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Simultaneous separation of *o*-, *m*-, *p*-fluoro-DL-phenylalanine and *o*-, *m*-, *p*-DL-tyrosine by ligand-exchange micellar electrokinetic capillary chromatography

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Abstract

The simultaneous separation of *o*-, *m*-, and *p*-fluoro-DL-phenylalanine and *o*-, *m*-, and *p*-hydroxy-DL-phenylalanine (tyrosine) by ligand-exchange micellar electrokinetic capillary chromatography (LE-MEKC) was carried out. A copper(II) complex of L-4-hydroxyproline was used as the chiral selector. The influence of the concentration of the chiral additive, organic modifiers such as methanol or acetonitrile, field strength and pH value on the selectivity was investigated. The relationship of electroosmotic flow with sodium dodecyl sulfate (SDS) concentration as well as the pH of electrolyte was discussed. The addition of SDS resulted in a significant improvement in the separation. It was interestingly observed that the migration order of D,L-enantiomers as well as *o*-, *m*-, *p*-enantiomers was reversed when the concentration of SDS exceeded a certain concentration. A possible mechanism of LE-MEKC was discussed. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Ligand-exchange micellar electrokinetic capillary chromatography; Enantiomer separation; Positional isomers; Fluorophenylalanine; Phenylalanine; Tyrosine; Amino acids

1. Introduction

Since the biological activities of many compounds closely relate to their optical activity [1], more attention has been paid to enantioseparation. Capillary electrophoresis (CE) is a powerful separation technique for many compounds. Compared to high-performance liquid chromatography (HPLC), where the chiral phase is chemically bonded to the silica gel, free-zone electrophoresis (CZE) is usually carried out with bare silica capillaries. For this reason, the chiral selector has to be added to the electrolyte to perform chiral discrimination. For example, chiral separations have been accomplished by the use of cyclodextrins [2,3], chiral surfactants [4,5], chiral micelle polymers [6,7], crown ethers [8,9] and chiral calix[4]arene derivatives [10,11]. In addition, ligand exchange is an effective method for chiral separation [12].

Ligand-exchange chromatography, a technique suggested by Helfferich [13] in 1961, has been developed into a powerful chiral chromatographic method [14,15]. A satisfactory result of enantioseparation based on ligand exchange has been achieved by HPLC. However, up to now, only a few papers have been published on the application of

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ligand exchange in CE. Gassmann et al. [16] first reported the separation of enantiomers by CE. They used Cu(II)-histidine [16] and Cu(II)-aspartame [17] complexes as additives to the electrophoretic electrolyte and successfully separated dansyl-D,L-amino acids. Cohen et al. [18] resolved the same group of analytes using a gel-packed capillary and *N*,*N*-dodecyl-L-alanine complexed with a Cu(II) ion. Fanali et al. [19] succeeded in the chiral recognition of some α-hydroxy acids using a Cu(II) complex of L-proline, L-4-hydroxy-proline and aspartame. Gubitz et al. [20] reported the direct enantiomer separation of underivatized amino acids using a Cu(II)-4-L-OH-proline complex. Horimai et al. [21] recently reported the resolution of new quinolone drugs using γ-CD-Zn(II)-D-phenylalanine solution as the running solution.

As described above, different amino acids and amino acid enantiomers can be resolved, due to the differences in complex formation constants. However, the difference in the complex formation constant is not enough to result in the complete simultaneous resolution of a mixture of *o*-, *m*- and *p*-DL-enantiomers which have both optical and positional differences. To achieve this simultaneous separation, ligand-exchange micellar electrokinetic capillary chromatography (LE-MEKC) was developed.

Micellar electrokinetic capillary chromatography (MEKC) introduced in 1984 by Terabe et al. [22] is based on the chromatographic principle of partitioning solutes between a pseudo-stationary phase and the mobile phases and was proven to be a powerful tool for the separation and analysis of a variety of analytes [23]. More and more attention is being paid to using surfactants in order to improve the separation efficiency and selectivity. LE-MEKC, as a method of combining the features of ligand exchange and micellar electrokinetic chromatography, will be a promising method for the separation of samples that cannot be separated by using only the mode of ligand exchange or micellar electrokinetic chromatography.

So far, there are few commercially available positional enantiomers and few studies on their physiological activities. It may be due to the lack of a powerful analytical method for positional enantiomers. Therefore, it is significant and useful to develop a simultaneous separation method for positional enantiomers. For example, it can be applied to determine the reagent purity of synthesized positional enantiomers and to determine the concentration of a positional enantiomer in the case of studying the physiological activity of positional enantiomers. *o*-, *m*-, *p*-Fluoro-DL-phenylalanine and *o*-, *m*-, *p*-DL-tyrosine (hydroxyphenylalanine) are a group of compounds having similar structures. Their simultaneous separation is hard and interesting work. In this paper, we describe their simultaneous separations by LE-MEKC. A copper(II) complex with L-4-hydroxy-proline was used as the chiral selector. The influence of the concentration of chiral additive, organic modifiers such as methanol or acetonitrile, field strength and pH value on the enantioselectivity was investigated. The addition of the micelle-forming surfactant, sodium dodecyl sulfate (SDS), resulted in a significant improvement in the separation and a interesting change of migration order and resolution. A possible model of LE-MEKC is proposed.

2. Experimental

2.1. Instrumentation

The capillary electrophoresis (CE) instrumental setup involves a HEL5-30P2-TTu high-voltage power supply (Matsusada Precision Devices, Japan), a CE-971 UV detector (Jasco, Japan) and a C-R6A Chromatopac Recorder (Simadza, Japan). The separations were carried out in fused-silica capillaries (0.050 mm I.D.×0.375 mm O.D.), from GL Sciences (Japan), with a total length of 56 cm and an effective length of 40 cm.

2.2. Chemicals

All chemicals were of reagent grade and used as received, unless otherwise stated. Cupric sulfate (GR), 4-OH-proline and SDS were from Wako (Tokyo, Japan). Ammonia solution (GR) from Kanto (Tokyo, Japan). All amino acid enantiomers were obtained from Sigma (USA) and Wako (Japan).

Samples were prepared by dissolving the amino acids in electrolyte containing 25 m*M* Cu(II) and 50 m*M* 4-L-OH-proline adjusted to pH 4.0 with ammonia at the concentration range of 5.0×10^{-4} and 1.0×10^{-3} mol/l. The running electrolyte was 25

m*M* $CuSO_4 \cdot 5H_2O$, 50 m*M* 4-OH-proline and 10 m*M* SDS adjusted to pH 4.0 with ammonia, unless otherwise stated. All solutions were filtered through 0.45-μm membrane (Nihon Millipore, Japan) and degassed by vacuum and ultrasonification. Water was purified by distillation apparatus (Advantec Tokyo, Japan).

2.3. Capillary electrophoresis

Before using, the capillary was washed with 0.1 mol/l NaOH, water and running electrolyte for 15, 30 and 20–30 min, respectively. When the electrolyte is changed, it is necessary to wash and equilibrate the capillary walls with new electrolyte. The sample is injected by the electrokinetic method. A typical injection condition is 5 s at 10 kV. UV detection was performed at 208 nm.

3. Result and discussion

3.1. Principle

The resolution principle of ligand exchange (LE) is based on the formation of a ternary complex of Cu(II) in the electrolyte solution, described as follows:

$$\text{Cu(II)[L]}_2 + \text{D,L-AAs} \rightleftharpoons [\text{D-AA}]\text{Cu(II)[L]} + [\text{L-AA}]\text{Cu(II)[L]} \quad (1)$$

where L is the ligand, L-hydroxyproline. D,L-AAs are the D-enantiomer and L-enantiomer of amino acids. In the electrophoretic electrolyte of the Cu(II)-4-OH-proline complex, Cu(II) forms a complex with 4-OH-proline in the ratio of 1 to 2. As DL-amino acids are injected into the capillary, D and L enantiomers form complexes with the Cu(II)-4-OH-proline complex by a ligand exchange mechanism, respectively [12]. The ligand-exchange products, ternary complexes of Cu(II) with 4-OH-proline and L-amino acid or D-amino acid, are diastereomeric, but they probably have close electrophoretic mobilities. The principle of this enantiomeric separation is the difference in the formation constants of the complexes.

The difference in the formation constants exists not only between enantiomers but also among different amino acids, resulting in the separation of both enantiomers and different amino acids [16,17]. However, the formation constants are not always different enough for the separation of *o*-, *m*-, *p*-positional enantiomers with structure difference in the *o*-, *m*-, *p*-position. To achieve the purpose of simultaneous separation of *o*-, *m*-, and *p*-stereoenantiomers, the LE–MEKC technique is proposed as follows.

The possible model of LE–MEKC is shown in Fig. 1. At low concentrations, the surfactant molecules are in the molecular-disperse stage, where they may be associated as dimers or trimers of oligomers depending on the type of surfactant. When the

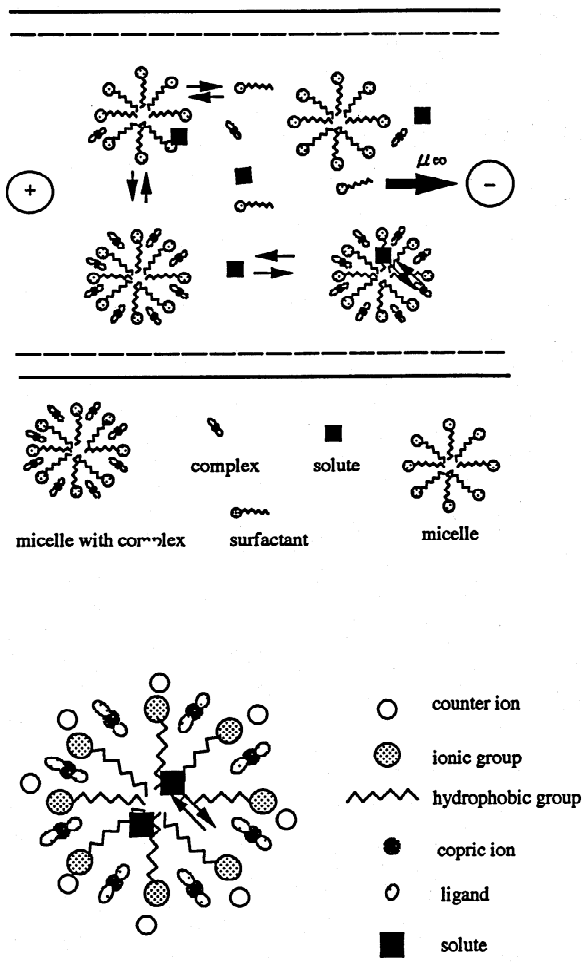


Fig. 1. (top) A possible model of LE–MEKC. (bottom) Simplified schematic representation of an ionic micelle associated with the complex.

concentration exceeds the critical micellar concentration (CMC), the molecules aggregate to form spherical micelles. Within the spherical structure, the hydrophobic moieties of surfactants are oriented toward the center of the spheres. The surface of the micelles is formed by hydrophilic groups which are in contact with the aqueous phase [24]. When the chiral recognition complex of $Cu(II)[L]_2$ exists in the electrolyte, the complex molecule will be distributed between micelles and bulk electrolyte solution. Due to the hydrophobic interaction, the hydrophobic moiety of the complex will orient toward the hydrophobic side of micelle. When solutes are introduced into a micellar system, they are partitioned between the hydrophobic micellar phase and the aqueous phase with a particular partition coefficient depending on the polarity of the solute. Meanwhile, solutes partitioned within micelles will undergo ligand exchange with one of the ligands of the complex. The combination of different partition coefficients resulting from different hydrophobicity, stereoconformation and different complex formation constants gives *o*-, *m*-, and *p*-D,L-enantiomers a better resolution.

The resolution, R_s and separation factor, α, were calculated by:

$$R_s = 2(t_2 - t_1)/(w_1 + w_2) \quad (2)$$

$$\alpha = t_2/t_1 \quad (3)$$

where t is the migration time and w is the peak bandwidth.

3.2. Effect of the concentration of SDS on separation

The concentration of SDS plays an important role in the separation. As the concentration of SDS increases, the separation of *o*-, *m*-, *p*-D,L-tyrosine and *o*-, *m*-, *p*-fluoro-DL-phenylalanine gradually becomes better. As shown in Fig. 2, without SDS in the electrolyte, these closely related compounds were not separated. With 10 m*M* of SDS in the electrolyte (CMC of SDS in water is 8.27 m*M*), a satisfactory resolution was obtained. As described in Section 3.1, the resolution principle is probably based on the differences in the partition coefficient and the complex formation constant of different positional enantiomers.

Fig. 3 shows the effect of SDS on the electroosmotic flow (EOF). Compared to the magnitude of the EOF in the absence of SDS, the EOF in the presence of SDS increases slightly. The is considered to be due to SDS adsorbed on the inner surface of capillary [26]. This generated a stronger zeta potential due to the strongly negative charge of the sulfate moiety of the detergent. However, upon increasing the concentration of SDS, especially above the CMC point, most of the SDS molecules are involved in micelle formation and as a consequence the EOF almost does not change.

The increase of EOF seems to cause shorter migration time. But Fig. 2 actually indicates that the migration time becomes longer after adding SDS, especially when the SDS concentration is above the CMC. The reason is considered to be that the negatively charged micelle phase distributed with the Cu(II) complex migrates toward the anode, the opposite direction of EOF.

3.3. Effect of pH

pH is one of the most important factors in chiral recognition. As shown in Fig. 4, pH leads to a great change in the separation time and the resolution. When the pH is 4.0, the separation time was about 48 min and the peak of L-tyrosine was ‘dropped into’ a negative system peak, shown in the zoom area in Fig. 4(a), due to the coelution with system peak. However, when the pH is 4.5, the separation time was shortened to about 23 min. Besides, it is satisfactory that the peak of L-tyrosine ‘moves out of’ the negative system peak as it does not coelute with negative system peak. But, when pH reaches 5.0, even though the migration time becomes shorter, some peaks become overlapped each other. Fig. 5 shows the relationship of the EOF to the pH. As pH increase makes EOF large, the migration time becomes shorter.

3.4. Optimization of the electrolyte composition

Since two ligands are required for a copper ion, the ratio between the chelating amino acids and copper was always kept 2:1 [20]. Fig. 6 shows the

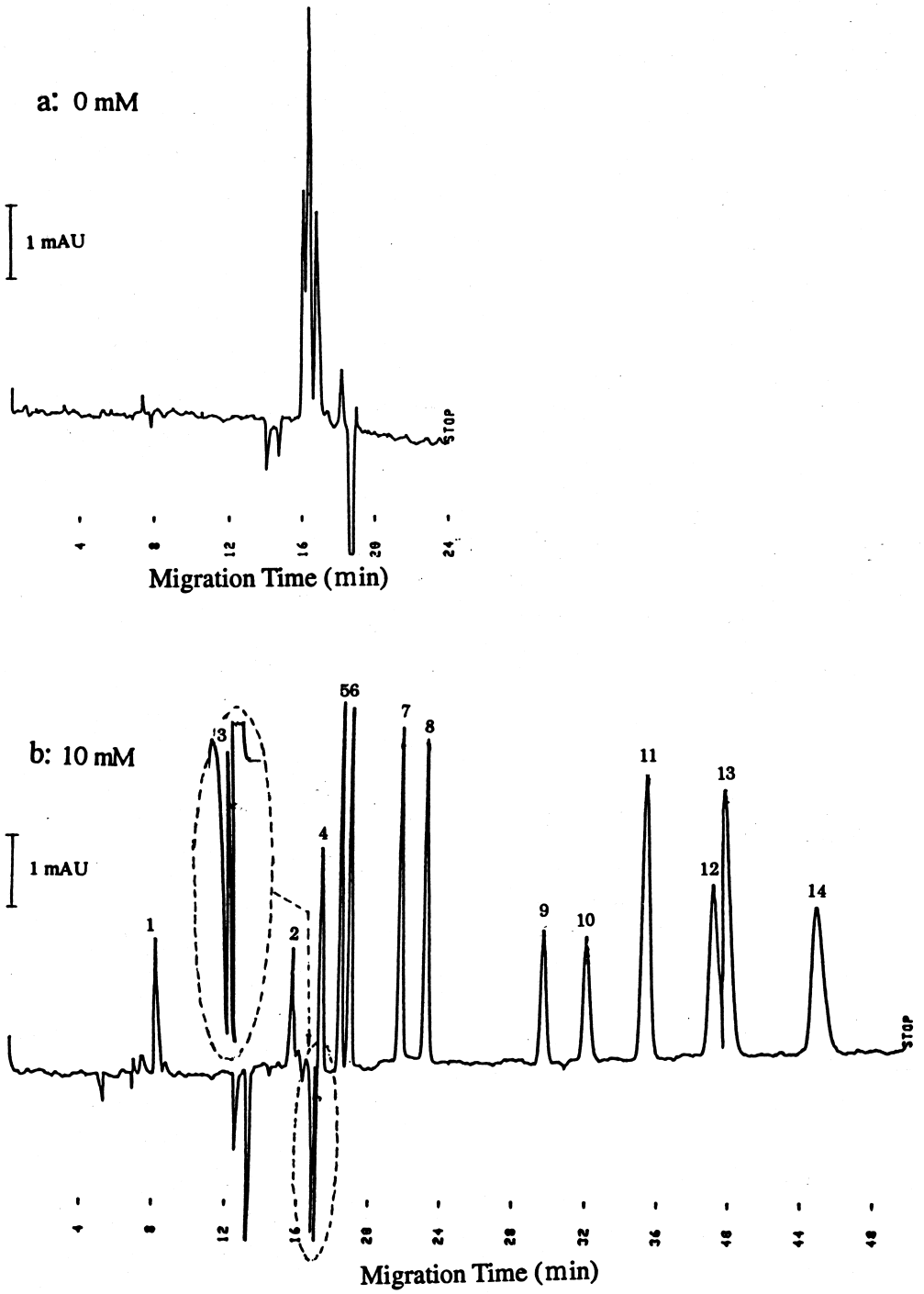


Fig. 2. Electropherograms of 12 positional and optical isomers of amino acids at different SDS concentrations. Running electrolyte kept at pH 4.0 contains 25 m*M* Cu(II), 50 m*M* 4-OH-proline and SDS. Other conditions are described in Section 2. In figure (b) peaks 1, 2 and negative peaks are system peaks, 3=*p*-L-tyrosine, 4=*p*-D-tyrosine, 5=*m*-L-tyrosine, 6=*m*-D-tyrosine, 7=*o*-L-tyrosine, 8=*o*-D-tyrosine, 9=*o*-fluoro-L-phenylalanine, 10=*o*-fluoro-D-phenylalanine, 11=*m*-fluoro-L-phenylalanine, 12=*p*-fluoro-L-phenylalanine, 13=*m*-fluoro-D-phenylalanine, 14=*p*-fluoro-D-phenylalanine.

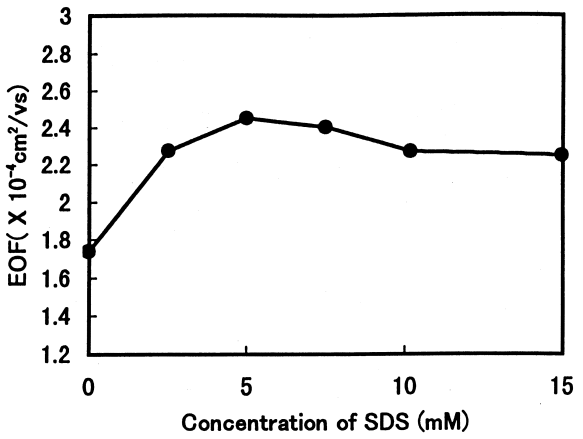


Fig. 3. The relationship of electroosmotic flow with SDS concentration. Acetone (10% in electrolyte without SDS) was used as marker. Other conditions are the same as in Fig. 2.

relationship of migration time and resolution with the concentration of Cu(II)/L-hydroxyproline. As shown in Fig. 6A, the migration time slowly increases with the increase in the concentration of L-hydroxyproline/Cu(II). The probable reason is that the increase of the concentration of L-hydroxyproline/Cu(II) in the electrolyte solution causes the increase in complex concentration distributed in the negatively charged micelle phase which migrates towards the anode. The decrease of the EOF resulting from the absorption of positively charged Cu(II) ions on the inner wall of the capillary may be another reason [25].

As shown in Fig. 6B, the increase of concentration of L-4-hydroxyproline/Cu(II) makes R_s higher. However, when the concentration of Cu(II)/L-4-hydroxyproline reaches 25/50 m*M*, R_s tends to a plateau. This means that the capacity of the complex is enough to achieve the separation. Increasing the concentration of Cu(II)/L-4-hydroxyproline results in an increase of current. It was shown that the current and the concentration of Cu(II) follow a linear relationship:

$$I\ (\mu A) = 2 + 0.8[\text{Cu(II)}\ (\text{m}M)](r = 1.0) \quad (4)$$

A high Joule heat resulting from the high current gives no benefit to the separation. Based on the above consideration, the optimum electrolyte composition was 25 m*M* Cu(II), 50 m*M* 4-OH-proline and 10 m*M* SDS.

3.5. Effect of field strength

The effect of the applied field strength on the resolution was examined in the range of 6 to 14 kV at pH 4.5. It was shown that although the resolution somehow decreases when the applied field strength was increased, a baseline separation can be achieved in all conditions. It has a large effect on the separation time. When the applied field strength was at 6 kV, the separation needs 32 min, when at 14 kV, the separation was finished within 12 min.

3.6. Effect of organic modifier

The separation behavior in the presence of an organic modifier like methanol or acetonitrile was examined. The results are shown in Fig. 7. It was shown that organic modifiers could not improve the separation, having a rather poor influence on separation. A possible reason is that organic modifiers change the CMC point of the surfactant.

3.7. The separation behavior in the absence of Cu(II) complex

In order to demonstrate the distribution of amino acids inside micelles, simultaneous separation of *o*-, *m*-, *p*-fluoro-D,L-phenylalanine in the absence of Cu(II) complex was carried out. Fig. 8 shows that positional isomers can be separated in the presence of SDS. It also indicates that amino acids can be distributed into micelles. Compared with Fig. 8(a), in Fig. 8(b) the migration time of peaks becomes shorter in the presence of SDS. This also supports the idea that amino acids distribute between the micelle phase and the aqueous phase, because negatively charged micelles migrate towards the opposite direction to the EOF, resulting in slower migration time.

3.8. Examination on the migration order of enantiomers

The migration time and resolution are listed in Tables 1 and 2. These experiments were conducted

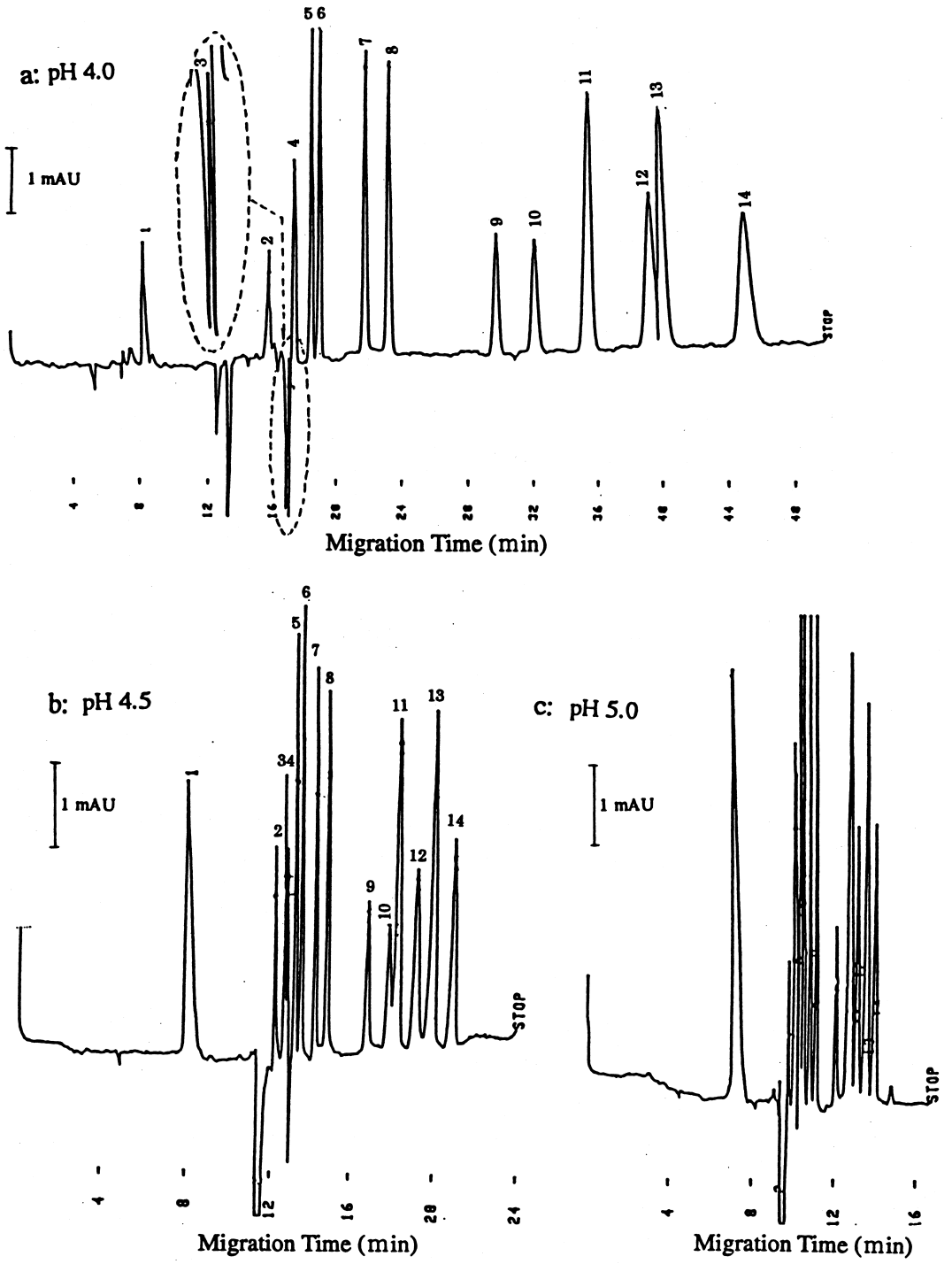


Fig. 4. Electropherograms of the mixture of 12 positional and optical isomers of fluorophenylalanine and tyrosine at different pHs. Running electrolyte contains 25 m*M* Cu(II), 50 m*M* 4-OH-proline and 10 m*M* SDS at different pH values. Peak identifications in figures (a) and (b) are the same as in Fig. 2(b).

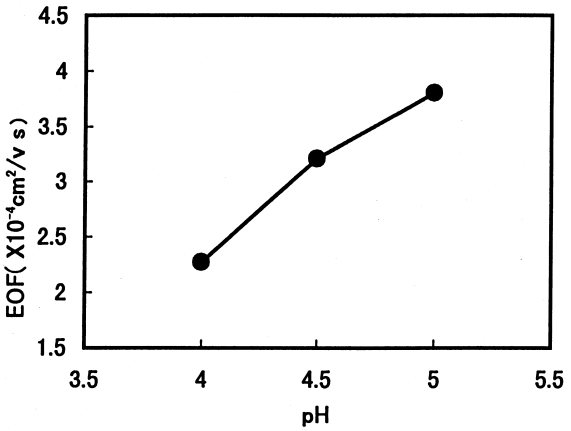
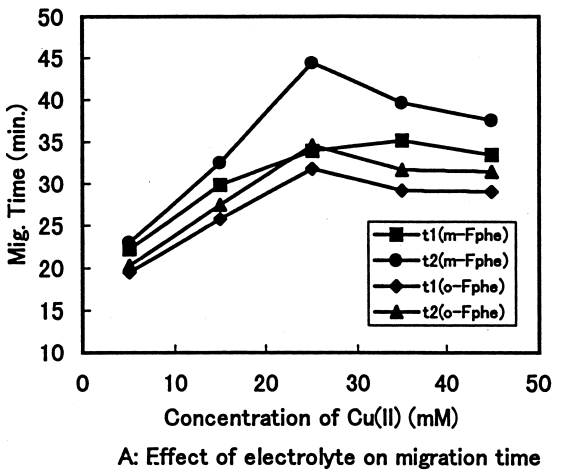


Fig. 5. Effect of pH on EOF. Conditions are the same as in Fig. 4. Marker: acetone.



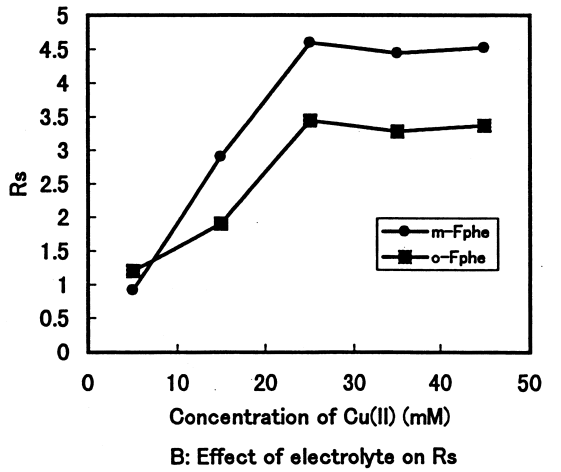


Fig. 6. Effect of electrolyte composition on separation. Electrolyte: ratio of Cu(II) to L-4-hydroxyproline was kept at 1:2, pH was adjusted to 4.0 with ammonia.

by sampling only one kind of D,L amino acid enantiomers. It was interestingly noted that the changes in both the migration time and the resolution follow a certain trend with and without the SDS in the electrolyte. Table 1 indicates that without SDS in the electrolyte, the migration time increases in the order of *o*-, *m*-, *p*- for tyrosine, but in the order of *p*-, *m*-, *o*- for fluorophenylalanine. Whereas as shown in Table 2 with 10 m*M* SDS in the electrolyte, the migration times increase in the order of *p*-, *m*-, *o*- for tyrosine, and in the order of *o*-, *m*-, *p*- for fluorophenylalanine. Besides, without SDS the D-enantiomer migrates faster, whereas, with 10 m*M* of SDS in the electrolyte, the L-enantiomer becomes faster. The possible reason for this reversal is that the mode of separation changes from ligand exchange into LE-MEKC. In the absence of SDS, solutes directly interact with the complex of chiral selector by ligand exchange. In the presence of SDS, solutes distribute into the micelles associated with the complex of chiral selector and exchange the ligands inside the micelles. Detailed studies on the changes in the order of migration time and resolution are under way.

4. Conclusion

We have demonstrated the ability of L-OH-proline-Cu(II) complexes as chiral selectors in the presence of SDS for the simultaneous separation of *o*-, *m*-, and *p*-positional enantiomers of fluorophenylalanine and tyrosine by LE-MEKC. It is shown that in addition to the concentration of the copper complex and the pH value of the electrolyte, the anionic surfactant SDS has a significant effect on the resolution. It is concluded that the optimum electrolyte was 25 m*M* Cu(II), 50 m*M* 4-OH-proline and 10 m*M* SDS adjusted to pH 4.5 with ammonia, whereas organic modifiers like methanol or acetonitrile result

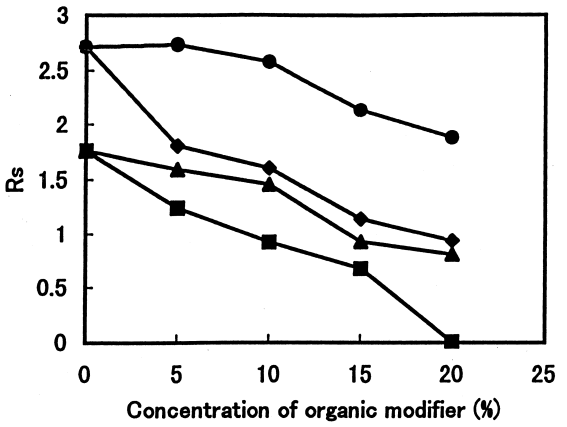


Fig. 7. Effect of organic modifiers on separation. *o*-Tyrosine in acetonitrile (■), *m*-fluorophenylalanine in acetonitrile (♦), *o*-tyrosine in methanol (▲), *m*-fluorophenylalanine in methanol (●).

Table 1
Separation in the absence of SDS

Sample	t_d (min)	t_1 (min)	α	R_s
o-Tyrosine	12.14	12.36	1.018	0.710
m-Tyrosine	12.16	12.40	1.020	0.960
p-Tyrosine	12.25	12.55	1.024	1.000
o-Fluorophenylalanine	12.49	12.62	1.011	0.528
m-Fluorophenylalanine	12.26	12.43	1.013	0.640
p-Fluorophenylalanine	12.13	12.29	1.013	0.644

in a worse effect on the separation. The order of migration time and resolution of *o*-, *m*-, *p*-positional DL-enantiomers was interestingly changed.

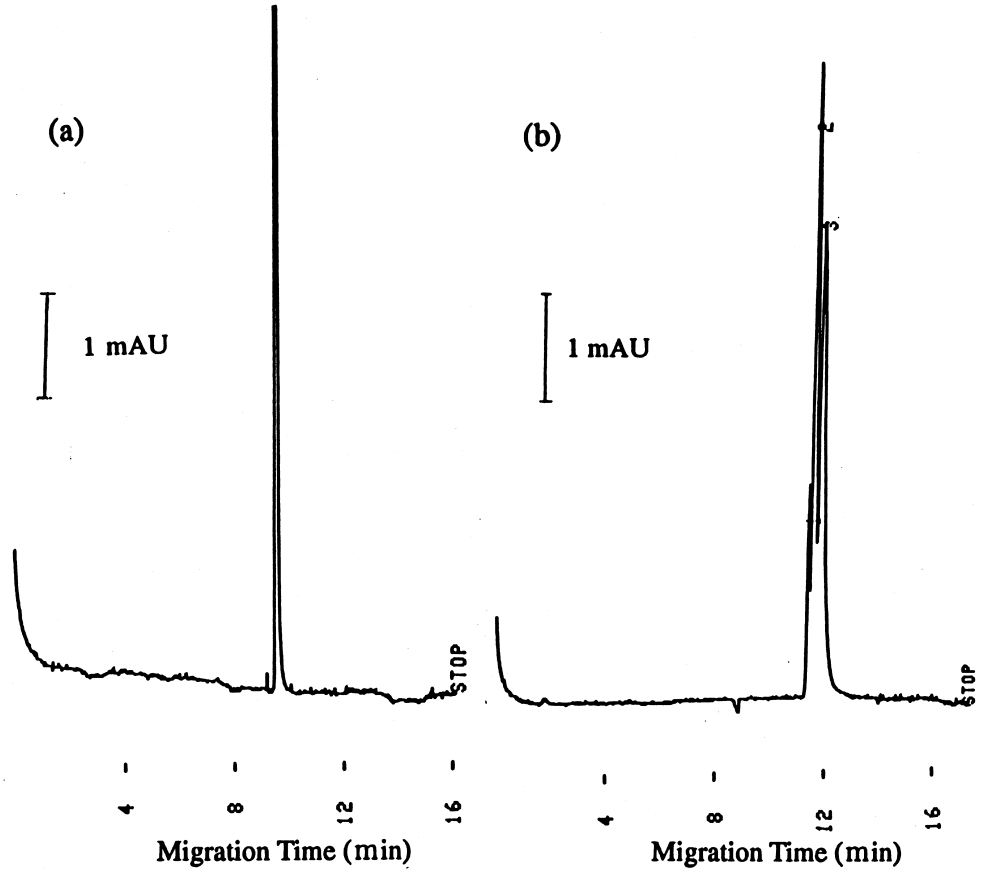


Fig. 8. Electropherograms of *o*-, *m*-, *p*-fluoro-DL-phenylalanine in the absence of the Cu(II) complex. Running electrolyte: (a) pH 4.0, 10 m*M* NH_4Ac, (b) pH 4.0, 10 m*M* SDS and 10 m*M* NH_4Ac. Sample was prepared in pH 4.0 NH_4Ac. Peak identification: 1=*o*-F-DL-phe, 2=*m*-F-DL-phe, 3=*p*-F-DL-phe.

Table 2
Separation in the presence of 10 m*M* SDS

Sample	t_1 (min)	t_d (min)	α	R_s
o-Tyrosine	20.47	21.76	1.063	2.87
m-Tyrosine	17.88	18.39	1.029	1.46
p-Tyrosine	16.70	16.98	1..017	0.923
o-Fluorophenylalanine	31.76	34.52	1.087	3.45
m-Fluorophenylalanine	38.88	44.40	1.142	4.60
p-Fluorophenylalanine	41.20	47.50	1.153	4.84

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