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Ligand Exchange Chromatography

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Introduction

Ligand exchange chromatography (LEC) was first introduced by Helfferich in 1961 as a general chromatographic technique to separate compounds which are able to form labile complexes with transition metal cations. The basic idea was to immobilize such ions as Cu(II) or Ni(II) on a stationary phase, in particular, a cation exchanger with sulfonic or carboxylic functional groups, and then let the ions form labile coordination compounds with analytes which possess electron-donating functional groups and can enter the coordination sphere of the metal ion, thus acting as ligands. Those analytes that form stronger complexes with the central ion, are retained longer on such a metal ion-incorporating stationary phase.

Starting with the idea that, in the densely packed coordination sphere, ligands enter multipoint interactions with each other and should therefore mutually recognize the spatial configuration of neighbours, Davankov and Rogozhin (1968–71) synthesized chiral complex-forming resins and further developed LEC into a powerful chiral chromatographic method. This technique, for the first time in liquid chromatography, resulted in a complete and reliable resolution of a racemate into constituent enantiomers. Typical analytes for enantioselective LEC are members of such important classes of chiral compounds as amino acids, hydroxy acids and amino alcohols. Having soon become one of the most extensively investigated methods for the direct resolution of enantiomers, LEC maintained for a long period of

time its leading positions in developing novel chiral chromatographic systems and evaluating mechanisms of chiral recognition and discrimination. This technique contributed much to the successful development of chiral silica-bonded stationary phases, both monomeric and polymeric, chiral coatings on column-packing materials and chiral mobile phase additives. In this last technique, it transpired that the central metal ion does not need to reside in the stationary phase, but, instead, can be a part of a chiral complex that is doped into the mobile phase to interact there with the analytes. This widens significantly the definition of LEC to a chromatographic process in which the formation and breaking of labile coordinate bonds to a central metal cation are responsible for the separation of complex forming analytes.

Ligand exchange has been realized in all known modes of enantioselective chromatography, including liquid chromatography, thin layer chromatography, gas chromatography, capillary electrophoresis and countercurrent liquid chromatography. In gas chromatography the principle of ligand exchange is more commonly known under the name ‘complexation chromatography’.

Theoretical Aspects of Chiral Discrimination in LEC

Being built of protons, neutrons and electrons, which basically are chiral elementary species, all atoms possess inherent chirality. Therefore, two enantiomeric molecules resulting from a mirror-symmetric arrangement of a certain ensemble of atoms in space, can differ in their thermodynamic stability. However, this difference may only amount to about $10^{-14} \text{ J mol}^{-1}$, which practically makes the enantiomers energetically identical and indistinguishable in any achiral environment. Using any chromatographic technique, two enantiomers can be recognized and

discriminated by the action of a chiral selector only. The latter has to be involved in interaction with the enantiomers, resulting in the formation of adducts which, being diastereomeric species, may differ in their stability. These adducts in LEC are ternary complexes composed of the molecule of the chiral selector, the complexing metal ion, and the enantiomer to be recognized. Thermodynamic enantioselectivity of the system, i.e. the difference in stability of the above two diastereomeric ternary complexes which incorporate (*R*) or (*S*) enantiomers of the analyte, represents the sole source of chiral discrimination of the enantiomers in LEC. For an analytical scale resolution of enantiomers, the enantioselectivity does not need to be high, since, in principle, enantioselectivity of $\delta\Delta G^0 = 25 \text{ J mol}^{-1}$ would correspond to a separation factor $\alpha = 1.01$, which is fully sufficient for two peaks to be resolved in a gas chromatographic capillary column generating about 10^5 theoretical plates. For a preparative scale liquid chromatography, one would prefer to deal with a separation factor of at least $\alpha = 1.5$, which would correspond to enantioselectivity of about 1 kJ mol^{-1} . Ligand exchange often generates a discrimination effect of this order of magnitude. With the $\delta\Delta G^0$ increasing further, the column selectivity, α , rises exponentially, according to the general relation $\delta\Delta G^0 = RT \ln \alpha$. In LEC systems with polydentate ligands, separation selectivities of up to $\alpha = 10$ are known.

As a matter of principle, a chiral selector has to enter into at least a three-point interaction with the enantiomers, in order to be able to recognize the difference in the spatial structure of the latter. This requirement is easily met with tridentate ligands forming ternary complexes with metal ions which have a coordination number of six. Among practically important classes of organic compounds, however, are molecules with two electron-donating heteroatoms, N and/or O, situated in a chain of carbon atoms in positions 1,2 or 1,3, e.g. in 1,2- and 1,3-diamines, 1,2- and 1,3-amino alcohols, α - and β -amino acids and α - and β -hydroxy acids. When forming complexes with metal ions, these compounds usually function as bidentate ligands with both heteroatoms coordinated to the same metal ion in the form of a five- or six-membered chelate ring.

It is very important that formation and dissociation of the bonds in the coordination sphere of the metal ion be fast. Otherwise, a slow rate of ligand exchange would degrade the efficiency of the chromatographic system. Therefore, only metals forming kinetically labile complexes can be employed in LEC, preferably Cu(II), Ni(II) and Zn(II). Even with the above metal ions, it has been repeatedly reported that the efficiency of ligand exchanging systems can be enhanced by

raising the column temperature to about 50°C , which speeds up the ligand exchange. Ions of Cr(III), Co(III), Pd(II) and the like, form kinetically inert, stable complexes unsuitable for LEC. However, the second coordination sphere of these complexes, which actually is their solvation shell, is well organized, too, and it does exchange its ligands readily, so that an enantioselective chromatography process can also be based on outer coordination sphere ligand exchange.

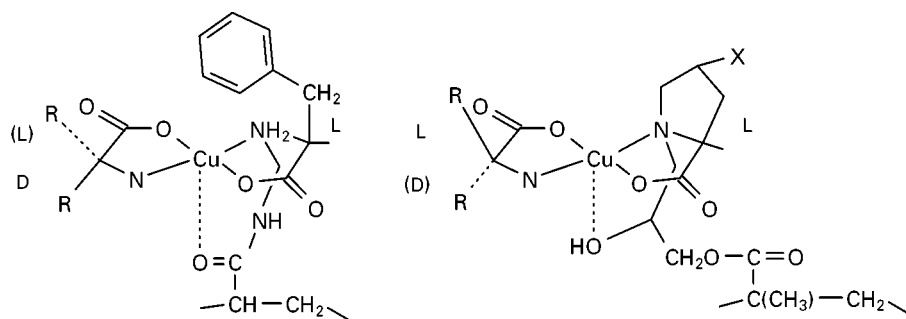
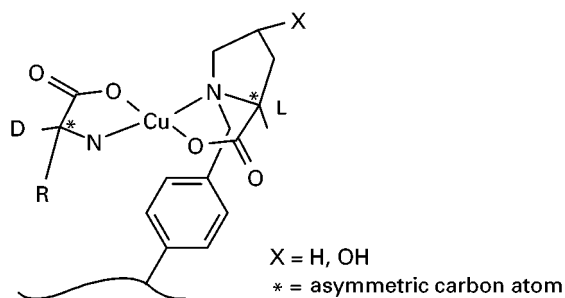
Zn(II) would usually coordinate four heteroatoms with lone electron pairs in the form of a tetrahedron. The coordination number of the Ni(II) ion is six and its ligands are usually arranged in an octahedron. Copper binds four ligands in its main coordination square, but offers two additional, more remote axial positions, so that a complex with the coordination number six adopts a distorted octahedral structure.

It is worth noting, that the optical purity of a complex-forming chiral selector employed in an LEC system does not need to be necessarily 100%. It is a distinguishing feature of all direct chromatographic separations of enantiomers that even a selector contaminated by its enantiomeric form can provide a complete resolution of racemic analytes. Enantiomeric impurities in the chiral selector do not diminish column efficiency but merely result in the two sharp enantiomeric peaks of the analyte approaching each other and completely coalescing into one sharp peak when the enantiomeric excess (e.e.) of the selector falls to zero. This phenomenon (which is not an issue, at all, with proteins or natural carbohydrates as the chiral selectors, since these selectors are available in only one enantiomeric form) has been repeatedly proven experimentally in chiral exchanging systems involving amino acids of known e.e. as the selector.

Chiral-Bonded Ligand Exchanging Stationary Phases

The first chiral ligand exchanging polystyrene-type stationary phases were synthesized and tested by Rogozhin and Davankov as early as 1968, while analogous silica-bonded phases emerged a decade later. Even nowadays, cross-linked polystyrene resins remain popular in preparative chromatography, mainly due to their excellent chemical durability and high loading capacity. To prepare such chiral packings, spherical particles of styrene-divinylbenzene copolymers are usually subjected to chloromethylation and the active chlorine atoms are then replaced by amino groups of chiral natural α -L-amino acids, in particular (*S*)-proline and (*S*)-hydroxyproline.

(According to modern stereochemical nomenclature, L- and D- α -amino acids belong to R and S configuration series, respectively, with a few exceptions, however.) The structure of a chiral selector immobilized in this way as well as its complex with a Cu(II) ion and an amino acid analyte, both of which bind to the selector during the process of LEC, can be represented by the following scheme:



Here, the most important features of the ternary mixed-ligand bis(amino acidato)copper complex are:

- the formation of two five-membered chelate rings;
- the occupation of the four positions of the Cu(II) ion main coordination square by four electron-donating atoms of the ligands;
- the *trans*-arrangement of the two negatively charged carboxyl groups and the bi-substituted nitrogen atoms, respectively, which minimizes both the electrostatic and sterical repulsion of the ligands in the complex.

The resins incorporating (*S*)-proline and (*S*)-hydroxyproline, when loaded with Cu(II) ions and eluted with ammonia solution, are usually found to retain α -amino acids of the opposite, (*R*)-configuration more effectively. In this case, the enantioselectivity of the system, when expressed as the ratio of retention factors of the (*R*)- and (*S*)-enantiomers of the analyte, $\alpha = k_R/k_S$, is larger than 1. With tridentate

amino acid-type ligands, like histidine or allo-hydroxyproline, the opposite elution order of the enantiomers is observed, thus giving α values smaller than 1. Many other types of chiral selectors have been similarly immobilized on chloromethylated cross-linked polystyrene beads, with the result that cyclic amino acids as well as benzyl-substituted chiral propan-1,2,-diamine display the highest selectivity and largest application range. Typical examples for resolving racemates of common α -amino acids on these phases are presented in **Table 1**.

Two additional polymeric matrixes, cross linked polyacrylamide and poly(glycidylmethacrylate), have also been shown to be useful for immobilizing chiral amino acid selectors. The mode of functionalizing of these resins, when they incorporate (*S*)-phenylalanine and (*S*)-proline as typical chiral selectors, can be represented by the following structures:

The first resin demonstrates higher affinity to (*R*)-isomers of all amino acids, without any exception, resolving all racemates with a selectivity of at least $\alpha = 1.3$.

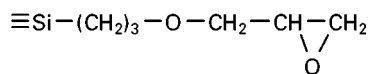
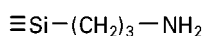
All the above polymeric ligand exchangers have been used successfully for both analytical and preparative resolution, e.g. for obtaining highly radioactive tritium-labelled optically active amino acids. In order to remove trace copper ions from enantiomers resolved in preparative experiments, the fractions of interest can be gravity filtered through a small additional column packed with silica, any chelating resin or even the same chiral resin, with a blue band of Cu(II) gradually forming at the top of the subsidiary column.

Silica-bonded chiral ligand exchangers were expected to demonstrate in analytical-scale separations still better column efficiency than polymer gels. Three types of bonded phases were suggested independently and simultaneously in 1979 by the groups of Foucault, Davankov and Guebitz, based on three different ways of activating the initial macroporous

Table 1 Enantioselectivity, $\alpha = k_p/k_r$, of resolution of racemic amino acids on the Cu(II) forms of polystyrene resins which incorporate residues of L-proline, L-hydroxyproline, L-allo-hydroxyproline, L-azetidone carboxylic acid and *N*-benzyl-(*R*)-propane-1,2-diamine. Mobile phases: aqueous ammonia solutions. Ambient temperature

Racemic amino acid	RPro	RHyp	RaHyp	RAzCA	RBzpn
Alanine	1.08	1.04	1.04	1.06	0.70
Aminobutyric acid	1.17	1.22	1.18	1.29	0.49
Norvaline	1.34	1.65	1.42	1.24	0.48
Norleucine	1.54	2.20	1.46	1.40	0.49
Valine	1.29	1.61	1.58	1.76	0.31
Isovaline	–	1.25	–	–	–
Leucine	1.27	1.70	1.54	1.24	0.49
Isoleucine	1.50	1.89	1.74	1.68	0.62
Serine	1.09	1.29	1.24	2.15	0.62
Threonine	1.38	1.52	1.48	0.78	0.44
Allothreonine	1.55	1.45	–	–	–
Homoserine	–	1.25	–	–	–
Methionine	1.04	1.22	1.52	1.29	0.60
Asparagine	1.18	1.17	1.20	1.44	0.70
Glutamine	1.20	1.50	1.40	1.25	–
Phenylglycine	1.67	2.22	1.78	1.38	0.49
Phenylalanine	1.61	2.89	3.10	1.86	0.52
α -Phenyl α -alanine	–	1.07	–	–	0.38
Tyrosine	2.46	2.23	2.36	1.78	–
Phenylserine	–	1.82	–	–	–
Proline	4.05	3.95	1.84	2.48	0.47
Hydroxyproline	3.85	3.17	1.63	2.25	–
Allo-hydroxyproline	0.43	0.61	1.48	1.46	–
Azetidine carboxylic acid	–	2.25	–	–	–
Ornithine	1.0	1.0	1.20	1.0	1.0
Lysine	1.10	1.22	1.33	1.06	1.0
Histidine	0.37	0.36	1.32	0.56	0.85
Tryptophan	1.40	1.77	1.10	1.13	–
Aspartic acid	0.91	1.0	0.81	0.88	1.0
Glutamic acid	0.62	0.82	0.69	0.77	–

microparticulate silica gel, namely, by first grafting silica with aminopropyl, chloroalkyl, and 3-glycidoxypropyl groups, respectively:



Whereas the aminopropyl spacer binds a chiral amino acid-type selector via the carboxyl group, the other two functions easily react with the amino group of the selector. The epoxy-activated and L-proline incorporating silica was the first chiral-bonded stationary phase on the market (Chiral ProCu, Serva, Heidelberg, Germany). Silica-bonded ligand exchangers have been used successfully to resolve racemates of numerous amino acids, hydroxy acids, *N*-dansyl- and *N*-alkyl-amino acids, α -trifluoromethyl- α -amino

acids, dipeptides, some β -amino acids, etc. Amino alcohols do not form stable chelates with Cu(II) ions, but are easily resolved after conversion into Schiff bases or, better still, after reaction with bromoacetic acid, which converts the amino group into a chelating glycine moiety.

The silica-bonded phases, however, still require elevated temperatures (usually 50°C) to generate sufficient column plate numbers. In addition only neutral or weakly acidic aqueous-organic eluents, in particular, in the pH range between 6.0 and 4.0 can be applied. Therefore, a much more hydrolytically stable packing was introduced by multiple-point binding of chloromethylated polystyrene chains to the silica matrix, followed by substituting the active chlorine atoms of the polymer for chiral selectors. This type of polymer-bonded phase can safely be used at elevated temperatures, and display high resolving power towards numerous racemates, as exemplified in Figure 1. In LEC, the elevated temperature does not noticeably affect the enantioselectivity of the column.

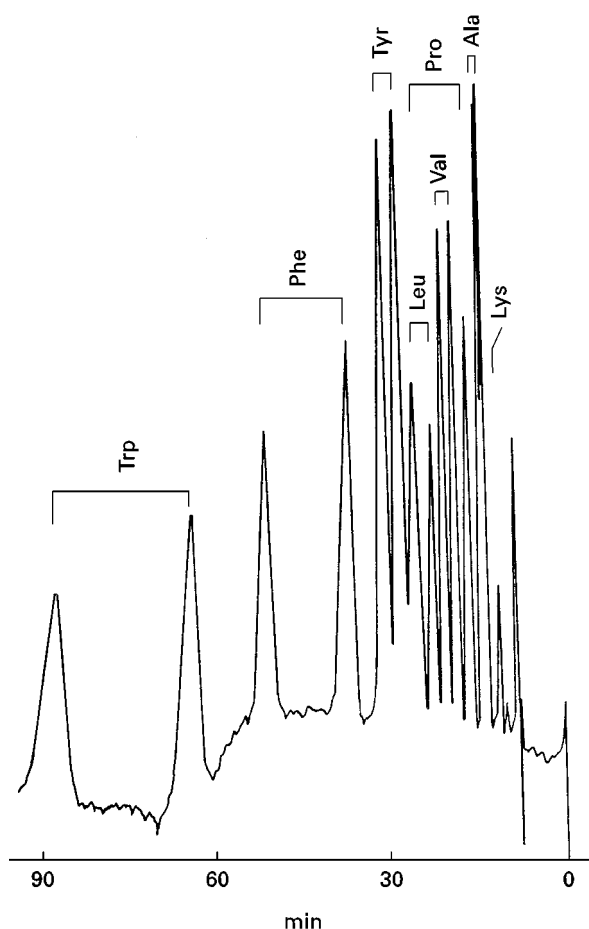


Figure 1 Enantiomeric resolution of a mixture of eight DL-amino acids with a chiral stationary phase, polystyrene grafted on silica gel and substituted with L-Hypro. Conditions: column, 250 × 4 mm i.d., dp 5 μm; eluent, 0.5 mM copper acetate, 10 mM ammonium acetate, pH 4.5, acetonitrile 30%; flow rate, 0.7 mL min⁻¹; temperature, 75°C; UV 254 nm. (Reprinted with permission from Davankov VA (1989) Chapter 15, Figure 5, p. 464. In: Krstulovic AM (ed.) *Chiral Separations by HPLC. Applications to Pharmaceutical Compounds*, pp. 446–475. Chichester, England: Ellis Horwood.)

When dealing with complicated analyte matrixes, it is often useful to combine in sequence a chiral column with a conventional achiral one, in order to supplement the resolution of enantiomeric pairs in the chiral column with the selectivity towards different analytes of the second column. Thus, chiral ligand exchanging bonded phases are easily compatible with achiral reversed phase columns, since both columns can be operated in a gradient mode with aqueous-organic eluents which contain about 0.1 mM copper acetate and about 0.25 M ammonium acetate.

Several silica-bonded and polymeric chiral ligand exchangers are commercially available, e.g. Chiral-Si 100 L-ProCu, Chiral-Si 100 L-ValCu, Chiral-Si 100 L-HyProCu (Serva, Heidelberg, Germany), Nucleosil Chiral-1 (Macherey-Nagel, Dueren, Germany),

Chiralpak WH and WM (Daicel, Japan), TSK gel Enantio L1 (Toso, Japan), MCI gel CRS 10W (Japan), Chirosolve L-proline, Chirosolve L-valine (JPS Chimie, Switzerland). Any scientific evaluation of results obtained by using these phases is, however, complicated, since the exact structure of immobilized chiral ligands on many commercially available chiral stationary phases is not specified by the manufacturers.

Chiral Dynamically Coated Stationary Phases

Ligand exchanging columns of an exceptional efficiency and enantioselectivity can be easily prepared from commercially available reversed-phase columns by dynamically coating the column with a hydrophobic chiral selector. According to the technique suggested by Davankov and Unger in 1980, percolating a solution of *N*-alkyl-L-hydroxyproline in aqueous methanol through the column results in depositing the chiral selector on the bonded alkyl stationary phase. After a subsequent saturation of the retained ligand with copper ions, the column is ready for racemate resolutions in water-rich eluents which do not desorb the chiral selector. Depending on the length of the *N*-alkyl group (heptyl, decyl or hexadecyl) and conditions of coating, the amount of the chiral selector deposited can be easily regulated over a very broad range. Such columns (also commercially available from Regis Technologies, USA, as 'Davankov columns') display a high resolving power toward numerous amino acids (Figure 2) and their derivatives, racemic glycid-di- and tripeptides, 3-amino- ϵ -caprolactam, as well as norephedrine and its analogous amino alcohols. Interestingly, the *N*-alkyl-L-hydroxyproline-coated columns, under severe overloading conditions, show a peculiar distortion of elution profiles of the enantiomers in that the first peak has no tail whereas the second peak shows a very sharp front, which enormously increased the column loadability, implying high preparative potential of the system.

Coating reversed-phase columns with *N,N*-dioctyl-L-alanine has proved especially efficient in resolving racemic α -hydroxy acids. Chiral coatings prepared with *N*²-decyl-L-histidine, *N*¹⁰-decyl-L-histidine, *N*²-octyl-(L)-phenylalaninamide, *N,S*-dioctyl-*N*-methyl-(D)-penicillamine, hydrophobic Schiff bases of chiral amino alcohols and *N*-dodecyl-*N*-carboxymethyl-(1*S*,2*R*)-norephedrine show high efficiency and selectivity. Reversed-phases coated with *N,S*-dioctyl-(D)-penicillamine and (*R,R*)-tartaric acid mono-(*R*)-1-(α -naphthyl)ethylamide are commercially available under the name Sumichiral OA-5000 and 6000 (Sumica Chemical Analysis Service, Japan).

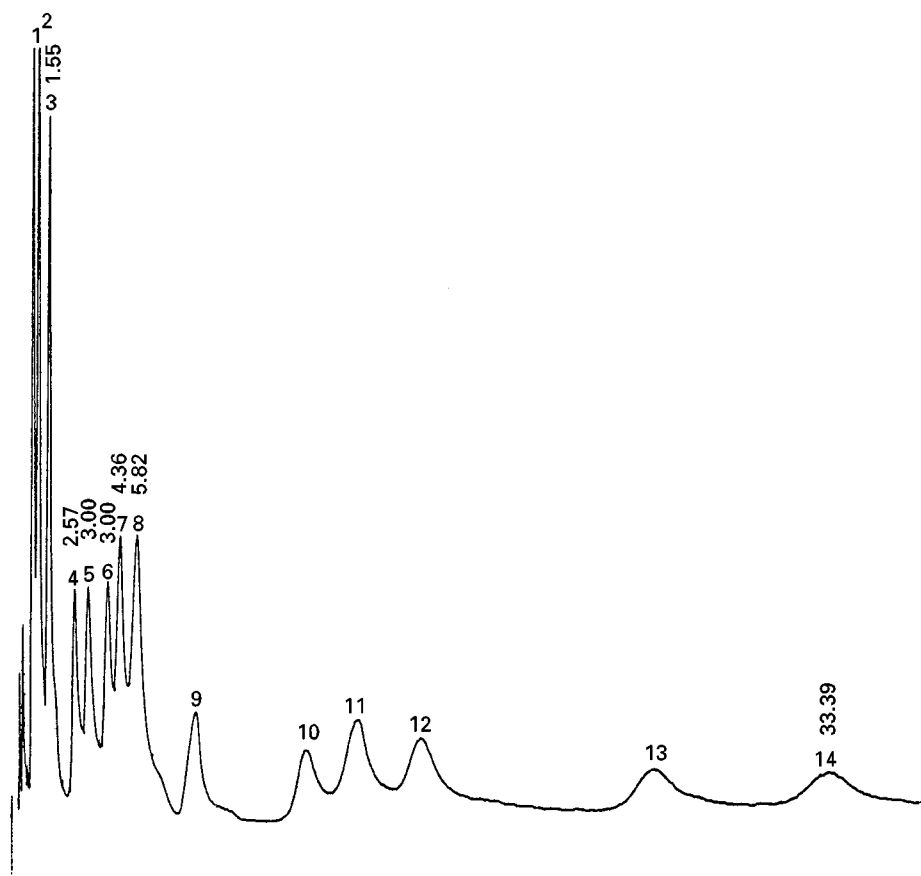


Figure 2 Enantiomeric resolution of a mixture of seven DL-amino acids with a chiral coating of *N*-decyl-L-Hydro on reverse phase material. Conditions: column, 100 × 4.2 mm, LiChrosorb RP 18, dp 5 μm; eluent, methanol-water (15 : 85), 0.1 mM Cu(II) acetate, pH 5.0; flow rate 2 mL min⁻¹; temperature 20°C, UV 254 nm; elution sequence; 1, L-Ala; 2, D-Ala; 3, L-Val; 4, L-Arg; 5, D-Arg; 6, L-Leu; 7, L-Nleu; 8, D-Val; 9, L-Phe; 10, D-Leu; 11, L-Trp; 12, D-Nleu; 13, D-Phe; 14, D-Trp. (From Davankov VA, Bochkov AS, Kurganov AA, Roumeliotis P and Unger KK (1980) Separation of unmodified α-amino acid enantiomers by reverse phase HPLC. *Chromatographia* 13: 677–685, Figure 2, p. 680.)

Whereas long *N*-alkyl substituents of the above chiral selectors are best suitable to bring about the intercalation of the latter between the surface alkyl groups of the reversed phase support, porous graphite offers a flat and rigid surface for the chiral selector to stick to. In this case, polyaromatic substituents show better retention and resolving power of the coated chiral selector, as is the case with *N*-naphthylmethyl-L-proline or *N*-(2-naphthalenesulfonyl)phenylalanine (see Figure 3).

Similarly, hydroxylated macroporous silica gel can be coated with hydrophilic chiral selectors such as silver(I)-*d*-camphor-10-sulfonate or (+)-cobalt(III)-tris-ethylenediamine trichloride. In non-aqueous media, these phases easily resolve racemates of some chiral diene and cyclopentadienylrhodium(I)-norbornadiene complexes. Since in the latter case ethylenediamine ligands of the inert cobalt complex are not exchanged for any new ligands, the separation

should be ascribed to ligand exchange in the outer, solvation shell of this complex.

Finally, polymeric chains of polyacrylamide grafted with L-proline can also be permanently adsorbed onto the surface of silica gel, to give a chiral ligand exchanger which resolves amino acids in polar media.

The ease of preparation of chiral-coated ligand exchanging phases, their exceptional efficiency, durability, wide application range and the opportunity of regulating the elution order of the enantiomers of interest by simply changing the configuration of the selector applied, make the technique increasingly popular.

Chiral Ligand Exchanging Mobile Phases

In the technique described in the previous section, the chiral selector permanently resides on the surface of

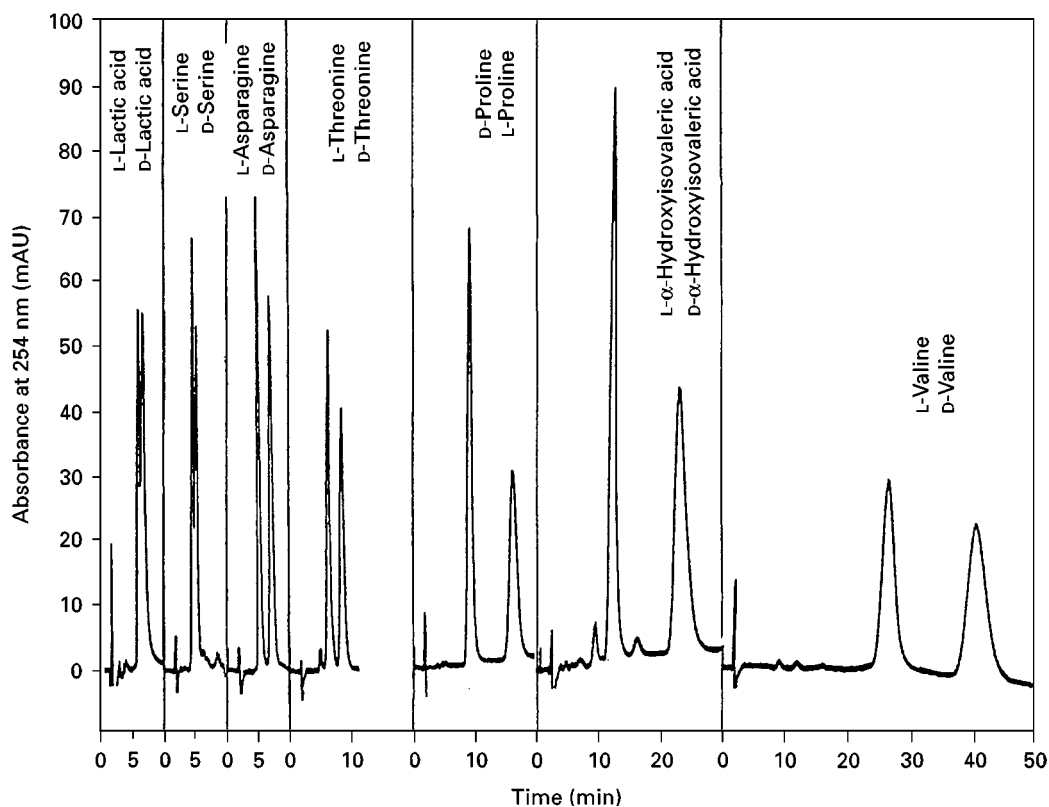


Figure 3 Enantiomeric separation of racemic amino acids and hydroxy acids with a chiral coating of *N*-(2-naphthalenesulfonyl)-*L*-phenylalanine on porous graphite. Conditions: column PGC 94F (5 μ m, 50 \times 4.6 mm), eluent 2.0 mM $\text{Cu}(\text{CH}_3\text{COO})_2$, pH 5.6, flow rate 0.5 mL min^{-1} , UV 254 nm, temperature 30°C. (Reprinted with permission from Knox JH and Wan Q-H (1995) *Chromatographia* 40: 9–14.)

the column packing. The coating is deposited prior to the chromatographic experiments and no eluents should be used which cause a leakage of the selector. A different approach, which was independently suggested in 1979 by the groups of Karger and Lindner and Hare and Gil-Av, consists in using chiral selectors which predominantly reside in the mobile phase. In this case, the selector complex has to be continuously introduced into the column. Analytes to be resolved in such a chiral eluant form mixed-ligand complexes with the selector, which are diastereomeric in their structure, and, therefore, may be differently retained through the interaction with the column packing. A great advantage of this technique is that many different chiral selectors can be easily tested with respect to the analyte to be resolved. Another advantage is that smaller ligands are usually applied as the eluent dopants, which results in enhanced rates of ligand exchange and better efficiency of the columns. A disadvantage is the loss of the chiral selector and problems of separating the selector from the enantiomers resolved in the case of preparative experiments. It should be emphasized also that the two enantiomers separated in the achiral column enter the de-

tector cell with the chiral eluent in the form of ternary complexes. These complexes are diastereomeric and can significantly differ in molar extinction. Indeed, a case of enantioselective fluorescence quenching of dansylamino acids by a chiral $\text{Cu}(\text{II})$ complex of *L*-phenylalanylamine in aqueous solutions has been reported, thus requiring two calibration curves for a quantitative determination of the two enantiomers.

In combination with $\text{Cu}(\text{II})$, $\text{Zn}(\text{II})$, $\text{Ni}(\text{II})$ and some other transition metal ions, many chiral selectors have been shown to function successfully in reversed phase systems as chiral dopants. They mainly belong to the following classes of chelating compounds:

- unsubstituted *L*- α -amino acids (proline, phenylalanine, isoleucine, histidine, etc.);
- *N*-alkyl-*L*-amino acids (*N*-benzyl-proline, *N,N*-dipropyl-alanine, *N,N*-dimethyl-valine);
- *L*- α -amino acid amides (valine amide, phenylalanine amide, prolyloctylamide, aspartyl-alkylamide);
- esters of *L*- α -amino acids (histidine methyl ester);
- peptides (prolyl-valine, aspartylphenylalanine methyl ester or aspartame);

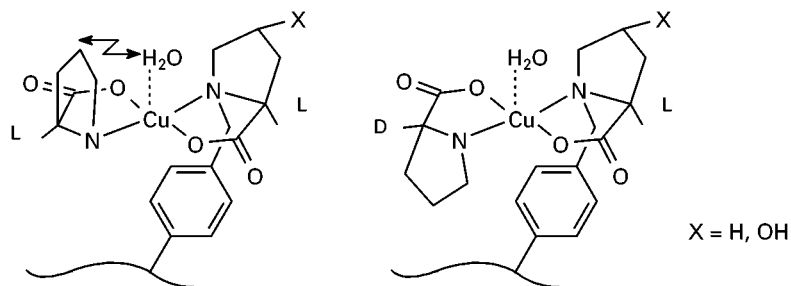
- chiral diamine (*N,N,N',N'*-tetramethyl-(*R*)-propanediamine-1,2-(*L*)-alkyl-4-octyl-diethylenetriamine);
- mandelic acid;
- derivatives of (*R,R*)-tartaric acid (tartaric acid mono-*n*-octylamide);
- nucleotides (adenosine diphosphate, β -nicotinamide, adenine dinucleotide and, in particular, flavine adenine dinucleotide).

Mainly racemic α -amino acids and their numerous derivatives, α -hydroxy acids and amino alcohols are successfully resolved. Less common, but nevertheless successful, are resolution of substituted pterins, phenylhydantoins and ornithine analogues.

Depending on the hydrophobicity of the analyte and chiral selector applied, the content of the organic modifier in the chiral mobile phase has to be adjusted in such a manner that the retention of enantiomers remains in the desired time window. In addition to this parameter, retention (and enantioselectivity) in LEC would slightly decrease with the rise in the ionic strength of the eluent and temperature of the column. Conversely, rising pH and concentration of the chiral additive in the mobile phase cause a clear increase in retention and a less marked increase in enantiomeric resolution.

Under normal phase conditions, with bare silica gel as the packing, copper complexes of *N,N,N',N'*-tetramethyl-(*R*)-propane-1,2-diamine can be used to resolve amino acids and mandelic acid. Bis(*L*-prolinato)copper is efficient even in combination with sulfonated polystyrene cation exchangers, which additionally demonstrates the versatility of the chiral mobile phase approach.

The high efficiency of the method is exemplified by Figure 4.



It is easy to imagine that, by using a mobile phase with a chiral selector of the opposite configuration, we reverse the elution order of the resolved enantio-

mers. This approach is useful in analysing enantiomeric impurities in chiral compounds, where it is always advisable to have the trace component eluting before the major enantiomer.

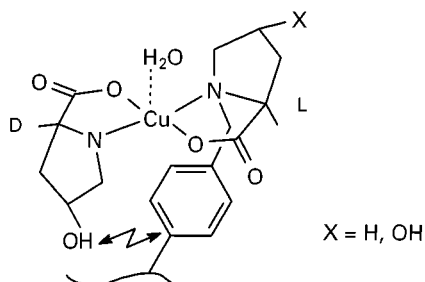
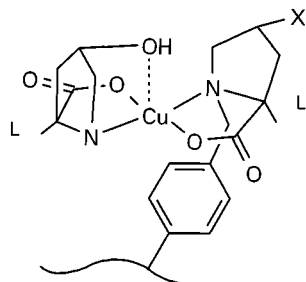
Finally, it is worth mentioning that the chiral mobile phase mode of LEC is very convenient to prove the reciprocity relations in chiral recognition, where the selector is exchanged for the analyte and vice versa. The enantioselectivity of the system should remain the same, unless some additional factors interfere with the essential interactions within the diastereomeric sorption complexes.

Mechanisms of Chiral Discrimination in LEC

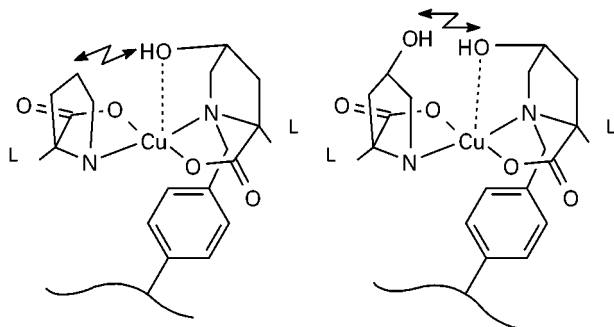
Of many enantioselective chromatographic systems developed thus far, ligand exchanging systems are the most examined. Understanding of the mechanism of chiral discrimination is greatly facilitated here by the fact that, of the three interactions needed for the chiral selector to recognize the solute enantiomers, two are well defined as the *trans*-coordination bonds of pairs of carboxylic and amino groups in the main coordination square of the Cu(II) ion. The third interaction should be looked for in the out-of-plane sterical interactions or coordination in remote axial positions of the coordination sphere. Thus with *L*-proline or *L*-hydroxyproline incorporating polystyrene-type resins, retention of *L*-isomers of amino acids is diminished by sterical interactions with the water molecule coordinated in one of the axial positions. On the other hand, the binding of *D*-isomers is facilitated by favourable hydrophobic interaction between the solute α -substituent and the polystyrene matrix, as shown below:

Tridentate amino acids, *L*-His, *L*-Asp, *L*-Glu, *L*-aHyp, manage to replace the water molecule from the axial position and form one additional coordination

bond, which logically explains the inverted elution order of these solutes:



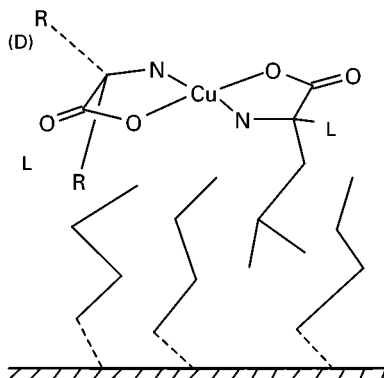
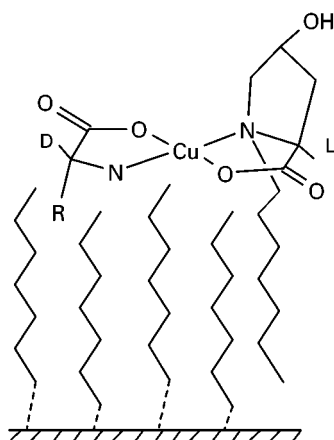
With tridentate L-*allo*-hydroxyproline as the chiral selector, both bifunctional and trifunctional solutes can form two coordinate bonds only and elute in the order L before D without exception:



shown above for the first resin. Conversely, with an L-phenylalanine-type chiral selector in a poly-

acrylamide matrix, the balance of steric interactions results in a reduction in the retention of L-amino acids (see above).

The decisive role of interactions with the achiral sorbent matrix in the enantiomer recognition was discovered by Davankov in chiral-coated and chiral mobile phase reversed phase systems. Here, only one of two solute enantiomers can realize favourable hydrophobic interactions with the silica immobilized alkyl groups, thus forming the stronger retained diastereomeric sorption complex.



With the L-Pro type selectors immobilized on polyglycidylmethacrylate, polyacrylamide, and polyvinylpyridine matrixes, which occupy three coordination positions of Cu(II), the steric repulsion diminishes retention of the D-amino acid isomers as compared to L-isomers, as this was

The mechanism of chiral discrimination is less clear with selectors and/or analytes other than α -amino or α -hydroxy acids, and metal ions other than Cu(II), since the coordination mode of ligands in these complexes is less well known.

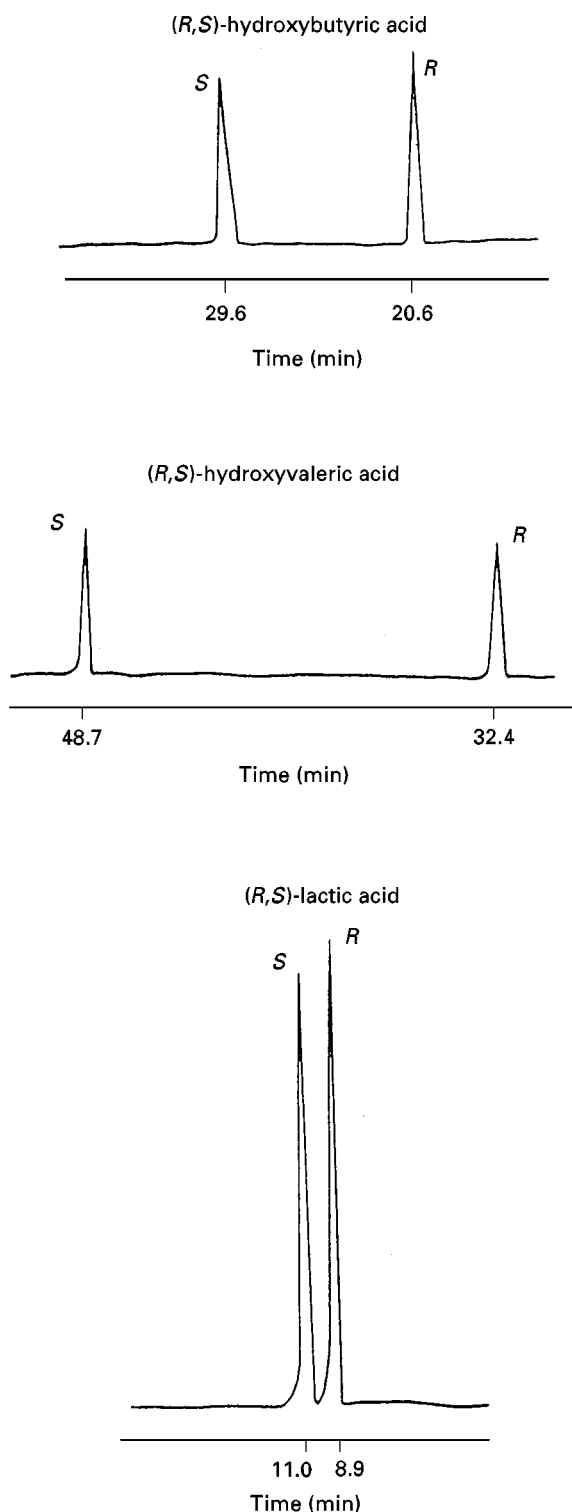


Figure 4 Enantiomeric separation of *R,S*-hydroxy acids with a chiral mobile phase. Conditions: 8 mM L-PheNH₂, 4 mM Cu(CH₃COO)₂, pH 6.0, ambient temperature, column Spherisorb 3 ODS-2 (3 μm, 150 × 4.6 mm), flow rate 1 mL min⁻¹, postcolumn derivatization with Fe(ClO₄)₃, UV 436 nm. (Reprinted with permission from Galaverna G, Panto F, Dossena A, Marchelli R and Bigi F (1995) *Chirality* 7: 331–336. Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

Other Chromatographic and Related Techniques of Resolving Enantiomers in Ligand Exchanging Systems

In gas chromatography, ligand exchange has acquired great importance since Schurig introduced metal complexes of chiral terpeneketonate (acylated camphor, carvone, pulegone, menthone) as additives to stationary phase in capillary GC in 1977. Ni(II), Mn(II), Cu(II), Zn(II), and Rh(I) proved especially useful in this 'complexation' chromatography. The resolution mechanism is ascribed in terms of the interaction between the above sterically or electronically unsaturated metal chelates with volatile ligands which offer lone electron pairs to form a labile mixed-ligand sorption complex. Enantioselectivity of this process is small, but, due to the extremely high plate numbers attainable with capillary GC columns, excellent resolution is achieved for many classes of alkenes, alcohols, ethers, oxirane, sulfur-containing compounds, aziridine, etc. It is important that even compounds containing one single electron-donating atom (O, N, S) are successfully resolved by GC, contrary to LC, where the presence of two donors to form a chelate with the central metal ion is highly desirable.

Similar bonded chiral selectors, e.g. polysiloxane-anchored Ni(II)-bis-[(3-heptafluorobutanoyl)-(1*R*)-camphorate], Chirasil-Nickel, can also be used in the supercritical fluid chromatography (SFC) mode. Although SFC cannot compete with GC with regard to efficiency, a significant increase in selectivity, due to a substantially lower temperature of the column, can compensate for the loss in plate number. Because of the high solvation strength of supercritical carbon dioxide, a number of low-volatility racemic alcohols, diols and esters have been resolved by ligand exchange SFC at temperatures as low as 40–70°C.

In capillary electrophoresis (CE) and other electromigration techniques, chiral ligand exchange can be involved in several ways. Cu(L-His)₂ has just been added to the ammonium acetate electrolyte to induce chiral resolution of dansylated amino acids. Analyte enantiomers which are strongly involved in the formation of positively charged mixed complexes with Cu(L-His) move toward the cathode at a higher rate than the uncomplexed ligands. Since the enantioselectivity of the ligand exchange reaction in the bulk solution is small, the resolution of enantiomers is also small. It is only slightly better with copper-aspartame as chiral additive. A significant improvement in selectivity can be obtained by adding tetradecyl sulfate, as a micelle-forming surfactant. Obviously, the enantioselectivity increases through the interaction of the diastereomeric ternary complexes

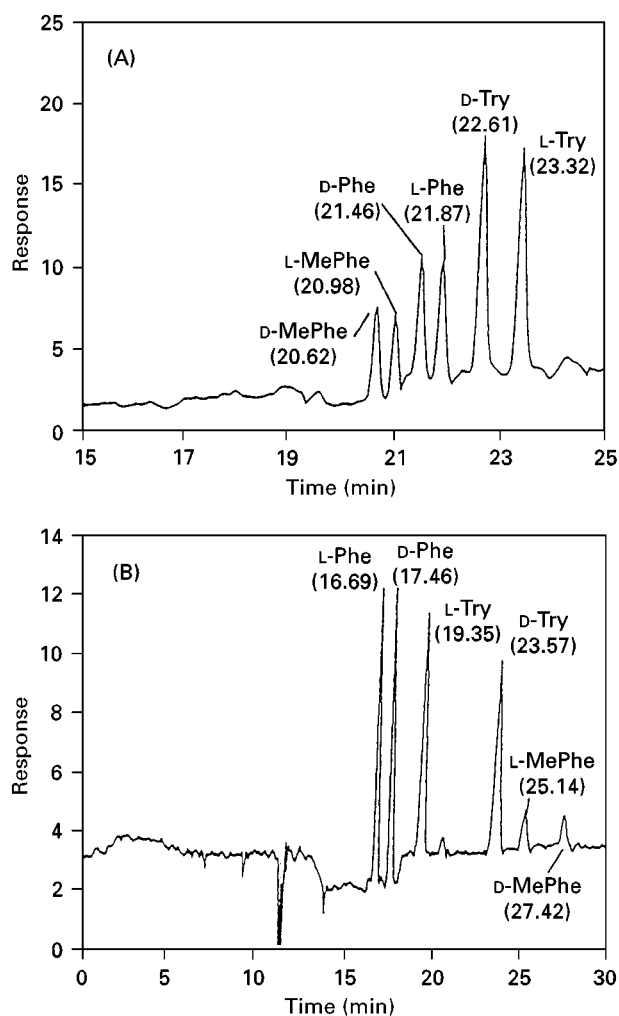


Figure 5 Electrophoregram of the enantiomer separation of DL-Phe, DL-Trp and DL-MeTrp with and without SDS. Conditions: (a) 80 mM L-Hyp, 40 mM Cu(II) sulfate, pH 4.0; (b) 50 mM L-Hyp, 25 mM Cu(II) sulfate, 15 mM SDS, 3 M urea, pH 4.0. Capillary: fused silica, 75 cm \times 75 μ m i.d. (effective length, 66 cm) $U = 27$ kV, UV 208 nm, ambient temperature. (Reproduced with permission from Schmid MG and Guebitz G (1996) *Enantiomer* 1: 23–27. Gordon and Breach Publishers.)

with the surface of the pseudo-stationary micelle phase. Good resolution for dansyl (DNS) amino acids has been obtained with mixed-micelle solutions containing sodium dodecyl sulfate (SDS) and copper complexes of *N,N*-didecyl-L-alanine. Figure 5 shows an example of CE resolution of underivatized racemic amino acids with Cu(L-Hyp)₂ in the support electrolyte, which is facilitated by SDS micelles. Note the inversion of the elution order of the enantiomers and solutes on transition from the homogeneous support electrolyte solution to the pseudo-heterogeneous micellar system.

In addition to paper chromatography which represents the oldest chiral planar chromatography tech-

nique, ligand exchange opened enormous possibilities for chiral thin-layer chromatography. Macherey-Nagel in cooperation with Degussa (Germany) developed reversed-phase plates coated with copper complexes of *N*-(2-hydroxydodecyl)-L-hydroxyproline in the manner described earlier by Davankov for chiral coating of RP-columns. The Chiralplate® has proved to be extremely versatile in the resolution of racemic α -amino acids, their *N*-methyl-, *N*-formyl-, α -alkyl-, and halogenated amino acids, dipeptides, α -hydroxy acids, thiazolidine derivatives, anomers of several nucleobases, etc. Copper complexes of *N,N*-dialkyl amino acids have also been tested as chiral coatings of plates, but so far have not found broad application.

Finally, enantiomeric separation of phenylalanine and lactic acid by diffusion through a liquid membrane was studied, using a solution of *N*-decyl-L-hydroxyproline copper complexes in hexanol-decane mixtures as the membrane. The rate of migration of the D-Phe was found to be about 2.4 times faster as compared to those of the L-isomer. Though the productivity potential of the technique is high, the above value of the kinetic enantioselectivity is still insufficient for practical use.

Applications

Two principal advantages of chiral LEC should be mentioned with respect to practical application of the method. Firstly, the detection of compounds which do not absorb in the near UV region is greatly facilitated by the fact that they partially elute from the LEC column in the form of metal complexes. Even having no strong chromophore in the molecule, they strongly absorb at 254 nm in the complexed form. When complexed to Fe(III), hydroxy acids can be selectively detected at 420 nm. The second great advantage of ligand exchange is that enantioselectivity of the separation is largely unaffected by the temperature of the column and the presence of buffer salts and organic modifier in the eluent. This makes the method easily compatible with numerous other separation techniques, including multidimensional and multicolumn chromatography.

In general, the efficiency and easiness of the direct resolution of amino acids and hydroxy acids, without any prior derivatization of the analyte, make LEC a technique of choice for serial determinations of enantiomeric compositions. This is the case, for example in fossil dating where, depending on the rate of racemization of the amino acid under investigation, the dating can be performed in the time range from several hundred years to one million years. Another application area of this kind is the

determination of D-amino acids in food products caused by partial racemization through heat or microwave treatment, and to fermentation processes. Occurrence of D-amino acids in biological fluids (e.g. serum, cerebrospinal fluid, urine), bone and tissues is also of great importance from a diagnostic point of view. Rapid enantiomeric analysis techniques are needed when asymmetric synthesis or chemical or enzymic racemate resolution processes are being developed. Examination of the enantiomeric composition of hydroxy acids, in particular malic acid, helps to investigate adulteration of apple juice and other soft drinks. Rapid preparation of enantiomers of compounds labeled with highly radioactive atoms, where only dilute solutions can be handled, is another excellent application area for chiral LEC.

Three decades of the existence and intensive development of enantioselective LEC have shown this technique to be an extremely versatile and productive general approach to resolving racemates of compounds that form complexes with metal ions. Other types of molecular interactions have been later found to provide alternative successful ways of organizing the formation of labile diastereomeric adducts with appropriate chiral selectors, e.g. formation of inclusion complexes of analytes with cyclodextrins and cyclic antibiotics or formation of charge-transfer complexes between electron-donating and electron-deficient aromatic groups in Pirkle-type chiral stationary phases. Designing novel chiral selectors which simultaneously offer to the analyte several different modes of interactions could probably result in developing chiral chromatographic systems with wider application areas or higher enantioselectivity with respect to particular important racemates. Thus first attempts of providing cyclodextrins with a copper complexed residue moiety have recently been reported in the literature. Another practically unexplored field for further development could be ligand exchange in nonaqueous and even nonpolar media. With the solvent molecules not competing for the coordination positions of the metal ion, much weaker electron donors than carboxylic or amino functional group of the analyte, should suffice for the complex formation. Ether, sulfide and carbon-carbon double bonds should thus be suitable for complexation. Achievements of complexing gas chromatography and 'argentation' thin-layer chromatography corroborate the feasibility of this development of

ligand exchanging enantioselective column liquid chromatography.

See also: **II/Chromatography: Liquid:** Chiral Separations in Liquid Chromatography: Mechanisms. **II/Chromatography: Thin-Layer (Planar):** Ion Pair Thin-Layer (Planar) Chromatography. **III/Amino Acids and Derivatives: Chiral Separations. Chiral Separations:** Capillary Electrophoresis; Cellulose and Cellulose Derived Phases; Chiral Derivatization; Cyclodextrins and Other Inclusion Complexation Approaches; Ion-Pair Chromatography; Liquid Chromatography; Molecular Imprints as Stationary Phases; Protein Stationary Phases; Synthetic Multiple Interaction ('Pirkle') Stationary Phases; Thin-Layer (Planar) Chromatography.

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