



Chiral separation of underivatized and dansyl amino acids by ligand-exchange micellar electrokinetic capillary chromatography using a copper(II)–L-valine complex as selector

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Abstract

Ligand-exchange micellar electrokinetic capillary chromatography was used for the chiral resolution of underivatized and dansyl amino acid enantiomers simultaneously. The separation was achieved by chiral copper(II)–L-valine complexes incorporated in micelles of sodium dodecyl sulfate (SDS). The enantioresolution was strongly affected by SDS and a concentration of 20 mM SDS was shown to be necessary for the separation. Other impacting factors were investigated including pH, the molar ratio of copper(II) to L-valine and the total concentration of complex. Using the proposed method, 11 different dansyl amino acids and two underivatized amino acids were separated successfully with a running electrolyte of 20 mM NH₄OAc, 4 mM CuSO₄, 8 mM L-valine and 20 mM SDS at pH 9.0 in less than 25 min. Experiments were also performed with other amino acid ligands in order to vary the stability and the sterical arrangement of the copper(II) complexes and the possible chiral recognition mechanism was also discussed briefly.

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1. Introduction

Amino acids are essential components of many natural molecules and frequently found in food, feeds, body fluids and tissues. Chiral analysis of DL-amino acids is of great interest and of increasing importance in life science and many other related fields. Enantioseparation of racemic amino acids has been extensively studied by gas chromatography (GC) [1] and high-performance liquid chromatog-

raphy (HPLC) [2] using chiral stationary phases or chiral mobile phase additives. In recent years, capillary electrophoresis (CE) has been receiving extensive attention for chiral analysis because of its high separation efficiency, short analysis times, feasibility in selectivity manipulation, low cost and relative simple instrument setup [3–5], compared with other chromatographic techniques. On the basis of the ligand-exchange (LE) mechanism introduced by Davankov and Rogozhin in the early 1970s for HPLC [6], chiral resolution of dansyl amino acids by CE was firstly reported by Zare and co-workers using L-histidine– [7] or aspartame–Cu(II) complexes [8] as chiral selectors. Subsequently, a great

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variety of papers dealing with LE-CE enantioseparation of amino acids have been published using Cu(II)–amino acid complexes as chiral selectors [9–14]. The authors observed a significant improvement in resolution when a micelle forming surfactant such as sodium dodecyl sulfate (SDS) was added to the electrolyte performing micellar electrokinetic chromatography (MEKC). Cohen et al. [15] and later, Sundin et al. [16] described the use of a complex of copper(II) and *N,N*-didecylalanine as chiral additive in combination with 20 mM SDS to facilitate separation of dansylated amino acids. Recently, Chen et al. [17,18] carefully studied chiral separation behaviors of the positional isomers and enantiomers of fluorophenylalanine and tyrosine with and without SDS addition. The authors called the method ligand-exchange micellar electrokinetic capillary chromatography. As a hybrid mode of possessing both the advantage of high enantioselectivity in ligand-exchange mechanism and the main advantages of MEKC, LE-MEKC allows the manipulation of the selectivity for large classes of neutral and charged compounds, making possible separation that otherwise are not feasible by using only the mode of LE or MEKC. Using this combined separation mode, hydroxy acids and dipeptides, as well as 16 positional and optical isomers of tryptophan derivatives could be optically resolved [19]. The same group also proposed a method for the determination of the critical micelle concentration (CMC) of anionic surfactants based on the LE-MEKC principle [5].

Separation of underivatized amino acid enantiomers by CE has always been a challenge. Although reports on CE separations of tagged amino acid enantiomers are numerous, only a few chiral CE procedures have been reported for native amino acids [20]. In the present work, we describe an optical resolution system for the simultaneous enantiomeric separation of native and dansyl amino acids by LE-MEKC. Using copper(II) complexes of L-valine as chiral selectors, the resolution of 11 dansyl amino acids and two unmodified amino acids is achieved. The mechanism of chiral discrimination is discussed on the base of the stabilities of the copper(II) binary complexes and of their ternary diastereomeric complexes with amino acids formed in solution and SDS micelles.

2. Experimental

2.1. Chemicals

All dansyl (Dns) DL-amino acids including aspartic acid (Asp), glutamic acid (Glu), leucine (Leu), methionine (Met), α -amino-*n*-butyric acid (Nbu), norleucine (Nle), norvaline (Nva), phenylalanine (Phe), threonine (Thr), tryptophan (Trp) and valine (Val) were purchased from Sigma (St. Louis, MO, USA). Underivatized DL-Phe was from Tokyo Kasei Kogyo (Tokyo, Japan); DL-Trp was from Nacalai Tesque (Kyoto, Japan). L-Val, L-Glu, L-alanine (Ala), L-serine (Ser) and L-ornithine (Orn) were of biochemical-reagent grade from the Institute of Shanghai Chemical Reagents Factory (Shanghai, China). Copper(II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) was from Kanto (Tokyo, Japan); SDS was obtained from Acros Organics (NJ, USA). Other chemicals were all of analytical-reagent grade obtained from Beijing Chemical Factory (China) and used as received. Standard solutions of amino acids were obtained by dissolving amino acids in 20 mM ammonium acetate (NH_4OAc) solution and the concentrations of all stock solutions were 1.0 mM. Sample solutions were prepared by dilution of stock solutions in 20 mM NH_4OAc at the concentration range of 10^{-4} M. The running electrolyte used for the enantiomeric separation, unless otherwise noted, consisted of 4 mM CuSO_4 , 8 mM L-Val and 20 mM SDS in 20 mM NH_4OAc adjusted to a desired pH by addition of 1 M aqueous ammonia or 1 M acetic acid. Water used to prepare sample and buffer solutions was freshly deionized by EASYpure water purification system with a 0.2- μm fiber filter (Barnstead, USA).

2.2. Instrumentation

All experiments were performed with a Beckman P/ACE MDQ CE system (Fullerton, CA, USA) equipped with a photodiode array detector. Sample detection was performed at 214 nm. Electropherograms were recorded and integrated by an IBM personal computer with Beckman 32 Karat software. Uncoated fused-silica capillaries (GL Sciences, Tokyo, Japan) with an effective length of 50 cm (57 cm \times 50 μm I.D. \times 375 μm O.D.) were used for

separation. The capillary temperature was maintained at 25 °C by the cooling system of the CE instrument. Samples were injected hydrodynamically at 0.5 p.s.i. for 5 s and separated at 20 kV (1 p.s.i.=6894.76 Pa). To clean and activate the inner surface, new capillaries were flushed successively for 5 min with methanol, 5 min with 0.1 M HCl, 10 min with 0.1 M NaOH, and 5 min with water. Daily before use, the capillary was rinsed for 2 min with 0.1 mM NaOH, 2 min with water and 4 min with running electrolyte. Between analyses, the capillaries were rinsed for 4 min with running electrolyte.

3. Results and discussion

Val proved to be a good chiral selector for HPLC [21]. However, until now, there has been no report in CE. Since the use of HPLC needs large consumption of chiral eluent, development and application of an L-Val–Cu(II) complex as a chiral selector in CE will supply a powerful alternative to HPLC. When such a copper(II) complex was used as chiral additive to the running electrolyte for capillary zone electrophoretic

analysis, resolution was not achieved for the racemic compounds studied (Fig. 1a), which suggests that the difference in stabilities of the ternary complexes is not enough for the separation. Therefore, the LE-MEKC mode was chosen, a hybrid method of electrophoresis and chromatography with SDS as a micelle-forming agent for increasing the separation selectivity (Fig. 1b).

3.1. Effect of SDS concentration

The influence of the concentration of SDS was studied in the range from 0 to 40 mM using the running electrolyte containing 20 mM NH₄OAc, 4 mM Cu(II) and 8 mM L-Val at pH 9.0. In this work, Dns-Nbu, Dns-Met, Dns-Leu and Dns-Nle were used as model compounds to study the effects on separation. The results are shown in Fig. 2. As expected, addition of SDS resulted in a significant improvement in resolution. When SDS concentration was lower than 8.1 mM, the separation could not be carried out well. The separation gradually became better when concentration of SDS increased to 10 mM and the resolution increased up to a con-

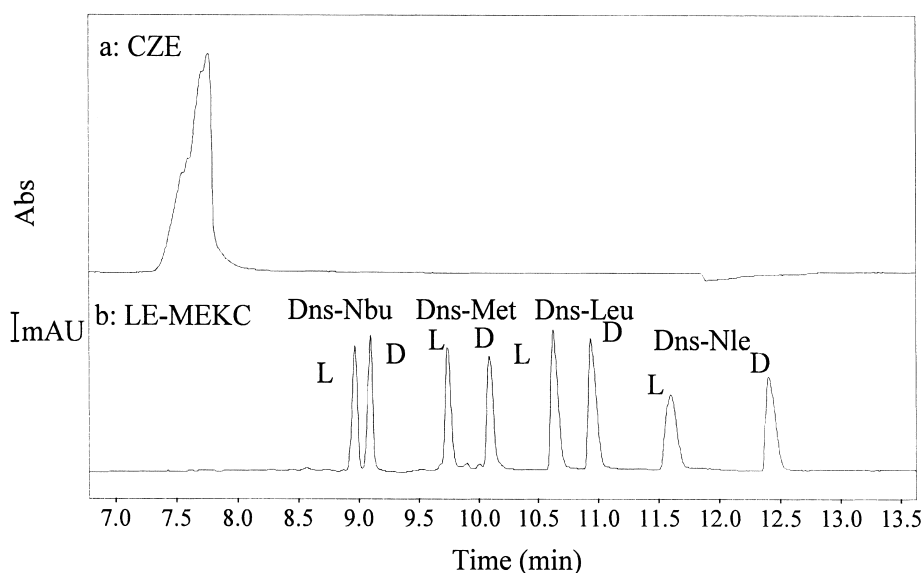


Fig. 1. Electropherograms of the separation of the mixture of Dns-Nbu, Dns-Met, Dns-Leu and Dns-Nle using L-Val as a chiral ligand without (a) or with (b) 20 mM SDS. Conditions: 4 mM CuSO₄, 8 mM L-Val, 20 mM NH₄OAc at pH 9.0; injection, 0.5 p.s.i., 5 s; separation voltage, 20 kV (350 V/cm); capillary, 57 cm×50 μm I.D.; capillary temperature, 25 °C.

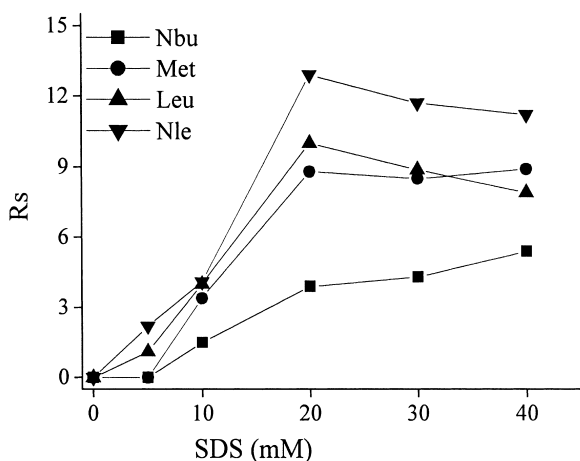


Fig. 2. Influence of SDS concentration on chiral separation in the presence of 4 mM CuSO_4 , 8 mM L-Val, 20 mM NH_4OAc at pH 9.0; other conditions as in Fig. 1.

centration of 20 mM. Above 20 mM SDS, the R_s values slightly decreased for Dns-Leu and Dns-Nle while leveled off for Dns-Nbu and Dns-Met. A too high concentration of SDS resulted in a marked increase in separation time and the running current. Therefore, 20 mM SDS was selected as the optimum condition.

Addition of SDS micelles plays a crucial role in separation. This is due to an additional separation mechanism described as micellar electrokinetic chromatography introduced by Terabe et al. [22]. The formation of SDS micelles in the electrolyte solution was regarded as hydrophobic pseudo-stationary phase and the enantiomers with different stereo-conformation and hydrophobicity will partition between aqueous buffer and micellar phase [16] associated with the chiral selector of the Cu(II)-Val complex with different partition coefficients. Meanwhile, enantiomers partitioned within micelles will undergo ligand exchange with one of the ligands of the complex [17]. Thus, a synergic effect of differences in the partition coefficient and complex formation constant may be generated between the chiral complex and SDS to enhance the resolution.

3.2. Effect of pH

An important factor in LE-MEKC is the pH of the

running electrolyte, since complex formation is strongly dependent on pH because of the different dissociation degree of both the amino acids and the chiral selector ligand. The effect of the pH value of the running electrolyte was investigated in this work. The dependence of resolution of DL-amino acids on the pH value of the running electrolyte was studied over the pH range of 6.0–9.5 using 20 mM NH_4OAc buffer consisting of 20 mM SDS, 4 mM Cu^{2+} and 8 mM L-Val. The pH value of buffer was adjusted by adding 1 M $\text{NH}_3 \cdot \text{H}_2\text{O}$ or acetic acid (HAc). The pH effects were not studied over pH 9.5 in order to avoid the precipitation of Cu(OH)_2 . As shown in Fig. 3, the separation of Dns-amino acids increased apparently with the increasing of pH range from 6.0 to 8.5, then decreased at the pH value above 8.5. The distinct maximum of the chiral separation could be achieved at pH 8.5. As pH increases from 6.0 to 8.5, the free L-Val is changed from the neutral form to negative ions [at its isoelectric point (pI) around 6.0]. As a result, the stability of the formed Cu(II)-L-Val complex will be increased. Thus, the chiral resolution should be enhanced. But further increasing of pH will lead to too high stability of the Cu(II)-ligand complex, which makes it difficult for the analyte enantiomers to replace ligands in the Cu(II)-ligand complex. These effects result in a maximum pH of 8.5 for the chiral separation. However, the

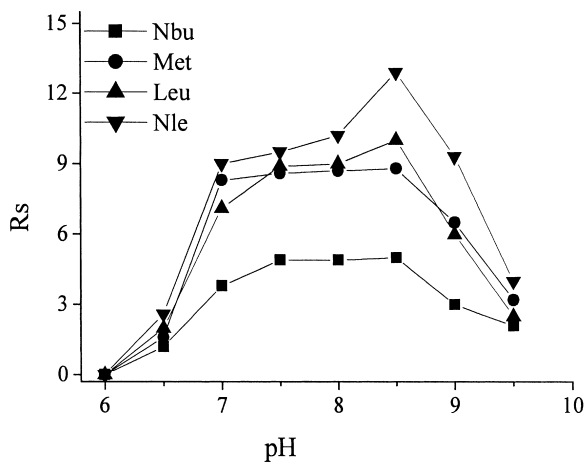


Fig. 3. Influence of pH on the resolution of Dns-amino acids. Buffer: 20 mM NH_4OAc containing 4 mM CuSO_4 , 8 mM L-Val and 20 mM SDS.

migration time decreased markedly at higher pH value resulting from the increase of electroosmotic mobility. Taking into consideration of resolution and migration time simultaneously, running buffer at pH 9.0 was selected to yield better resolution and shorter separation time.

3.3. Effect of the composition of the Cu(II)–L-Val complex

Achiral separation by MEKC was performed but the Dns-amino acids could not be resolved. When the Cu(II)–L-Val complex was present in the running electrolyte, the poor separation was greatly improved. To determine the optimum selector concentration, the Cu(II)–L-Val complex concentration was increased from 2 to 10 mM at an increment of 2 mM while the molar ratio between copper and the chelating amino acid was kept always at 1:2. The results reported in Table 1 show a general decrease of the migration time of the analyzed compounds by increasing the concentration of the Cu(II) complex. The resolution for the tested amino acids increased up to 4 mM Cu(II)–L-Val complex and then decreased at higher concentration. The results are different from those reported in LE-MEKC [23] using chiral complex as buffer additive, in which the resolution always increased steadily with the concentration of the copper(II) complex. In our experiment, a 4 mM selector concentration allowed the best resolution for the amino acids investigated.

The molar ratio of Cu(II) to L-Val has a strong influence on resolution. In order to make clear the effect of the molar ratio of metal ion to ligand on the

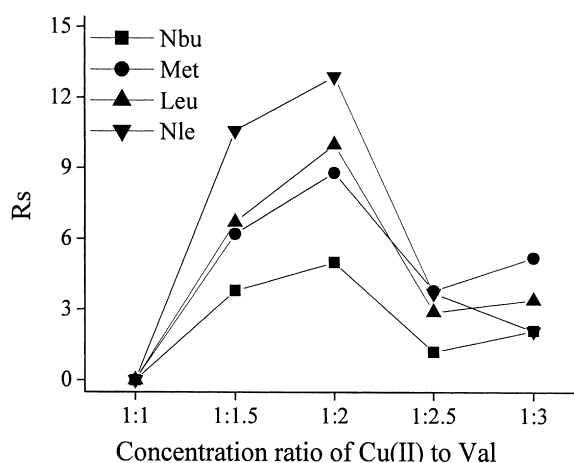


Fig. 4. Influence of the molar ratio of Cu^{2+} to L-Val on resolution. Buffer: 20 mM ammonium acetate, pH 9.0, 20 mM SDS, the total concentration of Cu^{2+} and L-Val is 12 mM.

enantiomeric separation, the relative amounts of the two components were varied between 1:1 to 1:3 ($[\text{Cu}^{2+}]:[\text{Val}]$, mM/mM), while the total concentration of Cu(II) and L-Val was kept constant at 12 mM. Fig. 4 indicates that the resolution of Dns-amino acids was very sensitive to the ratio and the separation increased rapidly as the ratio increased from 1:1 to 1:2. A large excess of L-Val dramatically reduced the resolutions, which may be due to the reduction in the competitive opportunity for the solutes to form complex with Cu^{2+} . Therefore, the optimum separation was obtained at a ratio of 1:2 ($[\text{Cu}^{2+}]:[\text{Val}]$), indicating that L-Val forms a bis-complex with Cu(II) ion.

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

C (mM:mM)	Dns-Nbu			Dns-Met			Dns-Leu			Dns-Nle		
	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	5.495	5.495	0	5.747	5.747	0	6.587	6.587	0	6.920	6.920	0
2:4	9.517	9.754	1.8	10.463	11.113	3.8	12.637	13.283	3.4	15.096	17.437	7.9
4:8	8.617	9.459	5.0	9.713	10.679	8.8	12.201	12.530	10.0	13.721	15.483	12.9
6:12	8.188	8.492	3.4	8.767	9.750	6.1	9.946	11.736	5.9	11.924	14.570	9.4
8:16	7.717	8.021	3.0	8.333	9.024	5.8	9.132	9.941	5.8	10.138	11.909	8.8
10:20	7.450	7.563	1.2	7.858	8.162	3.0	8.371	8.625	2.1	8.988	9.588	3.3

Running electrolyte: 20 mM NH_4OAc , 20 mM SDS and Cu(II)–L-Val (1:2); pH 9.0, other conditions as in Fig. 1. t_L and t_D are the migration times of the L- and D-enantiomers, respectively.

3.4. Enantioseparation of dansyl- and underivatized-DL-amino acids

The recommended experimental conditions were as follows: 20 mM SDS, 4 mM CuSO₄ and 8 mM L-Val, 20 mM NH₄OAc, pH 9.0. Fig. 5 shows the electropherogram of a mixture of underivatized Trp and seven different Dns-amino acid enantiomers. The results indicate that the L-enantiomers invariably migrated faster than the D-enantiomers. Under the proposed conditions, 11 pairs of different Dns-amino acids and two underivatized amino acids enantiomers could be successfully resolved and the migration time, separation factor and resolution are summarized in Table 2. As can be seen, the elution order of these amino acid derivatives simply agrees with the hydrophobic nature of side chains. In LE-MEKC, the elution order of enantiomers is basically dependent on both the stability of the ternary complexes and the partition coefficient of the enantiomers associated with the chiral selector of Cu(II)–L-Val complexes between the SDS micellar phase and the bulk electrolyte phase. As we have noted, the differences in stability between the ternary complexes formed by each of the enantiomers is difficult to determine in the Cu(II)–L-Val solution. And the migration order in

Table 2
Separation data for dansyl amino acids

Amino acid	t_L (min)	t_D (min)	α	R_s
Dns-Val	8.456	8.733	1.02	1.9
Dns-Thr	8.904	9.058	1.02	1.9
Dns-Nva	9.113	9.600	1.05	4.4
Dns-Nbu	9.537	9.908	1.04	4.9
Dns-Asp	10.050	10.262	1.02	1.5
Dns-Met	10.675	11.625	1.09	8.9
Dns-Leu	11.804	12.458	1.06	9.6
Dns-Glu	12.748	12.833	1.01	0.9
Dns-Nle	13.329	15.642	1.17	12.9
Dns-Phe	15.242	17.967	1.18	13.1
Dns-Trp	23.721	24.667	1.04	3.6
Phe	10.975	11.375	1.04	1.1
Trp	16.529	18.063	1.09	4.3

Migration time, separation factor (α) and resolution (R_s) are given.

Conditions: 20 mM NH₄OAc, 8 mM L-Val, 4 mM CuSO₄, 20 mM SDS, adjusted with NH₃·H₂O to pH 9.0.

the present system is thought to be dependent on the partition coefficient. From Table 2, the Dns-Trp derivative with the most hydrophobic side chain partitioned between the micelle and the buffer with the greatest distribution percentage in the micelle and showed the highest retention in the micellar phase and eluted lastly. These results suggest that the major

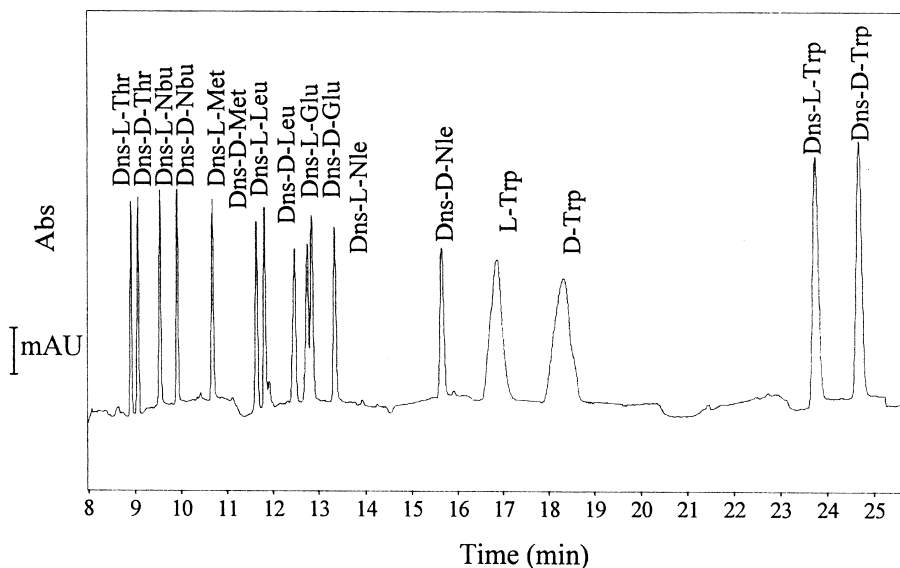


Fig. 5. Electropherogram of the optical resolution of underivatized and Dns-amino acid enantiomers. Running electrolyte contains 20 mM SDS, 20 mM NH₄OAc, 4 mM Cu²⁺ and 8 mM L-Val, pH was adjusted at 9.0.

factor involved in the recognition mechanism is the affinity of the complexes for the micellar phase, therefore, the distribution of an analyte between the micellar and aqueous phases plays the key role in the separation.

3.5. Comparison of other amino acid ligands with L-Val

An important component of the recognition process is the nature of the chiral ligand. In order to investigate whether chiral discrimination with copper(II) complexes of other amino acid ligands may occur, some other ligands, i.e., L-Ala, L-Glu, L-Orn and L-Ser were explored for the enantiomeric separation of Dns-amino acids under the same experimental condition and chiral separation ability was compared with that of L-Val. When L-Ala or L-Glu was used as chiral ligand, no chiral separation was achieved. L-Orn and L-Ser showed relative high resolution ability and the enantioseparation increases as a function of the hydrophobicity in the following order: L-Ser < L-Orn < L-Val. The higher hydrophobic the ligand, the better its resolution ability. This might be due to its high hydrophobicity which allows additional binding between the hydrophobic side chain of the selector and SDS micelle interior. As stated above, the stereoselectivity in solution contributes but a minimum to the overall discrimination process in LE-MEKC and that the major factor involved in the recognition mechanism is the affinity of the analytes for micellar phase. These results are similar to the recognition mechanism reported for HPLC using complex as chiral additives in mobile phase [24,25]. It was assumed that two factors were responsible for chiral recognition in ligand-exchange chromatography according to the three-point interaction rule: bidentate coordination of two amino acids to a metal ion and additional interactions of a substituent of the amino acid with the support. Finally, it was stated that the difference in stability of the diastereomeric ternary complexes in aqueous phase has a negligible influence on enantioselectivity and the chiral recognition takes place on the column where the initial complex is adsorbed. Our results shows clearly that the approach of LE-MEKC can be considered as a type of ligand-exchange chromatography from the nature of the recognition mechanism.

4. Conclusion

An optical resolution system for enantioseparation using a Cu(II)–L-Val complex as chiral selector is developed. The chiral Cu(II)–L-Val complex shows effective enantioresolution for the study of dansyl amino acids and underivatized amino acid enantiomers in combination of SDS as achiral micelle forming reagent. The procedure has been optimized in terms of SDS concentration, the running buffer pH, the ternary complex composition and the concentration of the complex. Under optimum conditions, enantioseparation of 11 Dns-amino acids as well as two underivatized amino acids was achieved (see Table 2). This work will make an inspiration to develop the more useful CE chiral separation system and further efforts will be made to extend the applications of this method in real samples, such as some chiral drugs containing amino alcohol structures.

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