1.16 (1.12)	2.23 (0.97)	15.12 (8.23)	1.22 (1.19)	3.06 (1.39)	11.58 (6.14)	1.20 (1.18)	2.30 (1.30)	10.90 (6.36)	1.12 (1.10)	1.76 (0.84)
	4.0	7.56 (5.80)	1.14 (1.14)	2.24 (1.07)	10.86 (8.09)	1.20 (1.21)	2.96 (1.64)	7.97 (6.03)	1.20 (1.17)	2.16 (1.23)	8.00 (6.04)	1.12 (1.11)	1.78 (1.06)
	4.5	5.70 (5.66)	1.08 (1.15)	1.41 (1.04)	7.94 (7.86)	1.13 (1.24)	2.03 (1.95)	22.85 (5.88)	1.27 (1.17)	3.04 (1.06)	6.22 (5.84)	1.06 (1.11)	1.22 (0.91)
B	10	7.72 (5.83)	1.05 (1.08)	1.03 (0.74)	11.09 (8.02)	1.09 (1.10)	1.60 (1.13)	8.30 (5.94)	1.07 (1.09)	1.36 (0.88)	8.42 (5.97)	1.04 (1.04)	0.95 (0.48)
	20	10.12 (6.01)	1.16 (1.12)	2.23 (0.97)	15.12 (8.23)	1.22 (1.19)	3.06 (1.39)	11.58 (6.14)	1.20 (1.18)	2.30 (1.30)	10.90 (6.36)	1.12 (1.10)	1.76 (0.84)
	30	12.34 (6.12)	1.14 (1.12)	1.53 (1.00)	16.34 (8.19)	1.17 (1.17)	2.60 (1.47)	13.74 (6.37)	1.18 (1.17)	2.40 (1.40)	12.35 (6.51)	1.12 (1.09)	2.04 (0.89)
	40	12.98 (6.63)	1.18 (1.16)	1.98 (1.16)	17.27 (8.73)	1.19 (1.21)	2.67 (1.53)	14.10 (6.68)	1.24 (1.19)	2.67 (1.23)	14.16 (6.66)	1.11 (1.12)	2.05 (1.11)
C	10	8.22 (4.24)	1.12 (1.16)	2.03 (1.14)	11.80 (5.73)	1.18 (1.20)	2.26 (1.39)	8.50 (4.08)	1.17 (1.15)	2.14 (1.03)	8.61 (4.03)	1.11 (1.09)	1.82 (0.78)
	20	9.58 (4.79)	1.07 (1.06)	1.36 (0.72)	12.77 (6.17)	1.12 (1.11)	1.86 (1.09)	9.84 (4.51)	1.13 (1.10)	1.81 (0.84)	10.10 (4.51)	1.08 (1.06)	1.59 (0.58)
	30	12.65 (5.90)	1.22 (1.16)	2.97 (1.20)	18.37 (8.12)	1.25 (1.18)	2.97 (1.36)	14.09 (6.11)	1.24 (1.18)	2.45 (1.22)	12.88 (5.90)	1.17 (1.10)	2.23 (0.85)
	50	15.91 (7.38)	1.25 (1.06)	3.00 (0.59)	18.54 (8.97)	1.20 (1.08)	2.60 (1.03)	17.06 (7.16)	1.29 (1.11)	2.43 (1.27)	16.97 (7.51)	1.19 (1.06)	2.28 (0.78)
	70	16.41 (10.57)	1.13 (1.12)	1.72 (0.95)	19.91 (11.02)	1.14 (1.11)	1.78 (1.01)	18.14 (9.97)	1.24 (1.18)	1.97 (1.43)	17.77 (9.83)	1.14 (1.09)	1.74 (0.98)

[a] The CLC separation data are given in parentheses. CEC conditions: column, length 360 mm (packed bed 200 mm; applied voltage, 20 kV (16.7 μA); temperature, 25 °C; injection, 15 kV for 5 s; detection, at 210 and 254 nm. CLC conditions are as given in Section 2. t_{R1}, retention time of the first-eluting enantiomer; α', apparent enantioselectivity (=t_{R2}/t_{R1}); R_s, resolution factor.

[b] Mobile phases: A, 20:80 (v/v%) MeOH/buffer (20 mM) with different pH; B, 20:80 (v/v%) MeOH/buffer (pH 3.5) with different buffer concentration (mM); C, MeOH/buffer (10 mM, pH 3.5) with different MeOH content (vol.%).

slow dissociation kinetics of the strongly interacting second eluting enantiomer.

While enantioselectivity and resolution show decreasing trends for all four analytes with pH in CEC, trends in α and R_s values in CLC are not wholly consistent for the four analytes. The reason for these resolution behaviors in CLC is not yet clear [16]. CEC and CLC separations were obtained with the same column and mobile phase compositions but the eluent flow rate was not adjusted to make the linear flow rate in CEC similar to that in CLC, due to practical difficulties. Linear flow rates in CEC were lower than those in CLC. Applying a voltage higher than 20 kV for faster linear velocity in CEC was accompanied by problem of excessive Joule heating, and decreasing volume flow rate for slower linear velocity in CLC was accompanied by excessive peak broadening of already broad peaks as seen in Fig. 2. Approximate comparison of performances of the two separation modes seems to be permissible yet although exact comparison is not quite possible due to different linear flow rates used. Resolution and enantioselectivity values are generally much better in CEC than in CLC, particularly in eluents of lower pH. The higher resolution observed in CEC is a consequence of the much higher plate numbers due to the flat EOF profile [10] while the higher enantioselectivity in CEC is not obvious yet. Better enantioseparation can be obtained in the eluent of lower pH at the expense of analysis time in CEC.

3.2. Influence of buffer concentration

EOF decreases with increasing buffer concentration as a consequence of decreasing double layer thickness and zeta potential [10]. The EOF values measured by uracil in 20:80 (v/v%) MeOH/Bis–Tris buffer (pH 3.5) were 1.16, 0.84, 0.70 and 0.68 [10^{-4} cm^2 s^{-1} V^{-1}] at the buffer concentration of 10, 20, 30 and 40 mM, respectively. An increase of the buffer concentration causes an increase in retention in CEC while retention in CLC is hardly changed (Table 1). As the concentration of the buffer increases the ionic strength also increases. The increasing ionic strength can cause the solubility of the amino acids with hydrophobic residue to decrease in the mobile phase and consequently result in increased retention. It seems that the extent of the effect of the change in the ionic strength on retention in CLC is small compared to the corresponding effect caused by decreasing zeta potential in CEC.

CEC enantioselectivity and resolution also show more or less increasing trends. The reason for increasing enantioselectivity with increasing buffer content in CEC is not clear. One plausible explanation is as follows: Although the cationic components of the Bis–Tris-citrate buffer are not to affect the enantioselectivity due to the negligible binding inside the cavity of crown ether [20], they can undergo ionic interactions with negatively charged surface silanol groups. This will in turn reduce undesirable non-stereoselective interactions between positively charged amino groups of the analyte and negatively charged silanol groups, thereby contributing to better enantioselectivity, and resolution as well as a consequence of the faster desorption equilibrium. Better enantioselectivity and resolution are in general obtained with higher buffer concentration in both CEC and LC again at the expense of separation time.

3.3. Influence of methanol content

EOF decreases with increasing MeOH concentration as expected from dielectric/viscosity ratios for MeOH–water mixtures [22]. The EOF values measured by uracil in MeOH/Bis–Tris buffer (10 mM, pH 3.5) were 1.10, 0.97, 0.74, 0.62 and 0.55 [10^{-4} cm^2 s^{-1} V^{-1}] in 10, 20, 30, 50 and 70 vol.% MeOH, respectively. An increase of the MeOH concentration thus causes an increase in retention in CEC. In CLC as the MeOH content increases the polarity of the eluent decreases and this will cause the solubility of polar-protonated analyte in the eluent to diminish, thereby increasing retention. In CEC on CSP **1** enantioselectivity and resolution for the amino acids are better in eluents of intermediate methanol composition than those of low and high end of the composition while in CLC the trends are similar but not as obvious as in CEC. The reason for better enantioselectivity and resolution in the eluent of intermediate MeOH content is not clear. For chiral separation of β-amino acids in HPLC better enantioselectivity and resolutions were also observed in eluents of intermediate MeOH content [23,24].

4. Conclusion

Four native α-amino acids were enantioseparated on a (−)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-bonded silica in MeOH/Bis–Tris buffers by CEC and CLC. Influences of pH (3.0–4.5), concentration of MeOH (20–70 vol.%) and buffer (10–40 mM) on retention, enantioselectivity and resolution were examined, and the CEC separation data were compared with the CLC data. Better enantioseparation was obtained on CSP **1** in the eluent of lower pH, higher buffer concentration and intermediate MeOH content, usually at the expense of analysis time. Generally CEC gave better enantioselectivity and resolutions than CLC.

Acknowledgment

This research was supported by the Yeungnam University research grant in 2008.

References

[1] R. Kuhn, F. Erni, T. Bereuter, J. Hausler, Anal. Chem. 64 (1992) 2815.
[2] R. Kuhn, Electrophoresis 20 (1999) 2605.
[3] W. Lee, S. La, Y.M. Choi, K.R. Kim, Bull. Korean Chem. Soc. 24 (2003) 1232.
[4] S.I. Cho, J. Shim, M.-S. Kim, Y.-K. Kim, D.S. Chung, J. Chromatogr. A 1055 (2004) 241.
[5] M.H. Hyun, J. Sep. Sci. 26 (2003) 242.
[6] M.H. Hyun, J. Sep. Sci. 29 (2006) 750.
[7] Y. Machida, H. Nishi, K. Nakamura, J. Chromatogr. A 810 (1998) 33.
[8] E. Bang, J.-W. Jung, W. Lee, D.W. Lee, W. Lee, J. Chem. Soc. Perkin Trans. 2 (2001) 1685.
[9] Y. Machida, H. Nishi, K. Nakamura, Chirality 11 (1999) 173.
[10] Z. Deyl, F. Svec (Eds.), Capillary Electrochromatography, J. Chromatogr. Library, vol. 62, Elsevier, Amsterdam, 2001.
[11] K.D. Bartle, P. Meyers (Eds.), Capillary Electrochromatography, Royal Society of Chemistry Monographs, Royal Society of Chemistry, London, 2001.
[12] Y. Gong, H.K. Lee, Helv. Chim. Acta 85 (2002) 3283.
[13] Y. Gong, G. Xue, J.S. Bradshaw, M.L. Lee, H.K. Lee, J. Heterocycl. Chem. 38 (2001) 1317.
[14] T. Koide, K. Ueno, J. Chromatogr. A 909 (2001) 305.
[15] M.H. Hyun, J.S. Jin, W. Lee, J. Chromatogr. A 822 (1998) 155.
[16] M.H. Hyun, J.S. Jin, H.J. Koo, W. Lee, J. Chromatogr. A 837 (1999) 75.
[17] A.E. Martell, R.J. Motekaitis, NIST Database 46, National Institute of Standards and Technology, 2001.
[18] K.K. Unger, Porous Silica, J. Chromatogr. Library, vol. 16, Elsevier, Amsterdam, 1979.
[19] P.J. Dutton, T.M. Fyles, S.J. McDermid, Can. J. Chem. 66 (1988) 1097.
[20] J. Jang, S.I. Cho, D.S. Chung, Electrophoresis 22 (2001) 4362.
[21] M. Bowser, G.M. Bebault, X. Peng, D.D.Y. Chen, Electrophoresis 18 (1997) 2928.
[22] C. Schwer, E. Kenndler, Anal. Chem. 63 (1991) 1801.
[23] R. Berkecz, A. Sztojkov-Ivanov, I. Ilisz, E. Forro, F. Fulop, M.H. Hyun, A. Peter, J. Chromatogr. A 1125 (2006) 138.
[24] R. Berkecz, I. Ilisz, F. Fulop, Z. Pataj, M.H. Hyun, A. Peter, J. Chromatogr. A 1189 (2008) 285.