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Review

Enantioselective ligand exchange in modern separation techniques

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Abstract

As a follow-up to a series of review articles on enantioselective ligand exchange chromatography, the present contribution critically evaluates achievements in this area of active and successful research which have been reported in the scientific literature since 1992. Also discussed is enantioselective ligand exchange in electromigration techniques which have developed especially fruitfully during the last decade.

Keywords: Reviews; Enantiomer separation; Chiral recognition; Electromigration techniques; Ligand exchange; Enantioselective ligand exchange

Contents

1. Introduction

Enantioselective ligand exchange chromatography (LEC), suggested by Davankov et al. [1–5] in the late 1960s–early 1970s for the resolution of racemic compounds into enantiomers, was the first liquid chromatography technique that provided a complete and reliable separation of stereoisomers of the then most important classes of natural and synthetic compounds, such as α-amino acids, hydroxy acids,

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amino alcohols and some others. The basic principle of enantioselective LEC was demonstrated by chemically binding a chiral selector, a Cu(II) complex of a natural amino acid, to the matrix of a polymeric particulate sorbent and conducting chromatography of the above mentioned racemates under conditions suitable for the formation of ternary complexes composed of the stationary chiral ligand, central Cu(II) ion and the analyte. Enantioselectivity of formation of the ternary complexes, i.e. the difference in the thermodynamic stabilities of two diastereomeric structures incorporating (*R*) or (*S*) enantiomers of the analyte, was found to be extremely high. It allowed both an analytical and a micropreparative scale separation of enantiomers, even at the relatively low efficiency of LC columns initially used. Together with the first success in gas chromatographic (GC) separations of enantiomers [6], which was achieved due to the outstanding efficiency of capillary GC columns introduced at that time, the above triumph of enantioselective LEC caused an outburst of research in stereochemistry, pharmacology, asymmetric synthesis, enantioselective catalysis, etc. Enantioselective LEC played a leading role in this progress and also contributed decisively to the emergence and development of basically new chromatographic techniques, including dynamic chiral coating of column packings, use of chiral mobile phase additives to the eluent, and preparative separations on simulated moving beds, as well as a series of electromigration techniques.

The progress of enantioselective LEC has been systematically reviewed by Davankov [7–15]. Reviews by Kurganov [16] and Gübitz et al. [17] specifically deal with the problems of peak shape analysis in LEC and the progress in electromigration techniques, respectively. Referring to our most detailed and latest reviews [9,12–15] which cover scientific publications up to 1992, we restrict ourselves here to more recent information on HPLC and electromigration techniques. Also, we do not discuss complexation gas chromatography, which is related to LEC, but developed parallel to it. This literature can be traced back from excellent reviews on GC [18] and supercritical fluid chromatography (SFC) [19] by Schurig, the main author of the complexation chromatography technique.

2. Resolutions aided by an electrical field: chromatography or electrophoresis?

The ligand exchange concept contributed much to the development of chiral electromigration techniques, with chiral capillary electrophoresis first of all. It appears appropriate here to analyze the principal distinguishing features of chromatographic and electrophoretic techniques, since in several chiral ligand exchange processes the above two separation techniques seem to interpenetrate and even sometimes to merge.

Basically, chromatography is a two-phase separation technique, where the ‘‘mobile’’ phase transports the enantiomeric analytes along the ‘‘stationary’’ phase, which provides a differential, enantioselective retention of the enantiomers. The movement of one phase relative to the other is thus the essential feature of all chromatographic techniques. (As discussed in Ref. [20], so-called hydrodynamic chromatography and all field flow fractionation approaches, which are sometimes considered to be one-phase separations, are in fact typical two-phase chromatographic techniques. The role of the ‘‘stationary’’ phase, in this case the walls of the capillary channel, is reduced here to the ‘‘retention’’ of the analytes through the retardation of the flow velocity of the mobile phase layers that adhere to the immobile solid surface. Nevertheless, the very presence of the immobile walls and the movement of the mobile phase along the walls are essential for the analytes to be separated.)

In contrast to chromatography, electrophoresis is a one-phase separation technique. Here, electrically charged analytes are self-transporting species and neither the movement of the bulk solution nor the presence of walls or any other stationary phase are needed for the separation to occur. The analytes separate due only to the difference in their movement velocity, their electrophoretic mobility. It is true that the movement of the bulk electrolyte due to the electroosmotic phenomena (or external pressure drop) can benefit the technique in many different ways, but this movement does not contribute to the separation of the analytes. Thus, electrophoresis (formerly ‘‘free zone electrophoresis’’) is not a chromatographic technique.

The basic difference between chromatography and electrophoresis becomes less transparent in capillary electrochromatography (CEC) techniques. Though the presence of a stationary phase (in the form of a column packing or wall coating) and the movement of the mobile phase along the stationary phase (due to a pressure gradient or electroosmosis) are essential features of chromatography in CEC, the electric field applied to the ends of the column can cause an electrophoretic contribution to the separation of the analytes, provided that the latter are charged and possess self-transporting properties relative to the mobile phase.

The electrophoretic contribution to analyte resolution becomes even more significant in micellar electrokinetic chromatography (MEKC) where the ''pseudo-stationary'' phase is represented by the micelles of a surfactant [21]. The micelles can be neutral or charged. In the first case charged analytes can be separated, in the second both neutral and charged can be separated. At least one of the components of the system, the micelles or analytes or both, must be charged, in order to provide the transportation of analytes. During their movement, the analytes partition between the bulk solution and the pseudo-stationary phase, mainly due to hydrophobic and electrostatic interactions. The phase partitioning process represents the chromatographic contribution to the enantiomeric separation in MEKC. Most probably, this contribution significantly exceeds that of the electrophoretic contribution. The enantioselectivity of formation of ternary complexes in the bulk solution is generally very small. This means that the extent of the involvement of two enantiomers into the complexation process is nearly identical, whereas the size and charge of the two diastereomeric ternary complexes formed are exactly identical. The two diastereomeric species thus can only differ in their shape. This difference, most probably, does not influence significantly the electrophoretic mobility of the species, but can affect most dramatically their adsorption on solid or pseudo-solid surfaces. This phenomenon of the decisive contribution of achiral solid surfaces to the chiral discrimination of enantiomers in their ternary complexes was discussed in detail by Davankov [22,23] regarding chromatography (on dynamically coated chiral stationary phases). The same phenomenon should be valid for the ''adsorption'' of ternary complexes on pseudo-stationary phases, i.e. on micelles in MEKC systems, largely explaining the success of this technique in chiral ligand exchange resolutions.

The balance between contributions from chromatography and electrophoresis must shift substantially towards electrophoresis in systems where a soluble polymeric selector is used, instead of a micelle-forming surfactant. The polymer solution must be considered as an isotropic, homogeneous phase. Through interaction with the analytes, by retarding or facilitating the movement of the latter in the electric field, the soluble polymeric molecules serve as ''pseudo-micelles'' (but no longer as a ''pseudo-stationary phase''). Here, as in MEKC, it is only important that at least one of the components of the system, either the analyte or the polymer molecules, or both, are charged and provide the movement of the analyte relative to the bulk solution. Whether the solution as a whole moves in the capillary or not, is irrelevant for the resolution of analytes, which is characteristic of all one-phase, non-chromatographic systems.

Finally, a typical electrophoretic separation operates in chiral systems with low-molecular-weight soluble selectors, as chiral metal complexes (or cyclodextrins). Through interacting with the chiral selectors of this type, the enantiomeric molecules differentially change their size or charge, thus changing their electrophoretic mobility.

The ligand exchange concept has been realized in all the above chromatographic and electrophoretic systems, thus contributing significantly to their emergence and development. It should be mentioned here that the mathematical description of resolutions is logically made either in terms of the analyte distribution between two chromatographic phases, or in terms of the electrophoretic mobility of the analytes and their labile adducts with the chiral selectors present in the one-phase electrophoretic separation system. The mathematical description of separation in systems where the contributions from both chromatography and electrophoresis are significant, still remain to be developed (first approaches can be found, for example, in Ref. [24]). At last, but not least, it should be mentioned here that in systems

where the chiral selector retards and in those where the selector accelerates transportation of two enantiomers toward the detector, opposite elution sequence of the enantiomers is generated. This regularity [25] must be valid for both chromatography and electrophoresis.

3. Enantioselective ligand exchange in combination with electromigration separation techniques

Several excellent reviews on chiral separations by electromigration techniques appeared in the last decade [26–34]. The last one [34] deals with ligand exchange separations. Accounting for the above significant differences in chromatographic and electrophoretic principles, we present below our critical evaluation of the literature on ligand exchange in electromigration techniques.

3.1. Capillary electrophoresis (CE)

Ligand exchange was the first enantioselective separation technique used in capillary electrophoresis. As early as 1985, Gassman et al. [35] reported on the use of 2.5 m*M* Cu(L-histidine)$_2$ in 10 m*M* ammonium acetate background electrolyte (BGE) for a successful separation of enantiomers of 12 *N*-dansyl-amino acids (Dns-AAs). In this one-phase system, the only driving force for chiral recognition is the difference in the thermodynamic stabilities of diastereomeric ternary complexes Cu(L-His) (L- or D-Dns-AA) formed by the analyte enantiomers with the chiral selector [Cu(L-His)$_2$]. This difference results in the different involvement of the two analyte enantiomers in the formation of ternary complexes. In spite of the low enantioselectivity of the complexation process in solution, the analyte enantiomers were completely separated, due to the extremely high efficiency of CE (a plate number of over 100 000 for a 75-cm capillary). Being incorporated into the ternary complex with the basic L-His, the analytes form a positively charged species and move faster toward the cathode and the detector (in the pH range of 7–8, the uncomplexed Dns-AA species are charged negatively). D-Isomers of Dns-AAs were found to appear first at the detector, implying that they bind preferably to the Cu/L-His selector. It is noteworthy that the enantiomers bound more strongly to the selector show a weaker fluorescence signal, which is the result of quenching from association with the copper ion. Replacement of Cu(II) with Co(II) ions resulted in a loss of enantioselectivity, whereas good resolutions were found to occur in the BGE containing Cu(aspartame)$_2$ (L-aspartyl-L-phenylalanine methyl ester) complexes [36] instead of Cu(L-His)$_2$. Both selector systems showed similar separation regularities and in both cases it was very difficult to resolve mixtures of several neutral amino acids (Fig. 1). Aspartame was also combined [37] with Mg(II), Cd(II) and Zn(II) in

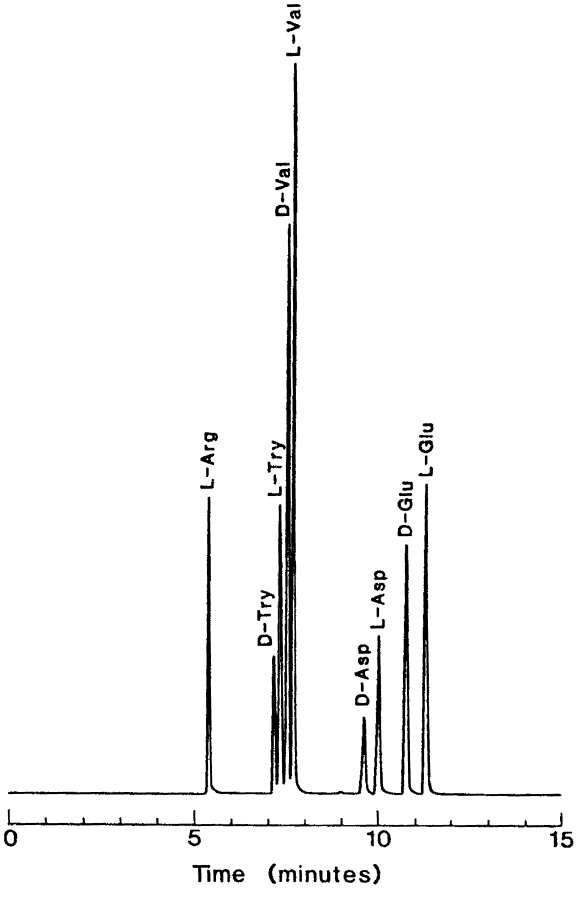


Fig. 1. Electropherogram of a mixture of four Dns-DL-AAs and Dns-L-Arg. BGE, 5.0 m*M* aspartame, 2.5 m*M* Cu(II), 10 m*M* NH_4OAc, pH 7.4; capillary, 100 cm 75 μm I.D. From Ref. [36].

the CE of Dns-AAs, but with no advantage over Cu(aspartame)$_2$.

Schmid and Gübitz [38] successfully resolved racemates of ten unmodified aromatic amino acids by CE in a fused-silica capillary with a hydrophobic wall coating, using 40 m*M* Cu(L-Pro)$_2$ or, better, Cu(L-Hypro)$_2$ adjusted to pH 4.0. Organic modifiers like methanol and acetonitrile were found to decrease the resolution. In all experiments the D-enantiomers migrated faster than the L-isomers. Nevertheless, in this case, the conclusion that the heterochiral Cu(L-Hypro) (D-AA) ternary complexes are more stable, needs to be carefully re-examined. At pH 4.0, the uncomplexed AAs could be expected to acquire a positive charge and migrate faster than the neutral ternary complexes. If D-enantiomers were more strongly involved in formation of neutral complexes with Cu and L-Hypro, they should migrate more slowly. Precisely this interpretation, namely that the heterochiral complexes Cu(L-Hypro) (D-Phe) and Cu(L-Hypro) (D-Trp) are the less stable species, is given for the same CE system in Ref. [39]. In this paper, Chen et al. interestingly report that the sign of enantioselectivity and the enantiomeric migration order (EMO) of aromatic AAs inverses, when L-Hypro (*trans*-arrangement of the carboxy and hydroxy groups, relative to the five-membered pyrrolidine ring) is replaced by *allo*-L-Hypro (or *cis*-L-Hypro). The latter ligand is tridentating, since its hydroxy group, in addition to the α-amino and carboxy functions, participates in the coordination to Cu(II).

Karbaum and Jira [40] applied copper complexes of L-proline and also L-isoleucine in a non-aqueous CE in the resolution of racemic unmodified AAs. In methanolic media containing 25 m*M* ammonium acetate and 1 *M* acetic acid and adjusted to apparent pH* value of 3.9 with NaOH and formic acid, eight investigated AAs were resolved. Interestingly, isomers of basic AAs, His and kynurenine were better separated with L-Ileu selector. In contrast to aqueous systems where the optimal ratio of Cu(II) to chiral amino acid was always found to be ~1:2, in methanol the optimal proportion of the selector amounts to 3. Increasing the total concentration of the selector complex in the BGE usually enhances selectivity of separation, but makes the detection of the resolved analytes more difficult. When Cu(II) was replaced by Co(II), no separation of the enantiomers could be observed.

A more efficient chiral selector was synthesized by the Gübitz group [41] by alkylating the secondary nitrogen atom of L-Hypro with 1,2-epoxyoctane in an alkaline media. The *N*-(2-hydroxyoctyl)-L-4-hydroxyproline, HO-L-Hypro, thus obtained is capable of functioning as a tridentate ligand at the copper ion, with the 2-hydroxy group (that originates from the opened epoxy ring) occupying an axial position at copper. Though the hydroxy group-containing 2-carbon atom of the *N*-octyl chain becomes asymmetric, with its configuration remaining unknown and probably irregular, its asymmetry obviously does not influence significantly the enantiorecognition capacity of the chiral selector, as this was the case in most chiral chromatographic systems that operate with similar kind of selectors [13]. Combined with the more dense packing of electron donating atoms in the coordination sphere of copper with the tridentate HO-L-Hypro, additional hydrophobic interactions between analyte enantiomers and the octyl group of the chiral selector may contribute to better mutual recognition of the chirality of the partners. Baseline separations of enantiomers of 12 aromatic amino acids (at pH 4.3) and the basic histidine (at pH 6.0) was achieved [41]. As little as 0.03% of D-DOPA could be detected in the L-preparation. The new selector *N*-(2-hydroxypropyl)-L-4-hydroxyproline, HP-L-Hypro, proved to be equally efficient having a shorter C3 substituent at the nitrogen of L-Hypro. A total of 13 underivatized aromatic and six aliphatic amino acids were successfully separated into enantiomers [42]. Since a smaller concentration of HP-L-Hypro was needed than that of L-Hypro, detection problems with aliphatic amino acids were minimized. Glycil-DL-phenylalanine was baseline resolved, whereas four DL-leucyl-DL-phenylalanine stereoisomers were split into three peaks only. In total only five among ten examined dipeptides displayed enantioseparation. In some cases, at high concentrations (20 m*M*) of HO-L-Hypro, 35% methanol was needed in the 5 m*M* phosphate BGE to overcame the low solubility of the selector complex.

In an ammonia containing BGE of pH 12, the Cu(HO-L-Hypro)$_2$ selector was found to baseline resolve nine out of 13 sympathomimetic amino alcohols of the ephedrine family [43]. The enantio-

meric analysis of these drugs is of great interest, since the *R*-(−)-enantiomer of epinephrine, for example, shows an ~50-fold higher activity. *R*-(−) enantiomers of both ephedrine and norephedrine were found to elute before the *S*-(+)-stereoisomers. Authors note that ''at high pH the selector is negatively charged and tends toward the opposite direction of the EOF. This retards the analytes, which migrate with lower velocity than the EOF''. Here again the conclusion that the more strongly retarded *S*-(+) isomer must show higher affinity to the selector, does not seem to be logical. The moment the selector forms a ternary copper complex with the analyte, the total charge of the new species becomes neutral or positive, depending on the dissociation of the amino alcohol hydroxyl group. Therefore, the stronger bound enantiomer should be expected to move faster, as a component of a neutral ternary complex, not as a free anion. In any case, one must consider relative charges and possible electrophoretic mobilities of the analyte-containing species only, not those of the selector itself. It is noteworthy that drugs having one (e.g. octopamine) or two (e.g. orciprenaline) dissociating phenolic groups migrate substantially slower. Using the same enantioselective system, six β-blockers, including propranolol and oxprenolol, were successfully resolved [44]. Their behavior in the CE experiments is typical for β-amino alcohols.

Fanali et al. [45] succeeded in the resolution of some racemic α-hydroxy acids using Cu(II) complexes of L-Pro, L-Hypro and aspartame. The influence of capillary temperature, applied voltage and pH of the BGE on the extent of the separation was studied. Krasensky et al. [46] used the same selectors for the resolution of α-hydroxy acids in a column coupling system, in order to avoid interference of the high UV absorption of the BGE with the detection. The detection limit for hydroxy acids could be improved to 10^{-18} *M* by this approach. When discussing the resolution of α-hydroxy acids in the Cu(HO-L-Hypro)$_2$ BGE of pH 4.3, Schmid et al. [44] reasonably notice that at that pH the selector complexes are neutral and move with EOF (indicated by DMSO), and amino acids are charged positively and elute earlier, whereas α-hydroxy acids bear a negative charge and remain much longer in the capillary. The latter resolve with very high enantioselectivity values, up to R_s=6.8 for 3-(4-hydroxyphenyl)lactic acid. Since the resolution was excellent with most of the hydroxy acids, increasing the voltage and shortening the effective length of the capillary allowed the analysis time to be reduced. In total, 11 racemic hydroxy acids were resolved.

A more complex task, separation of four stereoisomers of β-methyl substituted α-amino acids, has been addressed recently by the same research group [47]. Of the three chiral selectors tested in CE, Cu(II) complexes of L-Hypro, HP-L-Hypro and HO-L-Hypro, the second one was found to be superior to the others. Baseline separations were achieved at pH 4.3 for the enantiomers of β-Me derivatives of Phe, Tyr and Trp (Fig. 2), but not for the derivatives of 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.

An important theoretical paper was recently published by Chen et al. [48]. The authors derived equations that relate the electrophoretic mobility of the analytes to the stability constant of their ternary Cu(II) complex with the chiral selector (L-Hypro). By plotting mobility values as the function of the selector concentration in the BGE, it becomes possible to evaluate the thermodynamic stability constants of the hetero-ligand ternary complexes. CE seems to provide an unique possibility for calculating these barely available parameters. It turned out that heterochiral complexes Cu(L-Hypro) (D-AA), where AA is Phe or Trp, are less stable than the corresponding homochiral species. Accordingly, D-enantiomers are less involved in formation of neutral

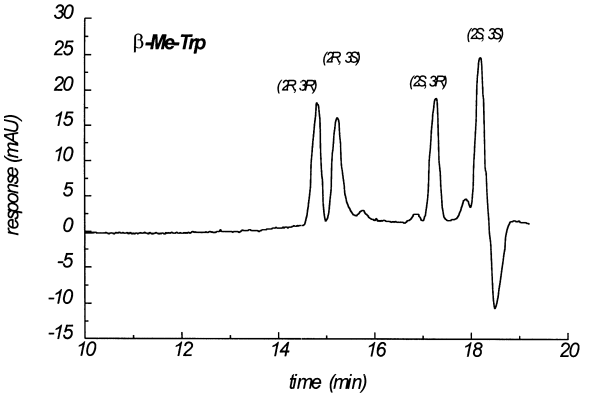


Fig. 2. Electropherogram of a mixture of four stereoisomers of β-methyl-tryptophan. BGE, 20 m*M* HP-L-Hypro, 10 m*M* Cu(II), in 5 m*M* phosphoric acid, pH 4.3. From Ref. [47].

ternary complexes and, being partially protonated species at the pH 4.0 of the experiment, move faster towards the cathode. It was also shown that higher enantioselectivity values can be obtained at the Cu(II) to selector ratio of 1:1, rather than at the ratio of 1:2, since, in the Cu(L-Hypro) $(H_2O)_2$ or Cu(L-Hypro) $(NH_3)_2$ complexes, water or ammonia molecules can be easily replaced by the bidentate analyte ligands to form the desired ternary complex, as compared to the ligand exchange in a more stable Cu(L-Hypro)$_2$ complex.

Recently [49], Cu(II) complexes of a basic AA, L-lysine were tested as the chiral selector in a BGE of pH 7.0. Unfortunately, the positively charged species were found to adsorb on the capillary walls, thus reducing the EOF, increasing the analysis time and diminishing the efficiency of resolution of free AAs. The resolution of Dns-AAs with another basic AA, L-arginine [50] proved to be much more efficient. Simultaneous resolution of six racemic Dns-AAs with this chiral selector in combination with Cu(II) ions at pH 7.0 was achieved (Fig. 3). Interestingly, combinations of Co(II), Zn(II), or Ni(II) with L-Asp, L-Glu or L-Ala resulted in a loss of chiral recognition in most cases.

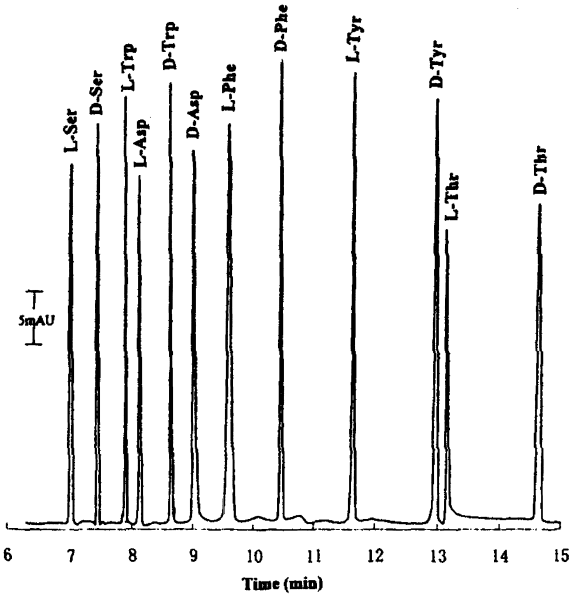


Fig. 3. Electropherogram of a mixture of six Dns-DL-AAs. BGE, 2.0 m*M* L-Arg, 1.0 m*M* Cu(II), 40 m*M* NH_4OAc, pH 4.0; capillary, 52 cm 50 μm I.D. From Ref. [50].

Besides various α-amino acids and their derivatives, another group of chiral selectors deserves to be examined in the ligand exchange CE, namely, α-hydroxy acids. Kodama et al. [51] succeeded in separating malic acid enantiomers by using Cu(II)–L-tartrate complexes in the BGE. Vice versa, applying 1 m*M* copper sulfate and 10 m*M* D-quinic acid (1,3,4,5-tetrahydroxy-cyclohexane-carboxylic acid) at pH 5.0, they developed a method for enantiomeric analysis of tartrate additives in grape juices, wines, jams, etc. (In Japan, both L- and racemic tartaric acids can be legally used in food products.) The authors employed a poly(vinylalcohol) coated capillary to suppress EOF. Since carboxylic groups of tartaric acid, with their pK_a 2.93 and 4.23, are dissociated in the BGE at pH 5.0, all complexes migrate to the anode. Under conditions of experiment, racemates of malic, lactic and glyceric acids showed no enantioseparation. Though the structure of ternary complexes that incorporate tartrate anions may be very complicated, using other than copper-amino acid complexes in the ligand exchange CE may extend the application of this technique.

A polycyclic antibiotic, Vancomycin, known to be an outstanding chiral selector both in HPLC and CE systems, was tested in the form of its Cu(II) complex in an aqueous–organic BGE. Partial resolutions of some non-steroidal anti-inflammatory drugs, Dns- and *N*-CBZ-amino acids, folic acid derivatives, etc., were obtained at pH of 5.0. However, Vancomycin itself resolved these analytes much more effectively. Obviously, the Cu(II)-ion blocks the most selective site of the polycyclic selector. On the other hand, functional groups of the selector occupy five coordination positions of the metal (the complex is purple in color), which prevents further ligand exchange around the metal. Most probably, only free Vancomycin molecules that form after dissociation of Cu(II) complexes at the low pH, not the complex itself, cause the chiral resolution of the analytes [52].

An interesting application of ligand exchange in the outer coordination sphere of kinetically inert Co(III) complexes with ethylenediamine and *o*-phenanthroline has been demonstrated by Fanali et al. [53]. Using solutions of L-(+)-tartrate, they were able to separate enantiomers of these and some other octahedral complexes.

An unexpected and important finding was made by

Horimai et al. [54], namely, that only a combination of the ligand exchange process with the formation of inclusion complexes provides a baseline separation of enantiomers of a series of quinolone drugs. These heterocyclic compounds belong to β-keto-carboxylic acids and form ternary complexes with divalent metal ions and α-amino acids. However, only inclusion of these ternary complexes into γ-cyclodextrin results in chiral differentiation in CE. Of α, β, γ, and dimethyl-β-cyclodextrins, Fe, Ni, Cu and Zn metal ions and the following chiral amino acids: Phe, Tyr, Ser, Thr, Val, Leu, Ile, Met, Asp, Lys, Arg and Pro tested, only a combination of γ-CD, Zn(II) and aromatic AAs, preferably Phe, was found to be effective and give adequate resolution. Replacing D-Phe for L-Phe inverted the EMO and slightly reduced the resolution. The resolution of a quinolone is considered to proceed by formation of the diastereomeric ternary complexes with Zn(II) and Phe and inclusion of the complexes into the hydrophobic cavity of the γ-CD. This report opens a new field of searching for synergetic combinations of several different chiral selectors in CE.

3.2. Micellar electrokinetic chromatography (MEKC)

In one of the very first reports [35], the Gassman/Zare group tested the addition of sodium tetradecyl sulfate micelles to the chiral Cu(aspartame)$_2$ containing BGE and observed an improvement of the resolution of enantiomeric pairs of Dns-AAs. In addition to this, the MEKC approach facilitated the separation of several equally charged analytes from each other. Under conditions of MEKC, all components of the system, including analytes and their ternary complexes, partition between the bulk solution and the ''pseudo-stationary phase'', micelles formed by the surfactant. This chromatographic component of the MEKC system significantly contributes to the resolving power of the system, also enhancing its enantioselectivity. Another early MEKC system contained mixed micelles of SDS that were doped with Cu(II) complexes of *N*,*N*-didecyl-L-alanine [55]. This chiral selector alone did not form individual micelles, but readily converted the SDS micelles into a chiral pseudo stationary phase and resolved a series of racemic Dns-AAs. The hydrophilic L-Ala part of the selector obviously remained exposed to the surface of the mixed micelles and readily formed ternary complexes with the Dns-AA analytes. Similar experiments on 11 Dns-AAs were later carried out by Sundin et al. [56].

An improvement in the enantiomeric resolution on switching from a CE to MEKC mode was also observed by Schmid and Gübitz [38] after adding SDS to the Cu(L-Hypro)$_2$ containing BGE. Interestingly, when the surfactant was added, the L-enantiomers of the 11 aromatic AAs tested started to migrate faster than their D-analogues (Fig. 4). The same changes were reported [49] to proceed on adding SDS to Cu(L-Lys)$_2$-based BGE.

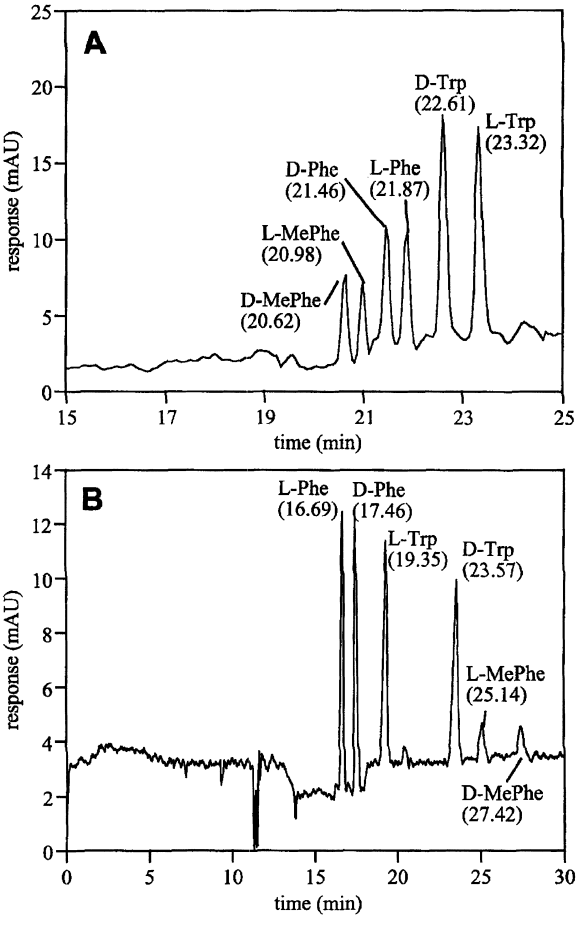


Fig. 4. (A) Electropherogram of a mixture of three AAs. BGE, 80 m*M* L-Hypro, 40 m*M* Cu(II), pH 4.0. (B) MEKC mode of separation of the same mixture. BGE, 50 m*M* L-Hypro, 25 m*M* Cu(II), 15 m*M* SDS and 3 m*M* urea, pH 4.0. From Ref. [38].

This important observation was used by Chen et al. [57] to develop an elegant method for the determination of the critical micelle concentration (CMC) of a series of anionic surfactants. Increasing the surfactant concentration in a Cu(L-Hypro)$_2$ containing BGE first results in the decrease of the enantiomeric resolution of DL-Phe and DL-Trp and then, after inversion of the elution order of the amino acids and their enantiomers, in a significant increase in the resolution. The inflection point at which resolution equals zero was considered as the CMC value of the surfactant. Thus, a 5 m*M* SDS concentration corresponds to the CMC of the surfactant [58]. In the same paper, the authors try to explain the inversion of EMO of analytes on addition of SDS by the inversion of the migration direction of analytes and complexes, when they enter the negatively charged micelle. This explanation, however, is a serious oversimplification, since all species acquire identical migration velocity with respect to the BGE, namely, the velocity of the micelle, when they enter this pseudo-stationary phase. Rather, one has to consider the extent of the phase partition of all components, as well as the enantioselectivity of complexation within the micelle. The latter may differ substantially from the enantioselectivity in bulk solution. This situation was discussed in more detail by Davankov et al. [25] in HPLC systems operating in accordance with the ligand exchange mechanism. A reasonable analysis of the inversion of EMO by adding SDS is given in Ref. [59], where the authors also observe an inversion of migration order of enantiomers on using tridentate *allo*-L-Hypro, instead of the bidentate L-Hypro ligand.

The same group of Chen et al. [60] described a series of practically important chiral resolutions using LE-MEKC technique. The simultaneous separation of 12 enantiomers of *o*-, *m*-, *p*-fluoro-DL-phenylalanine and *o*-, *m*-, *p*-tyrosine by using 25 m*M* Cu(L-Hypro)$_2$ chiral selector in the presence of 10 m*M* SDS at pH 4.5 was very impressive (Fig. 5). A total of 16 positional and optical isomers of the tryptophan family (having hydroxy, methyl or fluoro substituents) were successfully separated as well [61]. It is noteworthy that neutral surfactant Tween 20 suppressed the EOF and caused longer migration times. Cethyltrimethylammonium bromide (CTAB), a cationic surfactant inverted the direction of the

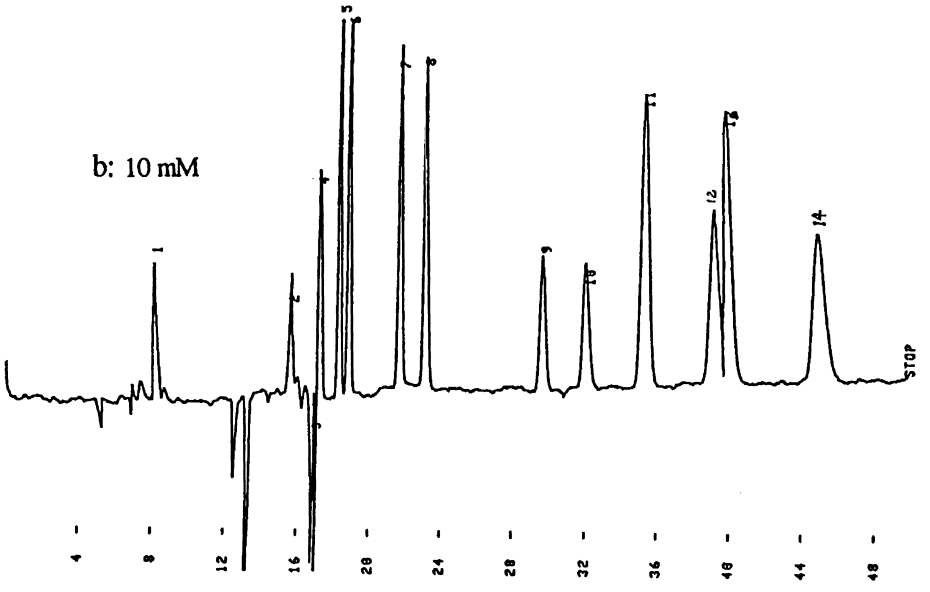


Fig. 5. MEKC mode of separation of a mixture of three positional isomers of tyrosine and three isomers of fluoro-phenylalanine. BGE, 50 m*M* L-Hypro, 25 m*M* Cu(II), 10 m*M* SDS, pH 4.0. Peaks: 1 and negative peaks are system peaks, 3=*p*-L-Tyr, 4=*p*-D-Tyr, 5=*m*-L-Tyr, 6=*m*-D-Tyr, 7=*o*-L-Tyr, 8=*o*-D-Tyr, 9=*o*-F-L-Phe, 10=*o*-F-D-Phe, 11=*m*-F-L-Phe, 12=*p*-F-L-Phe, 13=*m*-F-D-Phe, 14=*p*-F-D-Phe. From Ref. [60].

EOF, but did not improve the resolution [62]. Of other anionic surfactants, such as alkylbenzenesulfonates and straight-chain alkyl sulfates, SDS was the most efficient. Organic modifiers hinder the phase distribution of the ternary complexes and diminish the effect of resolution. This negative influence increases in the sequence DMSO, MeOH, EtOH, MeCN, acetone, 2-propanol and THF, in correlation with their distribution coefficients between water and *n*-octanol, log P_{ow}. Migration times of analytes in the MEKC systems also correlate with the log P_{ow} values of the analytes [63].

A series of racemic hydroxy acids (mandelic acid, β-phenyllactic acid, indole-3-lactic acid and their hydroxy and methoxy derivatives) were resolved in the same MEKC system. Interestingly, adding SDS to the Cu(L-Hypro)$_2$-based BGE does not change the enantiomeric migration order of hydroxy acids (L-enantiomers always migrate faster), whereas adding CTAB does [64].

3.3. Capillary electrochromatography (CEC)

Capillary electrochromatography (CEC), a modern separation technique, integrates major features of high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). As in HPLC, this technique can employ all kinds of packed and wall-coated capillaries and operate in chiral resolutions, depending on the location of the chiral selector, with all possible enantioselective systems, i.e. chiral stationary phase (CSP), chiral coated phase (CCP) and chiral mobile phase (CMP). The flow of the mobile phase is basically generated by the electroosmosis, though a certain pressure difference at the ends of the capillary can modulate the total flow. Thus far, only few reports have been published dealing with the combination of enantioselective ligand exchange and CEC, but all of them relate to the most progressive and fascinating type of CSP, namely continuous beds (or monolithic columns).

The first report on the preparation of a continuous polymeric bed designed for combining the LEC and CEC techniques appeared from the group of Schmid, Gübitz and Hjertén [65]. In a quartz capillary of 26 cm length and 75 μm I.D., authors in situ copolymerized an aqueous mixture of methacrylamide (monomer), piperazine diacrylamide (cross-linker), vinylsulfonic acid (introduction of a negative charge), and *N*-(2-hydoxy-3-allyloxypropyl)-L-4-hydroxyproline (chiral selector). For the fixation of the polymer to capillary walls, the latter were pretreated with γ-methacryloyloxypropyl-trimethoxysilane. A redox initiating system was used composed of ammonium peroxodisulfate and tetramethyl ethylenediamine, to result in a homogeneous packing of interconnected nodules of ~3000 Å in diameter. Since the negative charge of the bed originated from the strong sulfonic acid units, the EOF (from anode to cathode) was independent of the pH of the BGE. Nine racemic unmodified AAs were baseline resolved in the 50 m*M* sodium dihydrogenphosphate, pH 4.6/0.1 m*M* Cu(II) as BGE. Separation time of Phe enantiomers, with D-Phe eluting faster, could be reduced to 8 min by using a shorter (8.5 cm) continuous bed and a 12-bar pressure support at the column inlet. Pressure-driven HPLC on the same monoliths showed lower plate numbers. Comparable results were obtained when *N*-(2-hydroxyoctenyl)-L-4-hydroxyproline was used instead of the *N*-(2-hydoxy-3-allyloxypropyl)-L-Hypro chiral selector in the polymeric monolith. When L-Hypro was replaced by L-Pro, only partial resolution of racemic AAs was observed [66].

Whereas amino acids in weakly acidic solutions are positively charged and migrate along the EOF, anions of hydroxy acids migrate in the opposite direction and reside in the capillary for too long. Therefore, in the preparation of the monolithic bed for CEC of hydroxy acids, the vinylsulfonic acid was omitted in the polymerization mixture [67]. The transport of the analytes was thus accomplished primarily by their own electrophoresis toward the anode. Enantioselective interaction with the ligand exchanging CSP served for the separation of D- and L-stereoisomers. Of 11 racemic hydroxy acids tested, 11 were resolved, with baseline separation of all three lactic acid derivatives. Racemate of mandelic acid showed low resolution, whereas its derivatives were generally better resolved. Plate numbers for the best resolved 3-(4-hydroxyphenyl)lactic acid were found to be 1154 and 634 plate/m, which is lower than for the enantiomers of amino acids. The efficiency of this CEC system thus remains to be further investigated and improved.

A very detailed paper by Chen and Hobo [68] deals with chemically bonded L-phenylalaninamide monolithic silica capillary column. Polycondensation of tetramethoxysilane by a sol–gel process in a carefully cleaned 100 μm I.D. quartz capillary resulted in a continuous silica packing of high permeability. It was activated with (3-glycidoxypropyl)trimethoxysilane and finally treated with a 10% solution of L-phenylalaninamide in DMF at room temperature for 1 week. After complexing with Cu(II) ions, the packing was found to acquire a net positive charge counterbalanced with mobile anions which cause an electroosmotic flow toward the positive electrode (anode). The EOF logically increases with the Cu(II) concentration in the BGE (and the density of the positive charged complexes of the packing), but drops with increasing pH values where unmodified silanol groups begin to dissociate and reduce the positive charge of the surface. Dns-amino acids, as negatively charged species, were found to migrate in the same direction with the EOF, appearing at the detector (UV, 254 nm) in front of the acetone peak. Since L-enantiomers of all Dns-AAs tested showed longer retention times than D-analytes, the ternary complexes Cu(L-PheNH$_2$) (Dns-L-AA) must have higher stability than the corresponding heterochiral diastereomers, though the real structure of the sorption complexes cannot be evaluated from these data and remains largely unknown. The efficiency of the LE-CEC system under optimal conditions (Fig. 6) strongly depends on the type of the analyte and varies over a very broad range for reasons not yet known. No obvious decline in the column efficiency was observed after using the column for hundreds of operations. Interestingly, enantiomers of free amino acids were not separated on the Cu(L-PheNH$_2$)$_2$ CSP, whereas on an analogue Cu(L-ProNH$_2$)$_2$ monolithic column enantiomers of both hydroxy acids and Dns-AAs were separated [69].

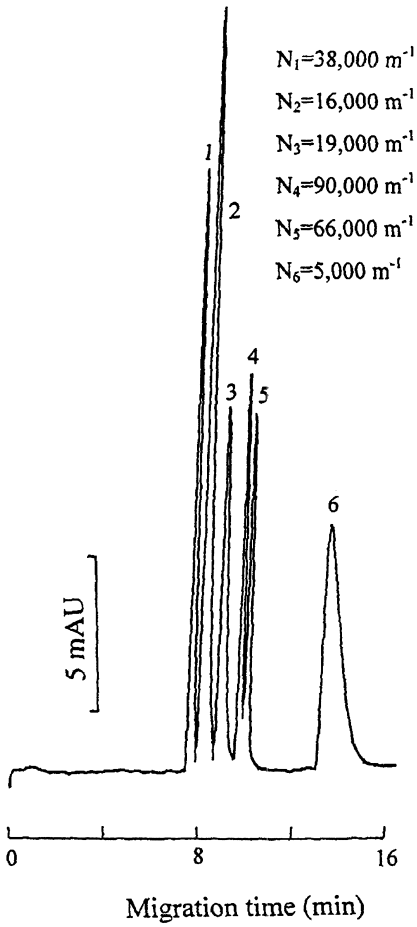


Fig. 6. CEC electropherogram of a mixture of three Dns-DL-AAs. Column: L-Phe-NH$_2$-modified monolithic silica capillary 26.5 cm, 100 μm I.D. BGE, acetonitrile/0.55 m*M* Cu(OAc)$_2$–40 m*M* NH$_4$OAc, pH 5.5. Peaks: 1=D-Thr, 2=D-Ser, 3=L-Thr, 4=D-Leu, 5=L-Leu, 6=L-Ser. From Ref. [68].

4. Enantioselective ligand exchange in combination with chromatographic techniques

4.1. Enantiomeric separations on chiral stationary phases (CSP)

Successful development over 30 years of chiral LEC technique has shown it to be especially effective for the separation of enantiomers of amino acids, numerous derivatives of AAs and hydroxy acids. It is only natural, therefore, that routine problems of enantiomeric analysis of these compounds are usually performed on commercially available ligand exchanging CSP. One typical application of this kind is the monitoring of the conversion of DL-aspartic acid into D-aspartic acid and L-alanine under the action of *Pseudomonas dacunhae*. Ligand exchanging column 5 μm TSK gel Enantio L1 (TOSO, Japan) was tested on 18 essential amino acids under varying tempera-

tures and in combination with Cu(II), Zn(II) and Ni(II) ions [70]. The latter were found to give acceptable results with aromatic AAs only. Zn(II) ions performed well with hydroxyl containing AAs. Most universal was Cu(II), whose use contributed to discovering and proving the formation of L-Thr as a by-product in the above process. A more advanced process, total enzymatic transformation of DL-aspartic acid into L-alanine by combined action of D-amino acid oxidase and aminotransferase, was monitored [71] on a Chiralpak WM (which is a L-Pro-Cu-complex bonded to silica gel) column (Daicel, Japan). The column permits convenient simultaneous determination of enantiomers of Ala and Asp in the fermentation mixture, using an 0.25 m*M* $CuSO_4$ aqueous eluent at pH 3.6 and 45 °C. However, sample pretreatment on a SPE cartridge was found to be essential, in order to remove the proteins and interfering matrix. The problem was solved by an automated column-switching technique.

In a process of converting *N*-acetyl-DL-methionine into L-methionine by the combined action of L-aminoacylase and *N*-acetyl amino acid racemase, the Chiralpak WH column separated enantiomers of both *N*-Ac-DL-Met and DL-Met [72], though still better resolutions of that mixture were provided by a Teicoplanine-based Chirobiotic T column. The LEC technique with Chiralpak WH also helped to prove that no racemization occurs, on storage in aqueous solutions, of the recently developed drug (SSS)-imidapril [73]. This complicated drug molecule contains three asymmetric carbons. A mixture of its eight stereoisomers produces six peaks on the chromatogram with good resolution of all enantiomeric pairs. Enantiomeric analysis of the antifungal agents, econazole, miconazole, and sulconazole, which have N, O or S atoms in their molecules, can be easily accomplished on Chiralpak WH [74]. MCI gel CRS10w column provided convenient analysis of DD-, meso- and LL-isomers of 2,6-diaminopimelic acid without derivatization [75].

Of a very great importance for synthetic peptide chemistry is the finding [76] that the LEC phase ChiralProCu (Serva, Germany) provides fast enantiomeric analysis of almost all N-protected AA derivatives, such as formyl-, acetyl-, monochloroacetyl-, trifluoroacetyl- (Tfa-), benzoyl-, benzyloxycarbonyl- (Z-), *tert*.-butyloxycarbonyl- (Boc-), *tert*.-butyl- (tBu-), 9-fluorenylmethyloxycarbonyl- (Fmoc-) and dansyl- (Dns-) derivatives (only Fmoc-Phe and Fmoc-Val were resolved incompletely). Enantiomers of α-hydroxymethyl AAs [77] also separated, as well as enantiomers of α-methyl AAs, as shown earlier [78,79]. All the above derivatives and unnatural AAs are widely used in peptide synthesis.

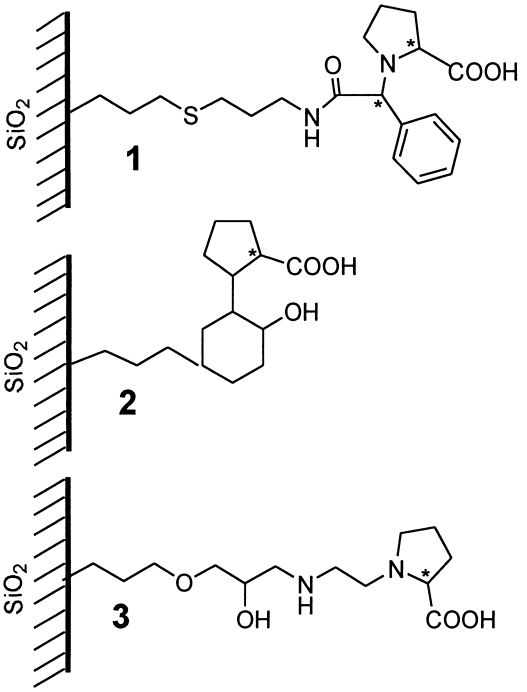
The same column also separates *cis*- and *trans*-isomers of enantiomerically pure proline-containing dipeptides, Leu–Pro and Ile–Pro [80]. Columns based on chiral Pro and especially Hypro and Val are well suited for the analysis of Phe and PhGly containing one or several fluorine atoms in the aromatic ring [81].

The Chiral-Si 100 L-Hypro-Cu (Serva, Germany) column readily resolves racemic α-hydroxy acids [82]. Thus, studies into asymmetric synthesis of 2-hydroxy-4-phenylbutyric acid were strongly facilitated by the enantiomeric excess (*ee*) analysis of the product on that column [83]. A very important experimental and theoretical report on LEC of hydroxy acids on Chiral-Si 100 L-Hypro-Cu appeared from the group of Chilmonczuk [84]. The authors successfully resolved 16 of 17 examined racemates of α-hydroxy acids and one β-hydroxy acid (only 2-hydroxy-2-methylbutyric acid was not resolved). Then, the authors analyzed the applicability of the general model of chiral recognition of amino acids in LEC to the recognition of hydroxy acids. These rules, most concisely formulated by Davankov in Refs. [9,12,13], are based on the distorted octahedral structure of bis(amino acidato)copper(II) complexes with two amino acid ligands situated in *trans*-position in the main coordination square of Cu(II) ion and two water molecules or other electron donating groups occupying two more remote axial positions. In this arrangement, heterochiral ternary complexes Cu(L-selector) (D-analyte) are generally the more stable diastereomers. This is the case with bidentate L-Pro and L-Hypro selectors as well as with tridentate L-*allo*-Hypro-incorporating polystyrene-type resins. For tridentate AA analytes, the enantiomeric elution order inverses. A different chiral discrimination mechanism operates in all polymeric and silica-type materials where L-Pro or L-Hypro are bonded to the sorbent matrix via a 2-hydroxyalkyl-type spacer (as in the above commercial Chiral-Si 100 L-Hypro-Cu). Here, the 2-

hydroxy group occupies an axial position in the coordination sphere and makes the binding of D-stereoisomers less probable. Only D-Pro, as an exception, forms a more stable ternary complex. After having examined the results of LEC of many hydroxy acids and after conducting extensive theoretical calculations within the ZINDO semiempirical quantum mechanical method, the authors [84] arrive at the conclusion that ''Davankov's selection rule, formulated originally to explain elution order of α-amino acids chromatographed on stationary phases employing ligand exchange mechanism, can be extended, after minor modifications, to α-hydroxy acids chromatographed on similar stationary phases . . . . . If we assume that the ternary complex adopts another structure in which the alcohol hydroxy and α-amino groups (of the analyte and selector, respectively) are in the *cis*-configuration (and not *trans*-), . . . then the reasoning provided by Davankov would also hold . . . ''. Thus, the important conclusion on the *cis*-arrangement of one amino acid and one hydroxy acid ligand in the ternary complex logically follows from chromatographic data and does not contradict the results of theoretical calculations; however, it still requires to be confirmed by other independent methods. As to the theoretical calculations, the authors [84] mention that a more advanced theoretical model of chiral recognition should include an improved representation of the spacer that connects the chiral selector to the sorbent matrix, and also considering the hydration shell around the basic ternary complex.

Besides the above mentioned ligand exchanging CSPs offered by Daicel, Toso (Japan) and Serva (Germany), Nucleosil Chiral-1 L-Hypro-Cu by Macherey-Nagel (Germany) and Astec CLC-L and CLC-D by Advanced Separation Technologies (USA) are also commercially available. The latter incorporate a proprietary Astec amine derivative and resolve racemates of free AAs, malic, lactic, tartaric acids, and 3-phenyllactate.

In spite of this fact, the search for new packings remains an area of constant activity. New binding chemistry for L-Pro and L-Lys as selectors was suggested [85]. The above amino acids have been provided with a chlorine-activated triazine substituent at the α- and ω-amino groups, respectively, and then bonded to aminopropylsilica. The rationale was to allow the amino groups of the triazine-type spacer to occupy one of the axial positions of the Cu(II) ion. Though the new material was shown to resolve racemates of some AAs, their *N*-(2,4-dinitrophenyl)- and Dns-derivatives, its overall performance was inferior to that of commercially available known materials having 2-hydroxyalkyl-type spacers. Another bulky substituent at L-proline nitrogen atom in combination with a polar amido group in close vicinity (structure 1 below) was tested by Veigl and Lindner [86]. The modified structure of the selector did not show any benefits and the sign of chirality of the chiral C-atom of the *N*-substituent was shown to play a minor role in the chiral recognition process. More successful was a similar attempt by Gübitz et al. [87], who fixed a rigid 2-hydroxy-cyclohexyl substituent at the N-atom of proline (structure 2). Alternatively, they also used a long and flexible spacer between the Pro moiety and silica matrix, 6-hydroxy-4-oxa-8-aza-*n*-decene (structure 3). Probably, due to the ability to supply an O or N heteroatom into the axial position of the Cu(II) complex, both the above chiral selectors showed enhanced selectivity toward isomers of AAs, their Dns-derivatives, hydroxy acids, and dipeptides, as well as some barbiturates.



After having tested several CSPs with cyclic amino acids as chiral selectors in an attempt to resolve racemates of *N*-alkylated barbiturates, drugs of the phenobarbital series, Gübitz et all. [88] decided that a less strongly complexing chiral selector was needed. They bonded L-prolinol, an analogue of L-proline with a hydroxymethyl group in place of the initial carboxy group, to the glycidoxypropyl-activated silica. The idea proved successful in that one of the two L-prolinol ligands in the initial Cu-bis-complex on the CSP could be better replaced by the barbiturate analyte to form a ternary complex. The result was a nice separation of enantiomers of all four barbiturates examined. Because both the selector and analyte represent relatively weak ligands, retention and resolution were found to rise with increasing pH, but, in order to prevent rapid destruction of the silica-type packing, pH of the eluent must be kept below 8.

Another successful new chiral LEC phase was prepared on the base of L-leucinol [89]. In this case, however, the amino group of the leucinol was provided with an additional carboxymethyl substituent, so that coordination to Cu(II) first of all resulted in an achiral five-membered chelate ring of glycine type. The hydroxy group of leucinol occupied an axial position of the copper ion. Nitrogen atom of the selector was connected to the silica matrix via a long undecenyl spacer. The new phase resolved racemates of 24 tested unmodified AAs, except asparagine, with good α-values, but relatively long retention times. L-Enantiomers were always retained longer. Enhanced temperature and organic modifiers in the eluent reduce the analysis time, but, also the enantioselectivity. Heating, however, enhances the peak shape and resolution factor. Importantly, the above *N*-carboxymethyl-L-leucinol phase resolved ten of 12 tested racemic β-amino acids [90].

An efficient new CSP was obtained by an Italian group [91] on treating the glycidoxypropyl-activated silica with L-phenylalanine-amide. It baseline resolved racemates of up to four dansyl- or dabsyl-amino acids (dabsyl, 4-(dimethylamino)azobenzene-4′-sulphonyl), taken in a mixture, as well as a cyclic sulphonamide, γ-sultam. Derivatives of polar AAs were separated with particularly high selectivity, up to $\alpha = 5.54$ for Dns-Ser. Reducing the pH (from 7.5 to 6.0) of the mobile phase enhanced the retention of solutes, opposite to the situation with AA-type selectors. The addition of acetonitrile allowed a faster separation and an increase of α for all analyzed Dns-AAs. The elution order was always $k_D < k_L$ and the authors suggest that the bulky Dns-group of the analyte coordinates *cis* relative to the amide group of the L-Phe-NH_2 selector.

CSP having the same chemical structure as the bonded ligand, L-Phe-NH_2, was also prepared on a monolithic silica column [92]. It showed similar selectivity with respect to Dns-AAs, but a lower efficiency. Bonded Ala-NH_2 was not selective, whereas L-Pro-NH_2 resolved several Dns-AAs and a few hydroxy acids.

New polymeric chiral LEC phases were also described during the last decade. A styrene-2%-divinylbenzene copolymer was substituted (to ~20%) with long decyl chains bearing active bromine at the chain end. Bromine was then replaced with L-Pro to give a resin with a relatively high exchange capacity, 1.43 mmol/g [93]. In aqueous-ethanolic Cu-containing eluents, of 15 free racemic AAs resolved, hydrophobic D-analytes were found to be retained longer, whereas hydrophilic amino acids showed opposite elution order, D before L. This pattern points to a stabilizing hydrophobic interaction of the alkyl or aryl substituents of D-AAs with the non-polar spacer and polymer styrene units, similar to the situation with polystyrene-type CSPs. A polymeric hydrophilic CSP was also prepared and compared with the above hydrophobic polystyrene resin. It had a polyvinylamine backbone with L-Pro bonded via glycidyl spacer [94]. As expected, the hydrophilic resin showed opposite elution sequence of enantiomers for most AAs. Interestingly, retention was reported to increase at higher temperatures. Hydrophilicity of the resin could be reduced by introducing benzyl substituents to the nitrogen atom of vinylamino units. This procedure inverted the enantioselectivity sign for Trp. Another hydrophilic resin with L-Pro selectors was made of crosslinked polyvinyl alcohol activated again with glycidyl functional groups [95]. It showed preference for the L-isomers of 15 tested AA analytes, in full accordance with Davankov's chiral recognition scheme mentioned above. The only exception, again, was the stronger binding of D-Pro. Highly porous microbeads

were prepared by a crosslinking polymerization of glycidylmethacrylate in the presence of porogens (cyclohexanol and dodecanol). L-Pro and L-Hypro residues were then introduced by opening the epoxy rings [96]. In the presence of Cu(II), the resins resolved DL-His. Providing chitosan, a natural polymer composed of glucosamino units, with ligand exchanging properties finally should be mentioned [97]. When coated on a silica gel carrier, the material was observed to resolve racemates of some AAs under conditions of LEC.

The first molecularly imprinted ligand exchange adsorbent was prepared by radical copolymerization of 10 parts of ethylene glycol dimethacrylate and 1 part of a chiral ternary complex (*N*-vinylbenzyliminodiacetate/copper/D-Phe) on the propylmethacrylate-derivatized surface of wide-porous silica gel [98]. After removing the chiral template, D-Phe, chiral cavities remain in the crosslinked polymeric matrix. These cavities in the chemically achiral material preserve the ability to preferably retain, via formation of ternary copper complexes with iminodiacetate, the isomers of the initial template or molecules of a similar spatial structure. Thus, the above material enantioselectively absorbs D-Phe ($\alpha = 1.65$), D-Tyr, and (*S*)-α-methylphenethylamine, but does not recognize enantiomers of aliphatic AAs. Preliminary experimental results on imbedding, into an electro-conductive polypyrrole, of D-Pro adsorbed on the Cu(II) form of a L-Pro-polystyrene resin were recently reported [99]. Though enantiomers of Pro, Lys and Asp were barely separated on that material, the elution order of the last two charged analytes could be changed by changing the sign of a potential applied to the conductive packing.

In conclusion to this section it can be noted that, in spite of many reports on preparing and using new CSPs, the highest resolving power in a LEC process was still displayed by the L-Hypro-incorporating polystyrene material that was bonded to silica gel surface, as described by Davankov and Kurganov [12]. Of eight pairs of AA enantiomers applied in a mixture, seven pairs were separated almost to the base line (Fig. 7). On a similar resin with N^1-benzyl-(*R*)-propanediamine-1,2 chiral selector, a mixture of 11 racemic AAs produced 20 chromatographic peaks [12].

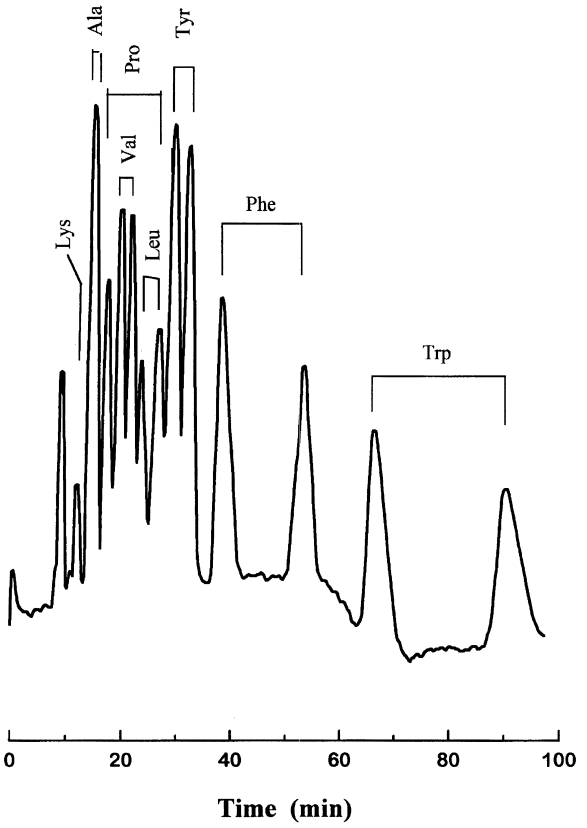


Fig. 7. Separation of a mixture of eight AAs on a CSP prepared by binding L-Hypro-modified polystyrene chains to silica. Column, 250×4 mm, 5 μm. Eluent, 0.5 m*M* Cu(II), 10 m*M* NH_4OAc, pH 4.5, flow rate 0.7 ml/min, temperature 75 °C. From Ref. [12].

4.2. Chiral coated phases (CCP) in ligand exchange chromatography

As early as 1980, Davankov et al. [100] described an extremely simple method of converting commercially available reversed-phase HPLC columns into highly efficient chiral ligand exchangers by a dynamic coating of the column with a hydrophobic derivative of complexing chiral selector, *N*-alkyl-L-Hypro. The long alkyl chain, from C7 to C18, provides permanent adsorption of the selector on the hydrophobic packing surface. The fact that mixtures of up to seven racemic amino acids could be baseline resolved on a 10-cm-long column [100] indicates the high selectivity and efficiency of such CCP systems.

Kurganov et al. [101] reported on fast chiral baseline separations within 30 s to 3 min. *N*-Decyl-L-hydroxyproline (C10-L-Hypro) coated onto RP C_{18} columns became available as the ''Davankov LEC column'' from Regis Technologies (USA). This efficient enantioselective separation technique became very popular not only in enantiomeric analysis, but also in micropreparative resolutions. Thus, $Cl_2C{=}C(CH_3){-}CH_2{-}C^*H(NH_2){-}COOH$ was separated into pure enantiomers, in order to convert them into chiral leucine of extremely high specific activity, (*S*)- and (*R*)-[4,5,5,5-^{3}H]-Leu [102]. More detailed examination of the preparative perspectives of the above CCPs showed that under optimized conditions up to 2 mg of a racemic AA can be baseline resolved on a 125×4 mm RP column [103]. The high loadability of the column becomes possible due to unusual and very favorable distortion of both peaks, fronting of the first and tailing of the second one. Interestingly, a similar unusual type of peak distortion was also observed [104] in capillary supercritical fluid chromatography (SFC) on ''Chirasil-Nickel'' in a process called complexation chromatography. The latter is closely related to LEC, since the both processes involve formation of ternary metal complexes with the selector and analyte. Initial attempts to explain this interesting and important phenomenon of ''enantioselective peak distortion'' are made by Kurganov [16] in terms of association-type secondary equilibria.

Another CCP recently became commercially available, Chiralpak MA(+) from Daicel (Japan) and is prepared by dynamic coating of *N*,*N*-dioctyl-L-alanine on 3 μm C_{18} RP material. According to information from the company, the new CCP is highly effective for resolution of racemates of hydroxy carboxylic acids.

In addition to the previously described *N*-decyl-L-His [105], another His derivative was synthesized and investigated, namely containing the *n*-decyl substituent at the imidazole N^τ atom [106]. Both the above His-based CCPs showed good enantioselectivity and general preference to D-enantiomers of AA analytes. More recently, Remelli et al. [107] described a new chiral ligand, N^τ-*n*-decyl-L-spinacine (structure 4) which is a condensation product of His with formaldehyde and contains a methylene bridge between the amino group and the imidazole ring. By coating of ~0.6 mmol of the ligand on a standard (4.6×250 mm) RP C_{18} column, an efficient LEC system was obtained for the resolution of racemic free AAs (with D-enantiomers retained longer, with the exception of His) as well as racemic Gly-containing dipeptides. The paper describes in detail relations between the retention, selectivity and efficiency of the column and such experimental parameters as pH, type and concentration of the buffer and organic modifier, temperature, flow rate, etc. These dependencies have been earlier studied with regard to the C10-L-Hypro CCP and are basically similar for all CCP systems.

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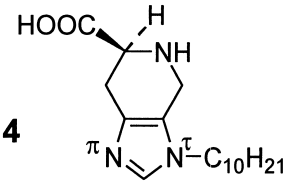
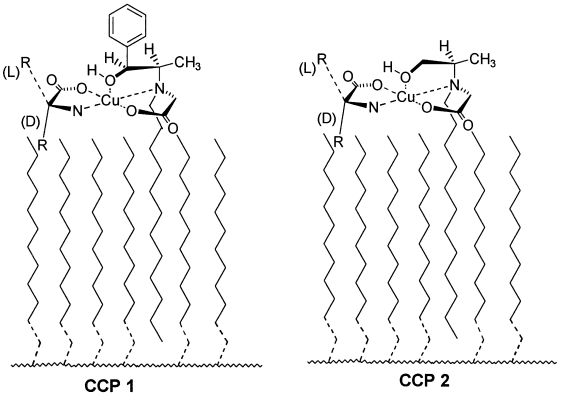


A promising approach, namely using *N*-alkyl-substituted amino acid amides for the preparation of CCPs, was suggested by an Italian group [108]. The most stable and efficient CCP involves C12-L-Phe-NH_2. It resolves ten of tested 11 racemic AAs (His is not resolved), five hydroxy acids, three amides and three methyl esters of AAs. Two diastereomeric dipeptides gave four peaks each. Interestingly, for faster elution of hydrophobic AAs, the authors prefer to employ enhanced concentrations of Cu(II) in the eluent, rather than using organic modifiers. In the first case, only retention is known to decrease substantially, whereas in the second both retention and selectivity decrease. The presence of the phenyl group in the selector seems not to be essential, since aliphatic Alk-L-Nleu-NH_2 generates comparable enantioselectivity values and the same elution order as AA enantiomers, L before D. Dipeptides elute in the following sequence: LD, DL, LL, DD.

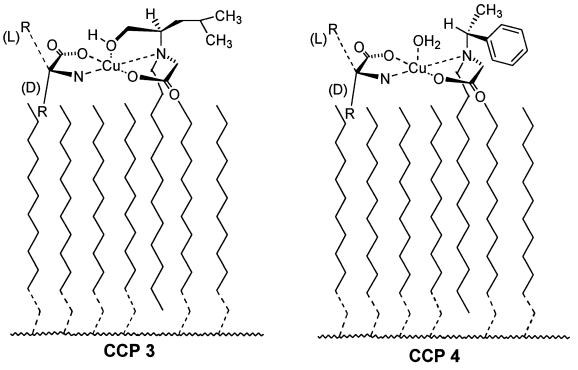
LEC is less suitable for chiral resolution of monodentate analytes that do not chelate Cu(II) ion. This follows from the three-point interaction model required for chiral recognition. In conventional LEC systems with AA-type ligands, two interaction points for each ligand are represented by two electron-donating heteroatoms (O and N) in the Cu(II) chelate, while the third point results from a steric,

hydrophobic or polar interaction that the side chain of the AA enters with the microenvironment of the ternary complex [7–9,100]. In order to add one missing interaction possibility to simple monoamines, Yamazaki et al. [109] suggest converting the amines, 1-phenylethylamine, 1-cyclohexylethylamine, 1-methylpropylamine into their *N*-carboxymethyl or even *N,N*,-bis(carboxymethyl) derivatives. These derivatives act now as bi- or even tridentate chelating ligands and easily resolve on the C12-L-Hypro CCP. Interestingly, the carboxymethylated amines form glycine-type copper chelates, with no asymmetric carbon atom within the chelate ring. The C* atom resides in the α-position in the *N*-substituent. Nevertheless, racemic analytes of this type resolve with high α-values.

A similar approach, namely, *N*-carboxymethylation, was taken by Hyun et al. [110,111], in order to enhance the complexing properties of the chiral selector. (1*S*,2*R*)-Norephedrine, as an α-hydroxy amine, does not bind Cu(II) sufficiently strongly, in contrast to its *N*-carboxymethyl derivative. The thus modified selector was further provided with an additional *N*-dodecyl chain and then dynamically coated on octadecyl silica from an aqueous/methanol mixture of 1:2 (v/v). The coated phase C12-NE showed excellent resolving ability with respect to all tested racemic AAs, with the exception of cystein. The chiral recognition model (CCP 1) suggested by the authors is almost identical to that suggested by Davankov et al. [100] for the C10-L-Hypro coating. The model implies *trans*-orientation of two amino groups in the coordination square of Cu(II) and a lipophilic interaction between the α-substituent of the D-amino acid analyte with the non-polar surface of the packing. This interaction stabilizes ternary sorption complexes of D-analytes having non-polar side chains R, but destabilizes complexes of polar D-AAs. Thus, first eluting enantiomers on CCP 1 are L-Ala, L-Val, L-Leu, but D-His, D-Ser, D-Thr, D-Tyr, D-Asp, D-Asn. However, Phe, Glu, Gln and Arg do not follow this simple elution rule on the CM-norephedrine CCP 1. As on Davankov's CCP columns, retention of AAs decreases at higher temperatures, higher Cu(II) concentrations in the mobile phase, at lower pH values and on adding organic modifiers. Enantioselectivity of resolution decreases with the content of organic modifier, which points out the importance of hydrophobic interactions in the chiral recognition mechanism.



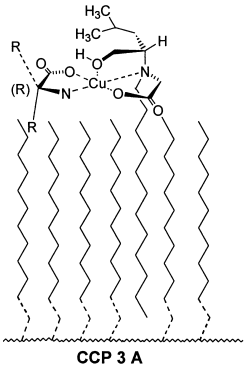
Two following important contributions from Hyun's group intended to generate more information on the mechanism of chiral recognition of amino acids on the CCP 1. New *N*-dodecyl-substituted derivatives of chiral *N*-carboxymethyl-alaninol, *N*-carboxymethyl-leucinol [113] and *N*-carboxymethyl-phenylethylamine [112] were synthesized for the preparation of CCP 2, CCP 3 and CCP 4, respectively.



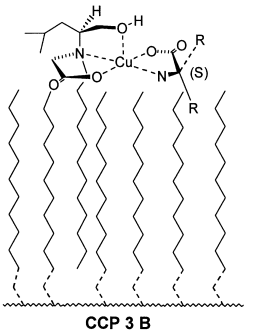
As compared to CCP 1, the alaninol-based CCP 2 did not contain the bulky phenyl substituent near the hydroxy group. The much better performance of the CCP 2 and its consistency in stronger retention of D-stereoisomers of all AAs (with the exception of His), regardless the lipophilicity or polarity of their side groups, leads to the conclusion that the phenyl functionality at the first chiral center of CCP 1 is not essential in chiral recognition and simply disturbs the axial coordination of the hydroxy group of the

stationary ligand. In this regard, CCP 3 prepared from leucinol and having one bulkier isobutyl group at the chiral C* atom displays even better enantioselectivity. Again, only His showed an inverted elution order of enantiomers. CCP 3 was also superior to CCP 2 in that it allowed use of a mobile phase containing up to 20% acetonitrile for faster elution of heavier AAs, without the α-values decreasing too much. In contrast to that, CCP 4 was found to perform badly with respect to its enantioselectivity and consistency of chiral recognition. This result proves unambiguously the importance of the occupation of the axial Cu(II) position by the hydroxy group of the chiral selector.

A further paper [113] is also important in that it pays attention to the fact that in all CCPs, 1 through 4, the coordination of Cu(II) to tertiary nitrogen of the selector makes that N atom chiral. Its absolute configuration, however, is not fixed and can invert on interaction with the analyte enantiomer on formation of the tertiary complex. This makes it possible that both the D-enantiomers of AA analytes and L-enantiomers, after a configurational inversion of N*, are in position to enter lipophilic interactions with the RP support. The only difference between the two diastereomeric ternary complexes in this case would consist of different orientation of three substituents at the chiral C* atom with respect to the glycine chelate ring below them (CCP 3A and 3B). This difference obviously becomes the only source of chiral discrimination in the CCPs of the type under discussion. It should be mentioned here that the configuration of the N* atom in Davankov's C10-L-Hypro chiral selector is always fixed by the cyclic nature of proline [100].



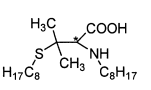
CCP 3 A



CCP 3 B

It has been found earlier that the C12-L-Hypro containing CCP separates enantiomers of underivatized aromatic β-amino alcohols and aliphatic β-amino alcohols of the 1-amino-2-ol type, such as 1-amino-2-butanol through 1-amino-2-hexanol, and *trans*-2-amino-cyclohexanol [114]. Thus, the importance of the location of the hydroxy group at the secondary C atom was established. An unexpected finding [115] was that these separations were greatly improved by the presence in the eluent of ~5 m*M* barbital, a six-membered heterocycle with two N atoms and three carbonyl groups. The role of this achiral additive was not clear. Later [116], it was found that, in the presence of barbital, aliphatic amino alcohol racemates also resolve on the C12-NE CCP. Even analytes with the hydroxy group at the primary carbon atom, 2-amino-1-propanol and 2-amino-1-butanol could be resolved in this manner.

N-Substituted (*S*)-phenylglycinole, structurally related to L-phenylglycine, has been synthesized, coated on C_{18} material, loaded with Cu(II) and tested as CCPs in the enantioseparation of five AAs [117]. The elution order of isomers was found to be D before L for all three aromatic *N*-substituents tested (4-methoxybenzyl-, 2-naphthylmethyl- and 9-anthrylmethyl-) and the aliphatic C12 chain. Surprisingly, the elution sequence inverted when shorter alkyls were attached to the amino group, C9 and C7. This finding remains to be examined more closely and explained.



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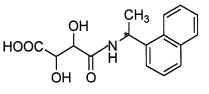

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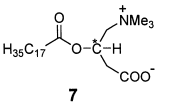


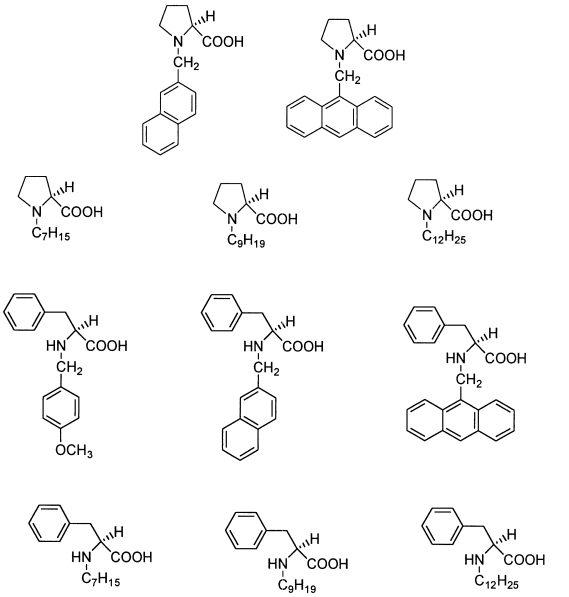
7

A CCP initially developed by Oi et al. [118], where a Cu(II) complex of *N*,*N*-dioctyl-D-penicillamine (structure 5) is embedded in RP silica, became available from Penomenex (Germany) as Chirex (D)-penicillamine. The chiral selector has three possible coordinating heteroatoms, N, O and S, in the molecule and can change the predominant chelating pattern as a function of experimental conditions and, possibly, the structure of the analyte. The system shows remarkable versatility and resolves racemates of amino acids, α-alkyl AAs [119], hydroxy acids, as well as *trans*-1,2 diaminocyclohexane, 1-aminoethylphosphonic acid, tetrahydro-2(or 3)-furoic acids, 3-aminopyrrolidine, etc. [120]. Chirex (D)-penicill-

amine was successfully used [121] for the separation of four stereoisomers of 1-amino-cyclohexane-carboxylic acid substituted in the 2-position with methyl-, hydroxy- or amino groups. An analogue methyl-substituted cyclopentane compound was also tested. Each of the above cyclic compounds contained the substituent in the *cis*- or *trans*-position to the carboxy group, hence two diastereomeric pairs. Since the analytes differed substantially in their polarity and retention, optimal separation conditions had to be found for each quadruple individually, by varying the concentration of methanol, propanol-2 or acetonitrile, pH, Cu(II) concentration, temperature, etc. Interestingly, the elution order of enantiomers was sometimes observed to change with the concentration or type of the organic modifier. A detailed thermodynamic investigation [122] of such cases showed that the temperature of inversion of elution order (''isoenantioselective'' temperature) strongly depends on the content of the organic modifier, which points toward the importance of hydrophobic interactions in chiral recognition. Similarly, enantiomers of *trans*-1-benzylcyclohexane-1,2-diamine were also separated [123] on the Chirex (D)-penicillamine.

Chiral coated phases with *N*,*N*-dioctyl-D-penicillamine and (*R*,*R*)-tartaric acid mono-(*R*)-1-(α-naphthylethylamide) (structure 6) are also available from Sumica Chemical Analysis Service (Osaka, Japan) as Sumichiral OA-5000 and OA-6000. Sumichiral OA-6000 was recently used for quantitation in urine of enantiomers of *p*-hydroxymandelic acid, a metabolite of synephrine contained in Chinese medicines [124]. Stearoyl-L-carnitine (structure 7) coated on RP was shown to resolve a series of racemic amino acids and hydroxy acids under conditions of LEC [125].

Aside from the above discussed alkyl-derivatized RP silica supports, another strongly hydrophobic material, porous graphite was recently suggested to be used for the immobilization of *N*-substituted amino acid-type chiral selectors in LEC [126]. First experiments showed good efficiency and enantioselectivity of L-phenylalanine bearing a 2-naphthalenesulphonyl anchor group at the nitrogen atom in the resolution of racemic Pro, Val, Thr, and Asn, as well as lactic and hydroxyisovaleric acids. Since the elution order was D before L (with the exception of Pro), i.e. opposite to that observed in systems RP/Alk-L-Hypro, it was decided to examine the mechanism of chiral recognition more closely. A series of L-Phe with alkyl- and aryl-type *N*-substituents were synthesized [127] and a similar series of substituted L-Pro derivatives [128] (see structures).



All selectors were found to strongly adsorb on the flat graphite surface and effectively resolve most racemic AAs in an aqueous 1 m*M* $Cu(OAc)_2$ eluent. Interestingly, the order of elution for AAs on C_7-L-Phe, C_9-L-Phe and C_{12}-L-Phe (with the exception of Thr and Arg) was D before L, whereas a reversed elution order was found with aryl-type substituents at the L-Phe (4-methoxybenzyl-, 2-naphthylmethyl- and 9-anthrylmethyl-). On all the *N*-alkyl- and *N*-aryl-substituted L-Pro phases, the L-enantiomer always eluted before the D-partner. Surprisingly, all alkyl-substituted CCPs showed a decrease in analyte retention as the alkyl chain length increased. This effect was especially dramatic with L-Pro phases. On the other hand, the selectivity of separation noticeably increased on the alkyl-L-Pro phases with the length of the alkyl. The authors try to explain the observed regularities in terms of hydrophobic interactions between substituents of both the analyte and selector in their ternary complexes. These considerations underestimate the interactions of the ternary complexes with the flat hydrophobic support. In these systems, all transformations take place on the graphite surface, not in bulk solution. Without understanding the conformation of the chiral selector that is strongly fixed on the hydrophobic surface, it is not

possible to discuss the structure of ternary complexes. The fact that hydrophilic Ser, Thr, Asp and hydrophobic Val, Met, etc. elute on L-Pro phases in the same L-D sequence, cannot be explained by internal hydrophobic interactions within the ternary complex structure. More likely, some repulsive steric effects on graphite surface play the decisive role in the chiral recognition.

4.3. Chiral mobile phase (CMP)-type separations

The combination of a conventional achiral RP column packing with an eluent containing a chiral selector, which was pioneered by Karger and Lindner [129], differs from the chiral coating-mode of enantioseparation only in that the selector predominantly resides in the stationary phase in the latter systems, whereas it predominantly moves with the mobile phase in CMP systems. The important consequence of this situation is that the enantiomer more strongly bound to the selector will be retained longer in the CCP column, whereas it will elute first with the CMP [25]. Of course, there is also a whole palette of intermediate systems where the chiral selector partitions between the mobile and stationary phases, acting in two opposite directions with respect to enantiomers of the analyte. For this reason, one should be extremely careful in coming to any conclusion on the relative stability of two diastereomeric ternary complexes in LEC, based on the elution order of enantiomers only. The situation is even more complicated in the case where one of the two competing diastereomers is more stable in the bulk solution, but represents the less stable species when in adsorbed state. (This combination is most productive with respect to chromatographic resolution of enantiomers.) Davankov postulated that the situation in the stationary phase is often the determining factor for the overall elution order, since the achiral sorbent surface largely enhances or even induces the enantioselective discrimination phenomenon [8,22,23,100]. Marchelli et al. [130] showed experimentally that stability constants of ternary Cu(II) complexes of Dns-AAs with *N*-(2-hydroxypropyl)-L-phenylalaninamide in solution do not correlate with the elution order of Dns-AA enantiomers from the RP column operated with a solution of that chiral selector as the mobile phase. Obviously, the differentiation was determined by the ''relative affinities of the ternary complexes for the column stationary phase'', or, as explained in Ref. [25], by the difference in the stabilities of the diastereomeric ternary complexes in the stationary phase. A similar contradiction between enantioselectivities in solution and on the surface was shown for several combinations of AAs and their amides [131].

Generally, amides of chiral AAs (Pro, Val, Phe, Trp) and their *N*-methyl derivatives (MeVal, Me_2-Val, MePhe, Me_2Phe) proved to be efficient chiral selectors for the resolution of racemates of both Dns-AAs [131] and free AAs [132]. Based on these experimental data, it would be interesting to reconsider the elution order of AA enantiomers by accounting quantitatively for the phase distribution of the above selectors. Interestingly, sufficient lipophilicity of MePhe-NH_2 and Me_2Phe-NH_2 (i.e. their predominant location in the stationary, rather than mobile phase) was found to be responsible for the special productivity of these selectors (Fig. 8). Their use permitted enantiomeric analysis of AAs in food products [133] to be performed and showed that fermentation, rather than heat treatment, causes the emergence of D-AA enantiomers in food [134].

L-Phe-NH_2 additive was found to be efficient in the resolution of racemic amino acids [135], a whole

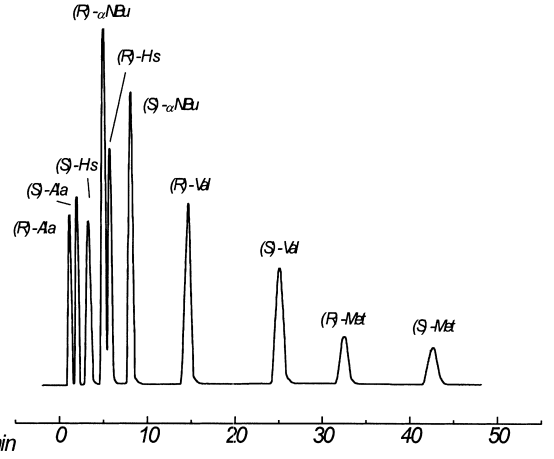


Fig. 8. CMP-type separation of a mixture of five AAs on a RP column Spherisorb-2 (3 μm, 150×4.6 mm). Eluent: 2.0 m*M* L-Me-Phe-NH_2, 1.0 m*M* Cu(II), pH 6.0, flow rate 0.5 ml/min. Fluorescence detection (post-column derivatization with OPA). From Ref. [136].

series of aliphatic and aromatic α-hydroxy acids and of dicarboxylic acids such as malic and tartaric acids [136]. Also the authors [136] showed that enantiomers elute in the form of diastereomeric ternary complexes, which means that a real ligand exchange equilibrium does exist in the system. This fact is further important in view of the necessity of obtaining individual calibration plots for each enantiomer [8] if their quantitative estimation in a CMP system is needed. That eluting diastereomeric species, indeed, have different optical properties, has been proven experimentally [130]. In a systematic approach, the same Italian group also examined terdentate and tetradentate chiral selectors based on L-Phe [137,138]. In the first, one amino group of ethylenediamine was condensed to amide with the carboxylic group of one L-Phe residue, while in the second both amino groups were converted to bis-(L-Phe-amide). The α-amino groups of the Phe-residues were additionally methylated. Of a series of derivatives examined, only the tetradentate $[Me_2Phe\text{-}NHCH_2\text{-}]_2$ displayed acceptable efficiency and selectivity. Being sufficiently hydrophobic, the latter selector could be easily operated as a permanent chiral coating on the RP material for the resolution of both polar and apolar AAs. Trying to understand the mechanism of chiral recognition, the authors measured stability constants of corresponding ternary complexes with the analytes in solution. No enantioselectivity could be found in the bulk solution, implying the important role of hydrophobic interactions of diastereomeric ternary structures with the hydrophobic surface in the emergence of enantioselectivity.

Based on the ''reciprocity rule of chiral recognition'' [25], Yamazaki et al. [139] showed that not only racemic hydroxy acids are resolved in a CMP based on chiral AAs, but also, vice versa, racemic amino acids and β-amino alcohols are resolved in an eluent containing 20 m*M* chiral mandelic acid and 2.0 m*M* Cu(II) at pH 6.5. Certainly, detection problems in this case must be overcome by post-column derivatization.

N,*N*-dimethyl-L-Phe in combination with Cu(II) and RP C_{18} packing provides a convenient method for the *ee* analysis of a whole series of α-alkyl-substituted α-amino acids and α-hydroxymethyl substituted AAs [77].

(+)-Monoethyl ester of *N*-(1′-hydroxymethyl)-propyl-α-aminobenzylphosphonic acid was shown to resolve racemic valine in combination with Cu(II) and RP packing [140]. Interesting chiral selectors for CMP chromatography, namely, a helically distorted square planar Ni(II) complex with tetradentate ligands (prepared by formation of Shiff bases of chiral 1,2-diamines with 1,3-diketones) were reported by Bazylak [141,142]. These selectors do not exchange their ligands with analyte enantiomers; rather, outer-sphere diastereomeric adducts are formed. This could be a reason for relatively rapid dissociation–association equilibrium, resulting in good system efficiency in the resolution of racemic amino alcohols and dipeptide-type sweeteners and diketopiperazines. The selector is also thought to form helical associates on the RP surface and offer chiral cavities for the enantiomers (e.g. clenbuterol) to be resolved [143].

A new concept for designing chiral selectors was suggested by an Italian group [144–146]. It consists of combining the ligand exchanging properties of the selector with its ability to form inclusion complexes. One of the six hydroxy groups of β-cyclodextrin was replaced by a histamine or 2-(aminomethyl)-pyridine residues. In both cases, the above diamino moieties are achiral, but, nevertheless, their ternary complexes formed with Cu(II) and an amino acid analyte display a distinct enantioselectivity, since only D-amino acids, after forming the ternary complex, can be included into the cyclodextrin cavity of the same selector molecule. The more stable complex with the D-enantiomer then resides in aqueous phase and emerges first from a RP column. Only aromatic AAs are recognized by the selectors, since Ala and aliphatic AAs are too small to be discriminated by the β-CD cavity. Rather than for ligand exchange chromatography, such selectors may prove useful for designing fluorescent enantioselective sensors of amino acids [147].

5. Enantioselective ligand exchange in liquid–liquid partitioning systems

Formation of a ternary metal complex with a chiral selector must affect distribution of two enantiomers of an analyte between two immiscible liquid phases. In a series of papers Pickering et al.

[148–152] examined in detail a composition of *N*-decyl-L-Hypro, copper(II) and DL-phenylalanine in a two-phase system (aqueous buffer/decane-hexanol) and considered the possibility of using liquid membrane technology for a preparative separation of Phe enantiomers. The process of liquid membrane extraction is analogous to the unit operation of solvent extraction. While the extraction step is identical for both processes, the stripping step of the former occurs simultaneously with extraction by using a second aqueous phase that has a composition favoring the re-extraction of Phe from the organic phase. This arrangement overcomes the problems of saturation of the organic phase with the analyte and provides significantly faster rates of transfer of the analyte from the first aqueous source phase through the organic membrane phase into the second aqueous receiving phase. A kinetic enantioselectivity of $\alpha = 1.65 \pm 0.31$ was found in the bulk extraction and up to $\alpha = 2.4$ in the emulsion liquid membrane system, with D-Phe enantiomer being extracted faster into the organic C10-L-Hypro-containing phase [149].

In a process called micelle-enhanced ultrafiltration [153,154], noticeable enantiomeric enrichment of Phe, up to *ee* 69%, was observed in the permeate chamber on ultrafiltration of a DL-Phe solution that contained Cu(II) ions and micelles of cholesteryl-L-glutamate. D-Phe was predominantly retained in the initial solution as a ternary complex inside the micelles which are too big to pass through membrane pores, whereas the predominantly free L-Phe migrated through the membrane. By the way, the *ee* analysis in this work was performed using enantioselective LEC on (D)-penicillamine coated column Chirex 3126 (Phenomenex, USA).

In the above cases, the *ee* of the received enantiomer is restricted by the enantioselectivity of the complexation process, unless a cascade of separation units is provided. Much higher purities result from multi-stage processes, e.g. from countercurrent solvent extraction which is closely related to a chromatographic process. An impressive result was presented in Ref. [155], where a solution of copper-C12-L-Pro in *n*-butanol was pumped in an opposite direction to a flow of aqueous solution of DL-Val. The racemate was completely resolved into enantiomers of optical purity of 99.5% and higher. In another example [156] DL-leucine was resolved using hollow fiber technique.

6. Conclusion

For more than three decades, ligand exchange chromatography has proved to be an extremely dynamic and fruitful field of research that has extended not only our fundamental knowledge of stereochemistry, coordination chemistry, chromatography and electrophoresis, but also our practical abilities in analytical, separation, and synthetic techniques. Its support of the fast development of the chemistry of natural compounds, synthetic organic chemistry and pharmaceutical science and industry can hardly be overestimated.

As with any other enantioselective separation technique, LEC has its own specific application area which are organic compounds capable of forming labile coordination compounds with transition metal cations. LEC is a technique of choice for all molecules having a pair of heteroatoms (N, O or S) with two to three carbon atoms in between. There is a reasonable understanding of separation mechanisms with these chelating molecules and some predictions of the elution sequence can be made. Cases of successful separation of enantiomers with more distant pairs or isolated heteroatoms are also known, but no systematic information for such compounds is available thus far. All types of chromatographic and electromigration techniques are suitable for analytical-scale separations, though the CSP approach displays lower column efficiency and requires enhanced column temperature, whereas the CMP systems, as well as the CE and MEKC approaches, require separate calibration for each enantiomer. Preparative separations are feasible with both CSP and CCP systems.

In spite of an extremely large amount of knowledge already generated in the field of enantioselective LEC, new approaches in research and application can still be explored. Thus, the design of new chiral chromatographic and electrophoretic ligand exchanging systems by involving new chiral selectors and new selector carriers, as a rather mature area of research and technology, has a relatively high chance of final success. In contrast, the search for systems with synergetic action of several different types of chiral selectors and systems where enantiorecognition is facilitated by an achiral auxiliary molecule remains an exciting field of empirical trial-and-error approach. The search for suitable chiral

kinetically stable complexes for an enantioselective ligand exchange in their outer coordination sphere, i.e. their solvation shell, should also be considered as a promising direction of development.

Acknowledgements

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