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Review

Chiral selectors with chelating properties in liquid chromatography: fundamental reflections and selective review of recent developments

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ABSTRACT

Taking ligand-exchange chromatography as a relatively well studied example, theoretically expected general correlations between retention, enantioselectivity and efficiency of chiral chromatographic systems are discussed. More recent practical achievements and the expansion of the application area of chiral ligand-exchange chromatography are also briefly reviewed.

CONTENTS

1. INTRODUCTION

Ligand-exchange chromatography (LEC), a technique suggested by Helfferich [1] in 1961, was further developed by Rogozhin and Davankov [2,3] into a powerful chiral chromatographic method by introducing chiral complex-forming synthetic resins. As early as 1968–71, this tech-

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nique, for the first time in liquid chromatography, resulted in a complete and reliable separation of enantiomers of amino acids and some other classes of chiral compounds that were able to form complexes with transition metal cations. Having since become one of the most extensively investigated methods for the direct resolution of enantiomers, LEC maintained for a long period of time its leading position in the development of novel chiral chromatographic systems (such as chiral silica-bonded phases, both monomeric and polymeric, chiral coatings, chiral mobile phase additives, chiral thin-layer chromatography (TLC) and chiral electrophoresis) and in evaluating mechanisms of chiral recognition and discrimination.

Chiral LEC has been the subject of several extensive reviews covering the fundamental principles [4–8] and outlining the preparation of chiral LEC packings [9,10], in addition to practical achievements in the separation of enantiomers [6–8] including the use of chiral mobile phase techniques [8], chiral TLC [8,11], preparative enantioseparations [12], capillary electrophoresis [13,14] and mechanisms of chiral recognition [15–17].

To avoid unnecessary overlap with the above publications, this selective review concentrates on the main theoretical problems of LEC, efficiency, retention and selectivity in chiral LEC systems and on more recent practical uses of the method.

2. COLUMN EFFICIENCY IN LIGAND-EXCHANGE CHROMATOGRAPHY

2.1. Performance of chromatographic systems

To achieve the generally desired results of a chromatographic experiment, namely the baseline separation of compounds of interest from all other components that are present in the initial mixture, it is often necessary to enhance the performance of the chromatographic system, *i.e.*, its efficiency and/or selectivity. Efficiency is concerned predominantly with the kinetics of all processes that take place inside the chromatographic column and involve molecules of compounds to be separated, the rate of approaching the equilibrium distribution of the analytes between the mobile and stationary phases determining the extent of broadening of the chromatographic zones in the column. Selectivity is concerned with the thermodynamics of intermolecular interactions of analytes with other components of the mobile and stationary phases, the position of the interaction equilibria for each analyte determining the residence time of their chromatographic zones in the column.

The efficiency of a column is usually expressed as plate number, N, or reduced plate heights, h. Both values should be calculated for each particular chromatographic peak of interest. These quantities are directly related to the intensity of dilution (dispersion) of chromatographic zones on passing through the column. A high efficiency of the systems facilitates clean resolution of neighbouring zones, enhances the detection precision of the components and, therefore, is especially important in the analytical-scale chromatography of chiral compounds, *e.g.*, in determining their enantiomeric composition.

The selectivity of a chiral chromatographic system towards two enantiomeric species of an analyte A is expressed as the enantioselectivity, α, which is the ratio of the capacity factors of the column with respect to the R and S enantiomers of that analyte. This quantity reflects the difference in the migration rates of the two zones through the column and the distance between the maxima of the two corresponding peaks on the final chromatograms. A high enantioselectivity of the system facilitates the separation of the enantiomers, enhances the loadability of the column and, therefore, is especially important in the preparative-scale resolution of racemic compounds.

Although valid for any type of chiral chromatographic systems, the above considerations should be further specified for the LEC by taking into consideration its distinguishing feature, the involvement of a transition metal cation in the interaction of the analyte with the sorption site of the packing material (Fig. 1). According to this peculiarity, the efficiency and selectivity of ligand-exchanging systems should depend on a very specific set of chromatographic parameters.

Generally, the enantioselectivity of chiral lig-

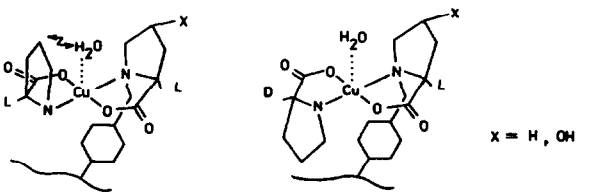


Fig. 1. Typical model for sorption complexes of proline enantiomers on L-proline or L-hydroxyproline incorporating polystyrene-type sorbents. Retention of L-Pro is diminished by the steric interaction with the water molecule coordinated in the axial position of the Cu(II) ion. Retention of D-Pro is enhanced by the (favourable in the aqueous mobile phase) hydrophobic interaction with the non-polar polystyrene chain.

and-exchanging chromatographic systems was found to be excellent, whereas the efficiency of the columns was often poorer than that of conventional HPLC columns, the operation of which did not involve the formation of complexes of the analyte species with metal cations.

2.2. Theoretical background for column efficiency and expectations for LEC

For ease of discussing the efficiency of a chromatographic column, the reduced plate height, h, which is the ratio of the height equivalent to a theoretical plate to the diameter of packing particles, is usually represented as the sum of contributions from several processes that cause broadening of a chromatographic zone in the column. In linear chromatography, these are dispersions due to axial diffusion, convection (including eddy dispersion), resistance to mass exchange in the mobile phase and resistance to mass exchange in the sorbent phase. In order to examine which of the above processes could be influenced unfavourably by the complex-formation reaction, we have to consider separately the ligand-exchanging chromatographic systems which operate with chiral stationary phases (CSPs) and systems exploiting chiral mobile phases (CMPs).

Chiral stationary phases in LEC represent either polymeric networks incorporating complex-forming chiral ligands or insoluble mineral porous materials bearing chiral complex-forming ligands on their surface. The latter can be chemically bonded to the matrix or permanently adsorbed on the surface. In any case, the chiral complex-forming selectors or ligands, L, reside entirely in the stationary phase. Therefore, ternary mixed-ligand complexes, AML, incorporating both the chiral ligand, L, and analyte molecule, A, can also form in the stationary phase alone:

$$\begin{array}{ll} \text{Mobile phase} & A^m \\ & \downarrow\uparrow \\ \text{Stationary phase} & A^s + ML^s \rightleftharpoons AML^s \end{array} \quad (1)$$

where M is a transition metal cation that is able to coordinate one or two (mobile or sorbent-fixed) ligands in its coordination sphere and the subscripts m and s denote the location of the species in the mobile and stationary phases, respectively.

The most important factor for LEC processes, namely the reversible conversion of the analyte, A, into the ternary sorption complex, AML^s, (either through the two-step process shown in eqn. 1 or by an immediate interaction of A^m with ML^s) may well happen to be a relatively slow process. It may well cause a significant resistance to mass transfer in the stationary phase, thus slowing the rate of establishing the interphase equilibrium. This would logically explain the above-mentioned decreased efficiency of many chiral ligand-exchanging stationary phases (CSPs).

In the systems considered, the transition metal cation, M, forms kinetically labile complexes that can readily dissociate. This implies the possibility for the metal ion to be desorbed from the stationary phase into the mobile phase and to form there complexes with the analyte molecules. In their turn, the latter would distribute between the two phases in the column. Rizzi [18] formulated this set of (secondary) equilibria as follows:

$$\begin{array}{lll} \text{Mobile phase} & A^m \rightleftharpoons AM^m \rightleftharpoons AMA^m \\ & \downarrow\uparrow \quad\quad \downarrow\uparrow \quad\quad \downarrow\uparrow \\ \text{Stationary phase} & A^s \rightleftharpoons AM^s \rightleftharpoons AMA^s \end{array} \quad (2)$$

One would expect that the phase distribution of all the above species would proceed with normal,

relatively high rates. Therefore, Rizzi [18] concluded, the possible slow conversion of one complex into another in the mobile phase should not further affect the column efficiency.

We shall comment on the last statement later, but it seemed to correlate with the often observed fact that chromatographic systems that operate in accordance with the chiral mobile phase mode usually appear more efficient than CSP columns where complexation takes place predominantly in the stationary phase.

Chiral mobile phases in LEC represent chiral complexes, ML or LML, that are added in the small concentrations to a conventional mobile phase. They enter ligand-exchange reactions with the analyte molecule, A, in free solution and the mixed-ligand complexes thus formed, AML, partition between the mobile phase and reversed-phase packing (which is usually taken in combination with the CMP):

$$\begin{array}{lc} \text{Mobile phase} & A^m + ML^m \rightleftharpoons AML^m \\ & \downuparrows \quad \downuparrows \quad \downuparrows \\ \text{Stationary phase} & A^s + ML^s \rightleftharpoons AML^s \end{array} \qquad (3)$$

Again, analyte molecules could also be involved in the formation of other complexes, *e.g.*, similarly to that shown in (2). However, all the "complexes are adsorbed and desorbed from the alkylsilica surface with the rapid kinetics typical of reversed-phase chromatography. Under these conditions slow ligand-exchange kinetics should not have much effect on h, in contrast to the case where the ligand is bounded at the surface" [18].

The above explanation of the difference in the efficiency of chiral ligand-exchanging systems which operate with CSPs and CMPs, although logical at first glance, cannot be fully accepted. Indeed, we have to give more consideration to the possible consequences of the slow interconversions of several forms of the analyte in the mobile phase. There are at least four types of species that incorporate the analyte A in the CMP: A, AM, AMA and AML, and they are in equilibrium with each other. Suppose first that the rate of interconversion of these species is extremely low, so low that there is no transformation at all during the chromatography experiment. In this ultimate case, the species would behave independently and produce four separate sharp peaks in the final chromatogram. In the alternative ultimate situation, that of extremely rapid equilibration of the mixture, the four peaks would coalesce into a single sharp peak situated close to the position of the species that predominates at the equilibrium. Now, it is understandable that any intermediate situation of a relatively slow interconversion of the analyte forms would result in a superposition of the above two ultimate chromatographic patterns, *i.e.*, that of four and one peak. Consequently, low rates of ligand exchange in the chiral mobile phase would unavoidably result in a significant peak dispersion. From this point of view, the CMP system could be as sensitive to the slow ligand exchange as is any CSP system.

We thus arrive at the important conclusion that the occasional poor efficiency of all chiral ligand-exchanging chromatographic systems may well be accounted for by the low rate of ligand-exchange reactions (if all other, more trivial, reasons for peak broadening are excluded). We also have to conclude that, in most instances, ligand exchange in free solution takes place at higher rates compared with the situation where one of the ligands, the chiral selector, L, is chemically bound at the sorbent surface or matrix.

2.3. Experimental proof of poor ligand-exchange kinetics

The above-discussed influence of slow ligand-exchange kinetics on the reduced efficiency of some ligand-exchanging chromatographic systems can be corroborated most reliably by the systematic observation by Rizzi [18] that "in all instances investigated, the enantiomer eluted later shows a larger value of h" (the reduced plate height). Indeed, two enantiomers A_R and A_S of compound A must be identical in all their properties and behaviour in the chromatographic system, with the only exception of processes which additionally involve the chiral selector L of the system. These processes are the formation of diastereomeric ternary sorption complexes A_RML^s and A_SML^s in the stationary phase (for both the CSP and CMP systems) and the forma-

tion of diastereomeric complexes A_RML^m and A_SML^m in the bulk solution (for CMP systems). Since, according to Rizzi, the reduced plate heights depend on the configuration of the analyte both in the CSP and CMP chromatographic systems, the kinetics of the ligand exchange are relatively slow in all the above HPLC systems. Obviously, the situation is especially severe for the enantiomer more strongly involved in complexation and in systems using CSPs.

2.4. Kinetics of ligand exchange in solution

Generally, the rate of ligand exchange in kinetically labile complexes of doubly charged cations of Cu, Ni, Co, Zn, Mn, Mg, Ca, Fe, Cd and some others is high and, therefore, difficult to measure. Thus, the rate of coordinating amine molecules to Ni ions was estimated to be of the order of 10^3–10^5 mol^{-1} s^{-1} and that to Cu ions even higher [19]. Unfortunately, no theoretical relationships have been established, so far, between the rate of a reversible transformation of analyte molecules in the column and the peak dispersion caused by this secondary equilibrium. Only simpler, first-order interconversions of one form of the analyte into another form, such as *cis–trans* proline peptide isomersization, RNase A denaturation–renaturation or pyranose sugar anomer mutarotation, have been examined (for a review, see ref. 20). For more complex secondary equilibria, one can generally predict that decreasing other contributions to the overall peak dispersion, *e.g.*, using non-porous surface-modified microparticulate column packings, would put more severe requirements on the rate of establishing all chemical equilibria with the analyte, if the latter should not deteriorate the column efficiency.

In 1987, studying thermodynamic enantioselectivity phenomena in ternary mixed-ligand copper(II) complexes in non-aqueous solutions, Kurganov *et al.* [21] discovered a whole series of systems with unusually slow kinetics of ligand exchange. The ligands, A and B, examined were 1,2-diaminopropane (pn) and 2-aminomethylpyrrolidine (amp), both of *R* and *S* configuration. Amino functions of these diamines were substituted with various numbers of benzyl (Bzl) groups. Each of the ligands was in a position to form bis-complexes with the Cu ions, $[CuA_2]^{2+}$ and $Cu[B_2]^{2+}$. On mixing solutions of the two complexes, ternary structures $[CuAB]^{2+}$ were also formed:

$$Cu(A_R)_2 + Cu(B_R)_2 \underset{k'_{RR}}{\overset{k_{RR}}{\rightleftharpoons}} 2Cu(A_RB_R)$$

$$K_{RR} = k_{RR}/k'_{RR} = \frac{[Cu(A_RB_R)]^2}{[Cu(A_R)_2][Cu(B_R)_2]} \quad (4)$$

$$Cu(A_R)_2 + Cu(B_S)_2 \underset{k'_{RS}}{\overset{k_{RS}}{\rightleftharpoons}} 2Cu(A_RB_S)$$

$$K_{RS} = k_{RS}/k'_{RS} = \frac{[Cu(A_RB_S)]^2}{[Cu(A_R)_2][Cu(B_S)_2]} \quad (5)$$

(charges are omitted in the above formulae). Depending on the *R* or *S* configuration of the ligand B used, two diastereomeric ternary structures are formed. Their stability was found to be different, as the equilibrium constants K_{RR} and K_{RS} differed from each other. Thermodynamic enantioselectivity in the system was expressed as the ratio of these formation constants of the two diastereomeric ternary complexes: $\alpha_K = K_{RR}/K_{RS}$.

Surprisingly, the rate of achieving the equilibrium state on mixing the solutions of the initial complexes, which was followed by registration of circular dichroism of the mixtures, was found to be very low. In many instances, the equilibration required dozens of minutes, which made it possible to investigate the kinetics of the ligand exchange and calculate the corresponding rate constants, k and k', of the forward and reverse reactions. In principle, the ratio of these two rate constants determines the position of the equilibrium of the reversible ligand-exchange reaction and coincides with the equilibrium constant, K, as shown in eqns. 4 and 5. Now, in most instances, the rate of interaction of the chiral complex $Cu(A_R)_2$ with the chiral complex $Cu(B_R)_2$ was found to differ from the rate of its interaction with the other enantiomer, $Cu(B_S)_2$. We can formulate the kinetic enantioselectivity of the forward and reverse reactions as follows:

$$\alpha_k = k_{RR}/k_{RS} \quad \text{and} \quad \alpha_{k'} = k'_{RR}/k'_{RS} \quad (6)$$

From eqns. 4, 5 and 6, it is easy to show that the thermodynamic enantioselectivity of the complexation reaction considered is equal to the ratio of the kinetic enantioselectivities of the forward and reverse reactions: $\alpha_K = \alpha_k/\alpha_{k'}$.

We thus arrive at a very important general conclusion: there is no thermodynamic enantioselectivity without kinetic enantioselectivity. Indeed, stability constants of two diastereomeric associates, K_{RR} and K_{RS}, can differ only in the situation where the formation rates, k_{RR} and k_{RS}, and/or the decomposition rates, k'_{RR} and k'_{RS}, of these diastereomers are different. In contrast, the presence of a kinetic enantioselectivity does not necessarily result in thermodynamic enantioselectivity.

Some representative examples of the ternary complexes examined should be mentioned here. For the pair of diastereomeric complexes $[Cu(N^1\text{-Bzl-amp})\ (N^1\text{-Bzl-pn})]^{2+}$ in nitrobenzene, there is no thermodynamic ($\alpha_K \approx 1$) and no kinetic enantioselectivity ($\alpha_k \approx 1$ and $\alpha_{k'} \approx 1$). Of the two diastereomeric complexes, $[Cu(N^2\text{-Bzl-}S\text{-amp})\ (N^1\text{-Bzl-}R\text{- or -}S\text{-pn})]^{2+}$, in nitrobenzene, the *S–R* isomer is more stable thermodynamically ($\alpha_K = K_{RR}/K_{RS} = 0.6$), as the rate of its decomposition is lower ($\alpha_{k'} = 2.20$), whereas the rates of formation of diastereomers are approximately equal ($\alpha_k = 1.27$). In contrast, the thermodynamic enantioselectivity in the system $[Cu(N^1\text{-Bzl-}S\text{-amp})\ (N^1\text{-Bzl-}R\text{- or -}S\text{-pn})]^{2+}$, where $\alpha_K = 2.40$ in acetonitrile, is mainly accounted for by the the difference in the formation rates of the diastereomers: $\alpha_k = 2.60$ and $\alpha_{k'} = 1.10$. Especially interesting is the system of complexes $[Cu(N^1\text{-Bzl-}S\text{-amp})\ (R\text{- or }S\text{-pn})]^{2+}$, where the thermodynamically preferred *S–S* complex ($\alpha_K = 4.50$) forms in nitrobenzene more slowly than the less stable *S–R* structure ($\alpha_k = 0.48$) (the reason is that this stable *S–S* complex decomposes considerably slower: $\alpha_{k'} = 0.11$). The slower rates of both the formation and decomposition of the stable *S–S* ternary complex imply a slow achievement of the equilibrium state in this system, which, in a chromatographic experiment, would definitely imply a larger reduced plate height value for the *S* enantiomer compared with that for the *R* enantiomer.

There are two additional important results in the paper by Kurganov *et al.* [21] that should be mentioned here, as they could be of great importance for ligand-exchanging chromatographic systems. The first observation is that ligand exchange takes place faster in acetonitrile than in nitrobenzene. The latter solvent proves unable to coordinate in the axial positions of the copper ion coordination sphere. Acetonitrile and probably other small ligands facilitate the ligand exchange. For this reason, aqueous mobile phases may be best suited for LEC, but the addition of alcohols, acetonitrile or other polar modifiers may prove useful in enhancing efficiency in chiral LEC.

The second observation is that conformationally flexible 1,2-propanediamine ligands participate in ligand-exchange processes at higher rates than the more ridged aminomethylpyrrolidine ligands. Obviously, ridged chiral selectors, although producing higher thermodynamic enantioselectivity, are less favorable from the point of view of kinetics and column efficiency. This statement corresponds to the observation that CMPs usually produce higher efficiency and lower enantioselectivity than similar CSP systems. This may result from the fact that binding the chiral selector to a polymeric network or silica surface causes significant steric constraints and reduces considerably the flexibility of the ligand.

2.5. Factors determining the peak dispersion in chiral LEC

One of rare systematic examinations of column efficiencies in chiral LEC was reported by Rizzi [18]. Two chiral stationary phases and one chiral mobile phase were tested (Fig. 2):

(i) CSP I: aminopropylsilica (7-μm particles) acylated by L-proline according to Lindner [22], column dimensions 125 mm × 4 mm I.D.

(ii) CSP II: chloropropylsilica (5-μm particles) aminated with L-hydroxyproline according to Unger and co-workers [23,24], column dimensions 125 mm × 4 mm I.D.

(iii) A CMP system consisting of an RP-2 or RP-8 column (125 mm × 4 mm I.D.) and a copper–L-proline-containing aqueous mobile phase.

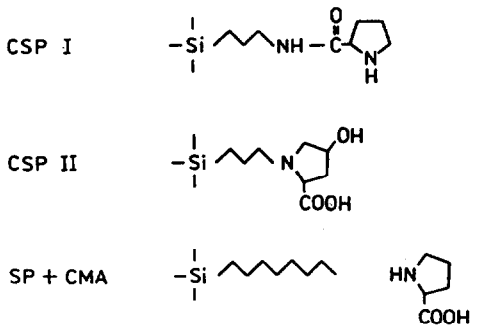


Fig. 2. Structures of the chiral stationary phases CSP I and II and the chiral mobile phase system. From ref. 18.

Various free and dansylated amino acids were resolved and reduced plate heights were calculated for each enantiomeric peak.

An important result was the dependence of the plate height on the flow-rate of the mobile phase for both the CSP's examined (Fig. 3). Extremely low flow-rates of 0.3 ml/min or less were required to produce an acceptable efficiency of the column with respect to bifunctional amino acid ligands. Histidine, which tends to act as a tridentate ligand, showed the highest peak dispersion. In contrast, a monodentate ligand, benzoic acid, displayed much sharper peaks and a lower peak shape dependence on the flow-rate.

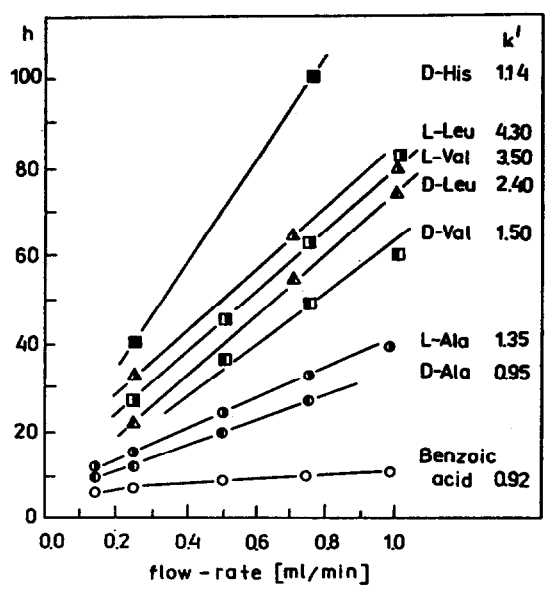


Fig. 3. Influence of flow-rate on the reduced plate height in the system CSP II with bonded L-Hyp. Solutes, free amino acids and benzoic acid. Mobile phase, aqueous solution of 4×10^{-4} *M* copper(II) acetate and 5×10^{-2} *M* ammonium acetate (pH 5.5); temperature, 30°C. From ref. 18.

For both bonded phases, an unexpected definite correlation of the plate height with the solute retention, k', was observed to exist (Fig. 4), although a strict dependence of peak dispersion on k' values is neither implied by theory nor found generally in HPLC experiments. Obviously, in a series of homologous amino acids, the free binding energy to the central copper ion (which determines the solute retention) could be positively correlated with the free energy of activation of complex dissociation (which determines the kinetics of ligand exchange and the efficiency of chromatography). One can expect, therefore, that under conditions of slow ligand

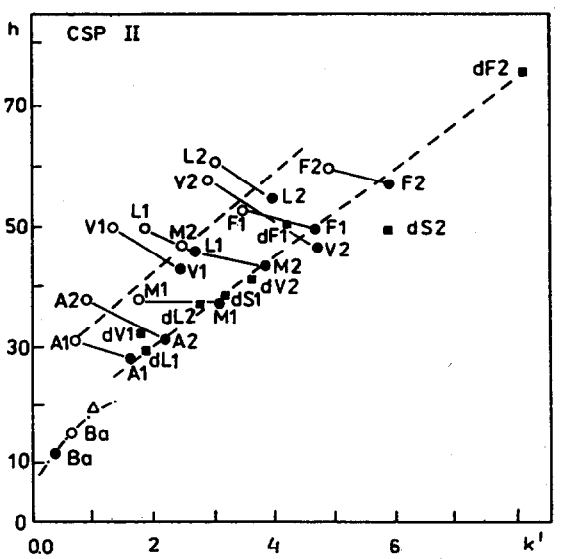


Fig. 4. Plate height *vs.* capacity factor correlation: influence of analyte structure and ethanol concentration in the mobile phase. Stationary phase, CSP II; mobile phase, aqueous solution of 4×10^{-4} *M* copper acetate and 5×10^{-2} *M* ammonium acetate (pH 5.5) with various concentrations of ethanol; temperature, 30°C; flow-rate, 1 ml/min. Solutes: circles, free amino acids; squares, dansylamino acids; open symbols, no ethanol present; full symbols, 20% (v/v) ethanol present. Analyte symbols: A = alanine; V = valine; L = leucine; S = serine; M = methionine; F = phenylalanine; W = tryptophan; T = threonine; Ba = benzoic acid; A1 = first-eluted enantiomer of alanine; A2 = second-eluted enantiomer of alanine, etc.; dA = dansylalanine; dV = dansylvaline, etc. Triangle: data point obtained for Dns-Phe in a phase system without copper ions. From ref. 18.

exchange, h will appear to correlate with k' values. As shown in Fig. 4, larger k' and h values are characteristic of bulky amino acids. Their mixed-ligand sorption complexes seem to be additionally stabilized by dispersion interactions of the amino acid side-groups with the organic microenvironment of the CSP. However, these interactions, just like the enhanced dentation number of the ligand, simultaneously create additional steric constrains and slow the process of the ligand exchange.

Thus, of many parameters examined, the following factors were found to influence in particular the efficiency of bonded chiral LEC phases: flow-rate of the mobile phase and structure of the analyte ligand (dentation number, steric shape, bulkiness and rigidity of the molecule, configuration). Compared with these factors, the pH of the aqueous–organic eluent and the type and content of its organic modifier seem mainly to influence the retention of the solutes.

According to Rizzi [18], in addition to many other observations, LEC systems using CMPs generally have better efficiencies, comparable to those in conventional reversed-phase chromatography. As shown in Fig. 5, sharp peaks and a low dependence of the peak width on the flow-rate is characteristic of dansylamino acids resolved with the bis(L-prolinato)copper-containing mobile phase system. Ligand exchange in the bulk solution obviously proceeds at higher rates, at least in the systems considered.

Whereas in the case of bonded CSPs and CMP systems the matrix-fixed chiral ligands are located at the surface of a macroporous mineral material and, therefore, are easily accessible to analyte molecules, with polymeric packing materials the diffusion of the analyte in the sorbent phase may acquire a dominant role in peak dispersion. This is the case with polymeric resins of low swelling ability. Here, the kinetics of the intra-particle diffusion and not the kinetics of ligand exchange may mainly deteriorate the column performance. Thus, no noticeable band resolution of DL-phenylalanine was reported on the Cu(II) form of a polystyrene-type resin incorporating L-proline ligands [25,26], whereas an earlier publication [27] claimed that the discriminating ability of a similar resin towards

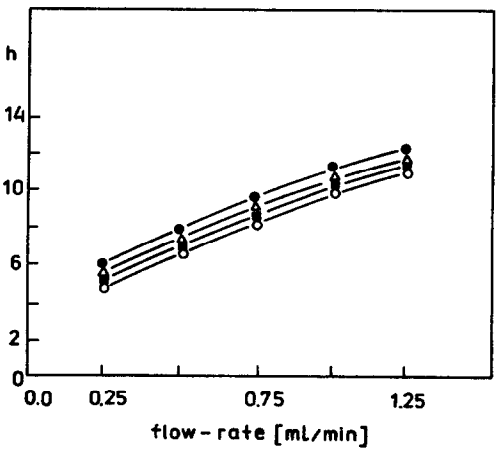


Fig. 5. Influence of flow-rate on the reduced plate height in the CMP system: RP-2 column and aqueous solution [5×10^{-3} M L-proline, 2.5×10^{-3} M copper sulphate and 1×10^{-3} M ammonium acetate (pH 7)]–acetonitrile (75:25 v/v); temperature, 30°C. Solutes, dansylamino acids and trifluoroanthrylethanol (TFAE) [mobile phase for TFAE, 10^{-3} M ammonium acetate–acetonitrile (60:40)]. ● = Dns-D-Trp ($k' = 2.0$); ○ = Dns-L-Trp ($k' = 3.0$); △ = Dns-L-Phe ($k' = 1.8$); ■ = TFAE ($k' = 3.9$). From ref. 18.

this racemate was sufficiently high ($\alpha = 1.63$). This discrepancy may result from the difference in the performances of the two resins considered: the first contained 1% of divinylbenzene and 1.53 mmol of L-proline per gram of dry resin and was swollen with water to only 60% [26,28]; the second resin had an enhanced macronet isoporous structure with a degree of cross-linking of 11%, an exchange capacity of 2.78 mmol g^{-1} and a swelling ability of 170% [27].

2.6. Improving column efficiency in chiral LEC

A general approach to enhancing the efficiency of LEC can be inferred from the assumption that ligand exchange may represent the slowest step in establishing interphase equilibrium in the column. If we also assume that the activation energy for ligand exchange is higher than that for diffusion and conventional adsorption–desorption processes, then we might expect that increasing the column temperature would enhance the ligand exchange rate to a greater extent than the rates of other processes. This idea was successfully exploited by Davankov and Kurganov [29], who examined systematically the

influence of column temperature and flow-rates on the reduced plate heights of a whole series of chiral bonded ligand-exchanging columns. Two test solutes were taken, L-proline and acetone, to compare the role of complex formation of the former with that of conventional phase distribution of the latter. The retention times of the solutes did not differ much, but the corresponding plate heights were found to differ dramatically. Fortunately, the column efficiency with respect to the ligand-exchanging sorbate improved considerably on running the columns at elevated temperatures. A representative two-dimensional response of the reduced plate height with varying temperature and decreased flow-rates is shown in Fig. 6. While many manufacturers suggest using chiral LEC columns at 40–50°C, Fig. 6 shows a further significant gain in the efficiency on increasing the temperature to 70–75°C.

Unfortunately, most chiral bonded LEC phases, like any other silica-based bonded phases, are not hydrolytically stable enough in aqueous and aqueous–organic media and lose their performance fairly rapidly at elevated temperatures.

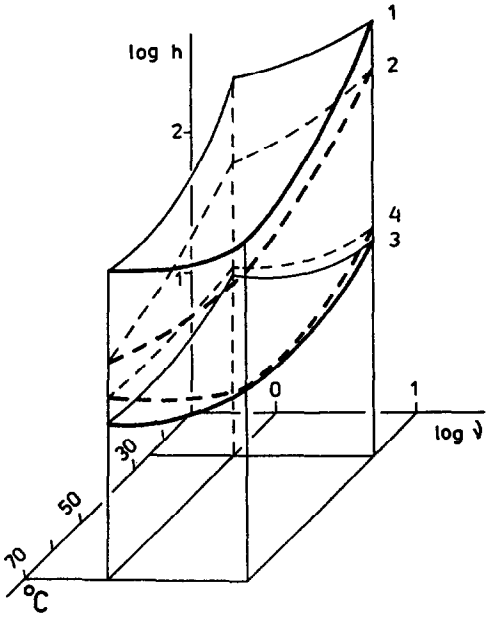


Fig. 6. Reduced plate heights as a function of column temperature and reduced flow-rates on chromatography of (1,2) L-proline and (3,4) acetone on two chiral bonded LEC phases prepared from Silasorb Si 600 by binding L-hydroxyproline ligands (2,4) via propylene C_3-spacer or (1,3) via bonded polystyrene chains.

To enhance considerably the durability of HPLC bonded phases, Kurganov *et al.* [30] in 1983 introduced polymeric bonded phases. They induced a low-molecular-mass copolymer of styrene with methylvinyldiethoxysilane to react with the surface silanol groups of a macroporous silica. The reaction conditions were selected such a way that the initial macromolecules were first adsorbed on the surface and then bonded to it via –O–Si–C–links. As each polymeric chain contained about ten ethoxysilane groups, a multi-point attachment of the chain was ensured. Additional intermolecular cross-linking of the polystyrene chains resulted in the formation of a monomolecular polymeric layer that could not be hydrolytically removed from the surface. By a standard chloromethylation of the bonded polystyrene followed by treatment with chiral amino acids, the authors arrived at the desired durable chiral LEC phases of the following structure:

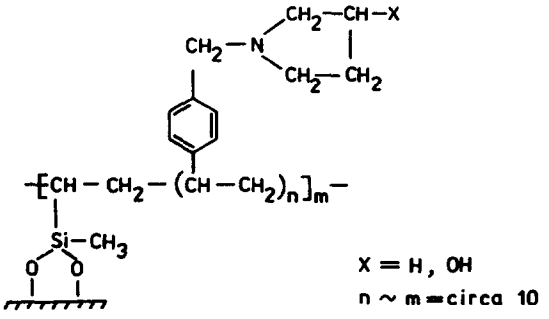


Fig. 7 represents an acceptably efficient chromatogram of a mixture of eight racemic amino acids that was obtained at a column temperature of 75°C [31]. Owing to the cross-linked polymeric nature of the bonded phase, the column could be used at that high temperature for a period of more than 1 month without losing its resolving power and efficiency (it is obvious that high-temperature LEC cannot be recommended for thermally unstable solutes).

In addition to the enhanced column temperature, a second suggestion could be made to improve the efficiency of CSPs in LEC, namely, introducing flexible spacers between the polymeric or mineral matrix of the column packing

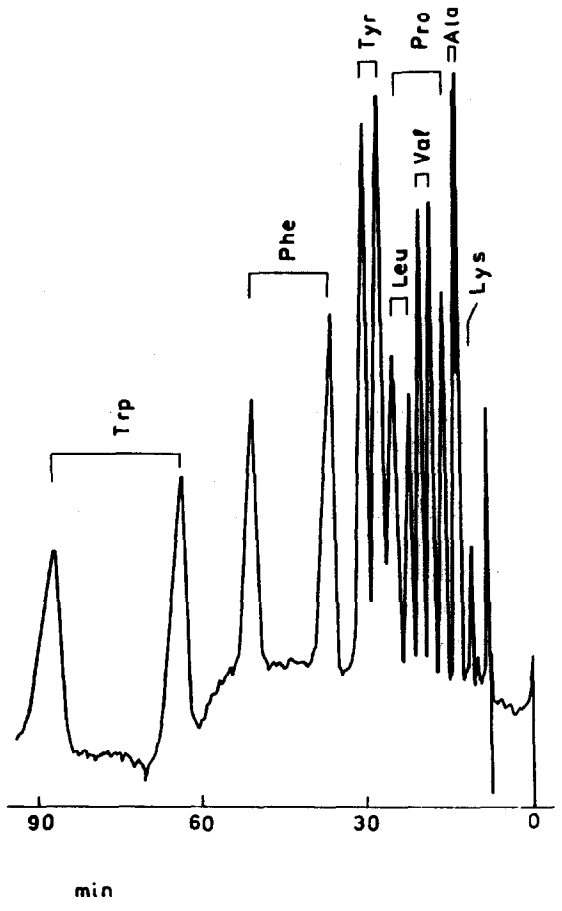


Fig. 7. Resolution of a mixture of eight amino acids on a polystyrene-bonded phase containing residues of L-hydroxyproline. Column, 250 mm × 4 mm I.D., d_p 5 μm; eluent, 5×10^{-4} *M* copper acetate and 10^{-2} *M* ammoniumn acetate (pH 4.5)–acetonitrile (70:30); flow-rate, 0.7 ml/min.; temperature, 75°C. From ref. 31.

and its complexing chiral selector L, which would minimize steric constraints for the ligand-exchange reactions. (In an aqueous eluent, alkylene-type spacers are not really flexible. Therefore, on increasing the length of the spacer to an L-Hyp-type selector from C_1 to C_3 and C_8, mainly an increase in hydrophobicity, rather than an improvement in efficiency of the sorbent, has been recorded [32].)

Many of the above considerations on the kinetic enantioselectivity phenomena and efficiency of enantiomeric resolution should be valid for all kinds of chiral chromatographic systems, not the ligand-exchanging mode alone. However, the efficiency of other types of chiral stationary and mobile phases still remains to be examined in more detail.

3. ENANTIOSELECTIVITY OF CHIRAL LIGAND-EXCHANGING CHROMATOGRAPHIC SYSTEMS

3.1. Enantoselectivity of complex formation in ligand-exchanging chromatographic systems

The only process that is responsible for the chiral recognition of two enantiomners of the analyte, A_R and A_S, is their participation in complex formation with the transition metal ion M and the chiral selector of the system, ligand L. The two labile ternary complexes formed, A_RML and A_SML, are diastereomeric and, therefore, may differ in their thermodynamic stability constants:

$$\beta_{A_RML} = \frac{[A_RML]}{[A_R][M][L]} \quad \text{and} \quad \beta_{A_SML} = \frac{[A_SML]}{[A_S][M][L]}$$

The ratio of these two constants, $\alpha^* = \beta_{A_RML}/\beta_{A_SML}$, is the thermodynamic enantioselectivity of the complex-formation process.

In chromatographic systems, this process takes place either (i) in the stationary phase alone when chiral ligands are chemically bonded to the sorbent matrix (CSP) or permanently adsorbed on it or (ii) in both the mobile and stationary phases when the chiral selector, ML_2, is added to the mobile phase (CMP) and partitions between the phases. In neither of the above situations is the enantioselectivity of the chromatographic system, which is the ratio of its capacity factors with respect to the enantiomers A_R and A_S, $\alpha = k'_R/k'_S$, equal to the enantioselectivity of the complex formation. Relationships between the α^* and α values have been considered [33] for M = Cu.

(i) In a CSP system, the solute A migrates from the mobile phase into stationary one where it partially forms ternary sorption complexes:

Mobile phase A^m

$\downarrow\uparrow$

Stationary phase $A^s + CuL^s \overset{K^s_{ACuL}}{\rightleftharpoons} ACuL^s$

where the superscripts m and s denote the location of the species in the mobile and stationary phase, respectively.

The capacity factor of the analyte depends on both adsorption and complexation of the latter:

$$k'_A = \phi \cdot \frac{[A^s] + [ACuL^s]}{[A^m]} \quad (7)$$

where ϕ is the phase ratio. By using the complex formation constant, K^s_{ACuL}, this expression can be rewritten as follows:

$$k'_A = \phi \cdot \frac{[A^s] + K^s_{ACuL}[A^s][CuL^s]}{[A^m]}$$

$$= \phi \cdot \frac{[A^s]}{[A^m]}(1 + K^s_{ACuL}[CuL^s])$$

$$= k''_A(1 + K^s_{ACuL}[CuL^s]) \quad (8)$$

The last equation is valid for all ligand-exchanging stationary phases. It demonstrates that compared with the analyte retention in the absence of complexation, k''_A, an increasing concentration of ligand exchanging sorption sites, $[CuL^s]$, and an increasing sorption power of the latter, K^s_{ACuL}, enhance the solute retention considerably.

The enantioselectivity of the column is given by

$$\alpha = \frac{k'_{A_R}}{k'_{A_S}} = \frac{k''_{A_R}}{k''_{A_S}} \cdot \frac{1 + K^s_{A_RCuL}[CuL^s]}{1 + K^s_{A_SCuL}[CuL^s]}$$

$$= \frac{1 + K^s_{A_RCuL}[CuL^s]}{1 + K^s_{A_SCuL}[CuL^s]} \quad (9)$$

since, in the absence of complexation reactions with the selector CuL^s, the retentions of both enantiomers are identical: $k''_{A_R} = k''_{A_S}$.

Eqns. 7–9 imply that enantioselectivity of the column with a ligand-exchanging CSP can only approach the enantioselectivity of the solute association with the chiral complexing sorption site if the non-selective affinity of the solute to the stationary phase remains negligible, *i.e.*, when $[A^s] \ll [ACuL^s]$ and $k'' \approx 0$. Generally, the enantioselectivity of the column appears to be lower than that of the chiral selector of the stationary phase:

$$\alpha < K^s_{A_RCuL}/K^s_{A_SCuL}, \quad \alpha < \alpha^*$$

Similar conclusions should be valid for all chiral stationary phases [34].

(ii) in a CMP system, the solute A adsorbs on the stationary phase as such as well as in the form of the ternary complex:

$$\begin{array}{lcccc} \text{Mobile phase} & A^m + CuL^m & \overset{K^m_{ACuL}}{\rightleftharpoons} & ACuL^m \\ & \downarrow\uparrow \quad \downarrow\uparrow & & \downarrow\uparrow \\ \text{Stationary phase} & A^s + CuL^s & \overset{K^s_{ACuL}}{\rightleftharpoons} & ACuL^s \end{array}$$

The capacity factor depends in a complicated manner on the phase partition coefficients of all the components and the stability constants of ternary complexes in the mobile and stationary phases:

$$k'_A = \phi \cdot \frac{[A^s] + [ACuL^s]}{[A^m] + [ACuL^m]}$$

$$= \phi \cdot \frac{[A^s] + K^s_{ACuL}[A^s][CuL^s]}{[A^m] + K^m_{ACuL}[A^m][CuL^m]}$$

$$= k''_A \cdot \frac{1 + K^s_{ACuL}[CuL^s]}{1 + K^m_{ACuL}[CuL^m]}$$

We note that a stronger complexation of the solute A in the mobile phase diminishes the retention of that solute in the chromatographic column. In contrast, stronger complexation in the stationary phase results in an increase in its k' value. It must be emphasized here that, because of the differing microenvironments, the formation constants of the ternary complex in the two phases are different. In addition, in most instances, the phase partition coefficient of the chiral selector varies with the varying concentration of the latter in the eluent. All these factors make any prediction of the retention of the solutes as a function of chromatographic parameters uncertain.

The same is valid for the enantioselectivity of the CMP chromatographic system. It can be easily shown that selectivity appears to be a complex function of the phase distribution of the chiral selector and the enantioselectivity of the latter in the mobile and stationary phases:

$$\alpha = \frac{k'_{A_R}}{k'_{A_S}} = \frac{k''_{A_R}}{k''_{A_S}}$$

$$\cdot \frac{(1 + K^{s}_{A_R CuB}[CuB^{s}])(1 + K^{m}_{A_S CuB}[CuB^{m}])}{(1 + K^{m}_{A_R CuB}[CuB^{m}])(1 + K^{s}_{A_S CuB}[CuB^{s}])}$$

If the formation constants of ternary complexes are sufficiently high, the enantioselectivity of the chromatographic system, α, is roughly given by the ratio of the complexation enantioselectivities in the stationary and mobile phases:

$$\alpha \approx \frac{K^{s}_{A_R CuB}}{K^{s}_{A_S CuB}} \Big/ \frac{K^{m}_{A_R CuB}}{K^{m}_{A_S CuB}} = \alpha^{*}_{s}/\alpha^{*}_{m}$$

Thus, the selector–analyte interaction selectivities in the mobile and stationary phases exert opposite effects on the total enantiomeric resolution in the chromatographic column. Therefore, it is due only to the occasional inequivalence of contributions of complexation reactions in the mobile and stationary phases to the retention of two enantiomers in the column that the chiral mobile phase system can function at all. It will be shown later that the complexation enantioselectivity in the stationary phase generally predominates over that in the bulk solution but, on the whole, the situation with the resolution and even elution order of the enantiomers in CMP systems appears to be unpredictable. (In capillary electrophoresis, where the theoretical treatment is simplified by the absence of phase-distribution phenomena, enantiomeric resolution exhibits a maximum at a certain optimum selector concentration in solution [35,36].)

It should also be noted that the most selective systems should be chiral chromatographic systems where one enantiomer binds to the immobilized chiral selector in the stationary phase, whereas the other enantiomer predominantly associates with the chiral mobile phase additive. With a certain probability this situation can be realized by combining in a push–pull mode a chiral bonded stationary phase with a chiral mobile phase, where two enantiomers of the chiral selector are involved, one for the CSP and the other for the CMP. First attempts at designing combined chiral chromatographic systems have already been reported [37–41]. Similarly, combinations of appropriate completely different chiral selectors [42] in the stationary and mobile phases, which would satisfy the above requirement of binding opposite enantiomers, are feasible, although not yet reported.

3.2. Three-point interaction model as a basis for evaluating retention, selectivity and efficiency in LEC

One of most important and productive stereochemical postulates, that of "three-point interaction", was formulated fairly early to account for the chiral recognition ability of biological receptors [43], enantiospecificity of enzymes [44] and chiral discrimination of racemic solutes on cellulose-type adsorbents [45]. It states that in order to recognize two enantiomers, the chiral selector has to enter a stereodependent three-point interaction with one of the enantiomers. In this case the corresponding appropriate interaction sites of the other enantiomer would appear in an incorrect orientation with respect to the selector. The latter would only be able to realize two-point interactions, thus giving a less stable diastereomeric associate. This three-point interaction requirement unavoidably results from geometric consideration of mutual chiral recognition of three-dimensional structures and has been best illustrated by Meyer and Rais [46], as shown in Fig. 8.

From Fig. 8, one can further infer that three combinations of two-point interactions are always available for the chiral selector with any of the two appropriate enantiomers. That pair, of the three possible, which produces the strongest binding will obviously predominate. If the chiral selector resides in the stationary phase, the above strongest pair of interactions will determine the retention of the more weakly bonded enantiomer in the column. The third, weakest interaction will be realized for the more strongly bonded enantiomer only, thus determining the surplus retention of the latter and, consequently, the resolution selectivity of the column.

In practice, when resolving two enantiomers,

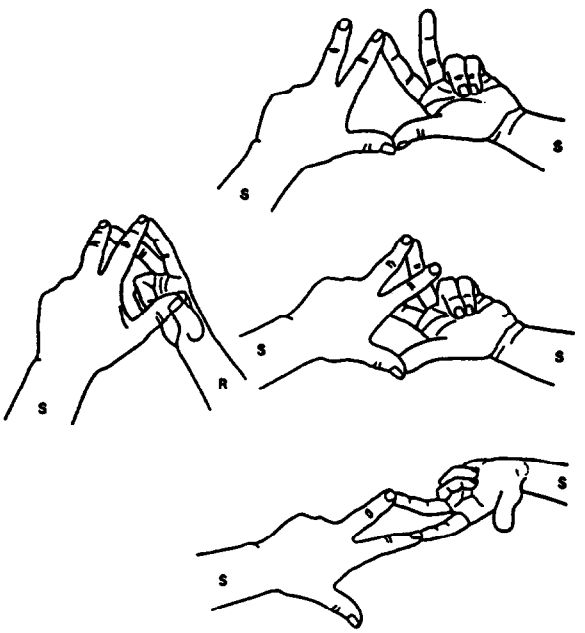


Fig. 8. Three active interaction sites of an *S*-chiral selector, the left hand, find three complementary interaction sites in the *R* structure of the selectand, but two sites, only, in the *S*-structure. From ref. 46.

one is looking for resolution, not retention, of the solutes. In an ideal situation, one would prefer to see the shortest possible retention of the first enantiomer and the largest possible retention of the second enantiomer. This implies that the ideal selector should produce the lowest possible binding energy due to the two strongest interaction points and the highest possible binding energy due to the third, weakest interaction. Obviously, this requirement would be met in the situation when all three interactions happen to be similar in their intensity. Then, expressed in energetic units, enantioselectivity may approach half of the value of binding of the more weakly retained enantiomer. [From the above point of view, the two main interaction sites in the LEC of amino acids, *i.e.*, the coordination bonds between the Cu(II) ion and amino and carboxylate groups, are probably exceedingly strong when compared with the small energy of steric interaction between α-substituents, which determines the selectivity.)

These considerations on selectivity and binding relationships recall the concepts of Lehmann [47,48], who described positive linear correlations between eudismic indexes (enantiomeric affinity ratios of several substrates towards biological receptors) and the total substrate–receptor affinity energies.

In the modern interpretation, the three interaction lines between the chiral selector and enantiomers considered do not need to be attractions, as some of them may be repulsive in nature. Only the resulting sum for one, favoured diastereomeric combination should produce binding, *i.e.*, formation of an associate, whereas the other associate does not need to form at all. With such a combination of attractive and repulsive interactions, the ratio between maximum selectivity and minimum binding energy may exceed the value of 1:2.

Finally, it should be added that the above three-point interaction rule only establishes the minimum number of interactions required for the chiral recognition. In fact, steric complementarity of the selector and enantiomers may produce much more contact points within the associate. Such a situation is probably realized with chiral cavities in a solid body, which are accessible for one enantiomer of the template used but not for the other.

When considering the structures of two diastereomeric associates formed by the chiral selector with the solute enantiomers, such as is depicted in Fig. 1, one can easily imagine how difficult it is for the interacting components to realize the required two- and three-point bindings. The molecules either have to try many collisions before the interaction is established, or the required bonds have to be formed one by one. In both instances, these processes must be time consuming.

The same should be valid for the dissociation of the adducts. Here again, a stepwise replacement of the interaction partner by solvent molecules is required, which is probably the case with flexible species, or an energy shock is needed, which should be more characteristic of conformationally rigid structures. (According to Rizzi [18], what really matters for kinetics is the free energy of activation of complex dissociation and not the binding energy of the complex. However, considering a series of homologous

compounds, these two values appear to be correlated.)

Obviously, the association–dissociation cycle would take more time for the more stable diastereomer, where three interaction sites are involved. Therefore, the above-discussed observation by Rizzi that the longer retained enantiomer generates less plate counts in a CSP column, can be considered as an experimental corroboration of the three-point interaction model.

The model further predicts that there must be two alternative ways to go in chiral chromatography: either one wants to achieve high selectivity and should be prepared to meet the problems of strong retention and relatively low efficiency, or one chooses high efficiency at the expense of selectivity and retention. One cannot have both high efficiency and high selectivity at the same time. The first of these two options would be the preferred way for preparative-scale separations and the second for analytical resolutions. We also may note that strong binding and high selectivity of a given selector can be achieved in combination with only a few selectands, whereas a low selectivity and low affinity could be a fairly universal feature.

3.3. Enantioselectivity induced by achiral structures

Of general importance for chiral separations is the observation, first made in ligand-exchanging systems, that achiral components of stationary or mobile phases may contribute substantially to chiral recognition of the enantiomers by being involved in the formation of diastereomeric selector–selectand complexes or by entering some interactions with the latter which stabilize diastereomers to different extents [17,49]. Thus, water molecules, when coordinated in axial positions of copper(II) ions, may mediate steric interactions between certain groups of the selector and enantiomer, if these groups are not large enough to interact immediately. This situation is obvious in Fig. 1, where the coordinated water molecule diminishes the stability of the L–L associate.

Much more abundant in chromatography should be the interaction of selector–selectand associates with the achiral matrix or surface of the sorbent. If fragments of both selector and selectand are involved simultaneously in the interaction with the matrix, the total interaction energy should be different for the two associates, as the latter are diastereomeric. It is important that the adsorption energy stabilizes the two associates to different extents in the stationary phase, thus contributing to the chiral recognition ability of the whole system. This contribution may even be decisive [49–51] for systems where no enantioselectivity can be expected to exist in the bulk solution. Thus, enantiomeric components involved in only a two-point interaction, with the chiral selector can be effectively discriminated owing to a cooperation of the achiral surface, as shown in Fig. 9.

Here, the achiral surface mediates the third required interaction site between the selector

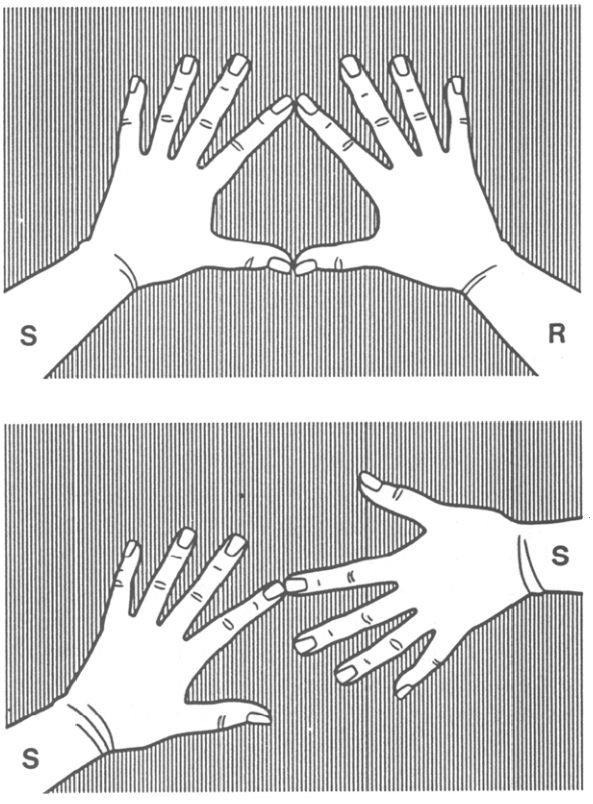


Fig. 9. With selector and selectand adsorbed on a surface, two points of interaction are sufficient for chiral recognition. From ref. 17.

and enantiomers to be recognized. When in the adsorbed state, the *S* selector finds two complementary active sites in the adsorbed *R* selectand, but only one site in the *S* isomer, which, therefore, will be retained more weakly. This type of chiral discrimination operates efficiently on reversed-phase packings modified by permanent adsorption of a hydrophobic chiral selector, *e.g.*, N-decyl-L-hydroxyproline [50].

Suppose now that the two-point interactions within the diastereomeric associates in the mobile phase are stronger than the energy of adsorption of these associates to the stationary phase. This is the case in the chromatography of copper complexes of relatively hydrophilic bifunctional amino acids on a reversed-phase column. Here, enantioselectivity will manifest itself during the realization of the weakest interaction in the system, which is the adsorption. In other words, the relatively stable diastereomeric two-point associates should exhibit different retention factors. This is exactly the reason for the interesting observation of Belov *et al.* [52] that in a copper(II)-containing eluent L-proline elutes with a smaller k' value from a reversed-phase column than does DL-proline, the difference amounting to up to about 20%. As the exchange of ligands in the bis(prolinato)copper is fast, the single elution peaks are sharp for both of the above solutes, chiral and racemic proline. Therefore, Belov *et al.* suggested that it should be possible to determine the enantiomeric composition of an unknown mixture of L- and D-proline, which should elute with a k' value between the extreme values characteristic of the optically pure and racemic prolines. It is remarkable that under the same conditions of chromatography, valine elutes with higher k' values and an inverted relationship, *i.e.*, L-valine with longer retention times than DL-valine.

4. RECENT ACHIEVEMENTS IN CHIRAL LIGAND-EXCHANGE CHROMATOGRAPHY

As mentioned in the introduction, several review papers have described practical achievements and development trends in the field of chiral separations using various LEC systems. Referring to our most detailed reviews [4,8], which cover publications up to 1989, we concentrate here on more recent information only.

4.1. Use of chiral stationary phases

The LEC technique has been generally shown to be especially effective for the enantiomeric resolution of amino acids, numerous amino acids derivatives and hydroxy acids. Thus, the commercially available Chiral ProCu, Chiral ValCu (Serva, Heidelberg, Germany) and Nucleosil Chiral-1 (Macherey–Nagel, Düren, Germany) are all suitable for the resolution of fluoro-substituted aromatic amino acids [53], α-trifluoromethyl-α-amino acids [54] and mono-, di- and trifluoro derivatives of alanine [55]. α-Amino-β-hydroxy acids, threonine and phenylserine, contain two asymmetric atoms and resolve into four components, in particular, on a Chiral ProCu column [56]. As the asymmetric β-carbon atom in these compounds appears in the side-chain and does not participate in the formation of the five-membered chelate ring on binding a Cu(II) ion through the amino and carboxy functions of the amino acid, its configuration exerts a much weaker (although still sufficient) influence on the resolution of the four components than does the configuration of the asymmetric α-carbon atom. Introduction of a fluorine atom into the *o*- or *p*-position of the remote phenyl ring of phenylserine has little effect on the relative retention of the isomers.

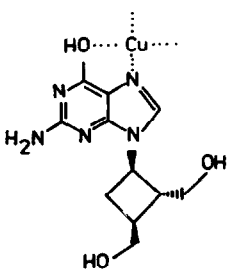
In general, the efficiency and ease of the direct resolution of amino acids, without any prior derivatization of the latter, make LEC a technique of choice for serial determinations of enantiomeric compositions, *e.g.*, in fossil dating [57]. Depending on the rate of racemization of the amino acid under investigation, the dating can be performed in the time range from several hundred years ago back to one million years.

As shown above with threonine and phenylserine, asymmetric atoms outside the five-membered chelate ring of the copper–amino acid chelate still bring about a safe enantiomeric resolution in LEC. This allowed Gübitz *et al.* [58] to suggest a general approach to resolving amino alcohols after derivatization of their amino function with bromoacetic acid, which

converts the latter into glycine. The achiral glycine coordinates to copper, whereas the asymmetric atom in the N-substituent provides for the enantiodifferentiation. A whole range of important adrenergic drugs and β-blocking agents having a β-amino alcohol structure with secondary amino groups were resolved in this manner with α-values up to 2.6 (sunephrine). Phosphate buffer (pH 4.5) containing 0.1 mmol Cu(II) and 20% methanol was found to be the most suitable mobile phase for these separations. Operating the column at 50°C improved its efficiency.

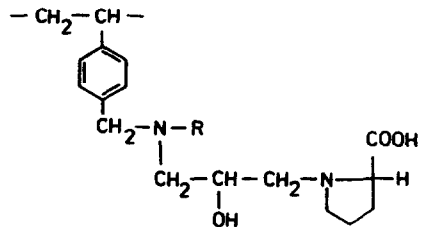
Similarly, glycine acylated with 2-phenylpropionic acid was resolved using a Ciralpak WH ligand-exchange column (Daicel, Tokyo, Japan) in aqueous 0.3 m*M* $CuSO_4$ [59]. This finding was used in examining enantioselective metabolism pathways of 2-arylpropionic acids in dogs.

The first use of chiral ligand-exchange HPLC for the resolution of enantiomeric nucleosides was presented recently [60]. In contrast to many other types of chiral HPLC columns examined, only Nucleosil Chiral-1 column resolved enantiomers of an HIV anti-infective nucleoside consisting of a guanine nucleus attached to a pseudosugar of the following structure:



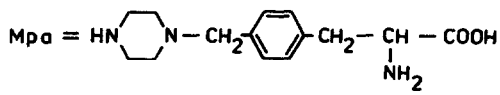
The silica-bonded L-hydroxyproline ligand of the packing is assumed to form a mixed-ligand Cu(II) complex with guanine oxygen and nitrogen atoms, as shown above. Micropreparative separations of the ^3H-labelled preparation were also performed. The copper ions were then efficiently removed from the fractions of interest by using a gravity silica column, with a blue band forming at the top of the column.

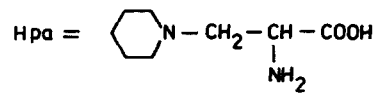
In addition to the above silica-bonded chiral ligand exchangers, materials based on organic polymers are still popular in LEC. In addition to a series of cross-linked polystyrene resins incorporating various amino acids as chiral selectors in LEC packings (for reviews, see refs. 4 and 8), new polymers were synthesized [61] with L-proline (or L-hydroxyproline) bound through a longer spacer of the structure:



R = **1** CH_3 **5** C_2H_4OH
2 C_2H_5 **6** i-C_3H_7
3 C_3H_7 **7** cyclo-C_6H_{11}
4 C_4H_9

Polymers **3**, **6** and **7** in the Cu(II) form were found to resolve racemic His, Pro, Val, Thr, Tyr, Mpa and Hpa, where





Usually D-forms of the amino acids eluted ahead of the L-forms (except for Pro). This elution sequence corresponds to a general prediction [4] for all L-Pro-type stationary ligands having in the N-substituent an additional functional (particularly hydroxy) group which is capable of coordinating in the axial position of the central Cu(II) ion.

A new chiral ligand exchanger, TSK gel Enantio L1 (Tosoh, Tokyo, Japan) was successfully used in determining the absolute configuration of proline and pipecolic acid in the cyclotetrapeptide antibiotics trapoxin A and B [62], and the configuration of Asp, Pro, Val and Phe in the lanthionine-containing peptide antibiotic duramycin [63]. Pantoic acid and calcium pantothenate were resolved on ligand-exchanging Mcl gel CRS 10W (Mitsubishi, Tokyo, Japan) in 2 m*M* $Cu(SO_4)$ solution modified with 10% acetonitrile

[64]. Any scientific evaluation of these results is complicated, as the exact structure of immobilized chiral ligands on many commercially available CSPs in not specified by the manufacturers.

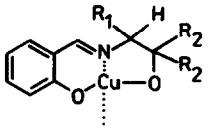
Finally, an interesting suggestion was made [65] of employing cross-linked polygalactomannan, guaran, with boron-complexed D-mannose units for the resolution of mandelic acid. Here, two *cis*-hydroxy groups of the saccharide participate in the formation of ternary sorption complexes with boron atoms and hydroxy and carboxy groups of mandelic acid. The enantioselectivity of the column was observed to be very high. This appears to be the first example of the use of boron as a complexing ion in chiral LEC.

4.2. Use of chiral coated phases

In 1980, Davankov *et al.* [50] reported the direct enantiomeric separation of amino acids by LEC using reversed-phase ODS columns coated with N-*n*-alkyl-L-hydroxyproline as a CSP and acetate buffer containing copper(II) ions. Here the chiral selector resided permanently on the surface of the packing due to strong hydrophobic interactions between the N-alkyl substitutent of the selector and silica-bonded alkyl groups, whereas the hydrophilic part of the selector remained exposed at the interphase layer to the aqueous eluent and solutes molecules. C_{10}-L-Hyp-coated reversed-phase columns are now available from Regis (Morton Grove, IL, USA). Yamazaki *et al.* [66] demonstrated the usefulness of a similar C_{12}-L-Hyp-modified ODS column by resolving successfully racemic norephedrine and thirteen of its analogous. All the amino alcohols having various substituents in the aromatic ring and in the amino group were resolved, provided that the pH of the eluent was increased to 6.0 and the concentration of copper was kept as high as 5 m*M*, which reflects the lower complexing ability of the amino alcohols compared with that of amino acids. Owing to the high efficiency of the technique, baseline resolution was observed for all the aromatic amino alcohols examined.

Similar efficiency and selectivity are exhibited by chiral coatings prepared with N^{α}-*n*-decyl-L-histidine [67] and N^{τ}-*n*-decyl-L-histidine [68].

Oi and co-workers [69,70] introduced new types of chiral ligands that can be adsorbed permanently on reversed-phase materials. These are Schiff bases of chiral amino alcohols, N-salicylidene-(*R*)-amino-1,1-bis(2-butoxy-5-*tert.*-butylphenyl)-3-phenyl-1-propanol and N-salicylidene-(*R*)-2-amino-1,1-bis(5-*tert.*-butyl-2-octyloxyphenyl)-1-propanol, with the following structures:

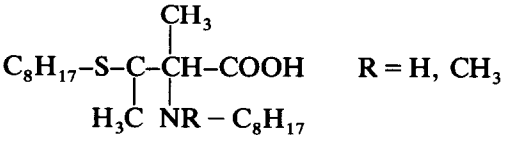


R_1 = benzyl
R_2 = 2-butoxy-5-*tert.*-butylphenyl

R_1 = methyl
R_2 = 5-*tert.*-butyl-2-octyloxyphenyl

The coating was accomplished by passing 0.05% tetrahydrofuran–water (50:50) solutions of the above selectors through the columns followed by a 1 m*M* aqueous solution of copper acetate. The packings obtained, particularly the first one, were found to resolve efficiently racemic amino acids, hydroxy acids, amines and important amino alcohols, some of which are listed in Table 1 [70].

The same group [71] employed N,S-dioctyl-D-penicillamine and N,S-dioctyl-N-methyl-D-penicillamine:



R = H, CH_3

as chiral coatings on ODS packings to obtain chiral columns capable of resolving more than twenty amino acids, N-acetylamino acids, racemic glycyldi- and -tripeptides, 3-amino-ε-caprolactam and a series of amino alcohols (which required a higher pH of the eluent of about 6). With the exception of histidine, D-isomers of amino acids were observed to be retained longer on the columns prepared with D-penicillamine. However, introduction of a methyl substituent into the amino group of the selector changed the elution order of the en-

TABLE 1

ENANTIOMERIC SEPARATIONS ON AN RP-HPLC COLUMN COATED WITH A CHIRAL SCHIFF BASE [70]

Mobile phases: (A) 1 m*M* $CuSO_4$ in water; (B) 2 m*M* $CuSO_4$ in water–acetonitrile (85:15); (C) 2 m*M* $CuSO_4$ in water–acetonitrile (80:20). A flow-rate of 1 ml/min was typically used for the 150 × 4.6 mm I.D. column at room temperature.

Compound	k'_1	k'_2	α	Mobile phase
Amino alcohols				
Norphenylephrine	2.90	3.57	1.23	A
Normethanephrine	6.25	7.19	1.15	A
Norephedrine	3.85	4.27	1.11	A
p-Hydroxynorephedrine	1.56	1.76	1.13	A
Atenolol	5.98	6.40	1.07	A
Propranolol	9.85	10.44	1.06	B
Phenylalaniol	5.43	11.07	2.04	A
Amines				
1-Phenyl-(*p*-tolyl)ethylamine	17.60	28.51	1.62	C
α-Amino-ε-caprolactam	0.70	1.34	1.91	A
Homocysteine thiolactone	6.03	7.19	1.19	A
Ketamine	1.47	1.85	1.26	B
Amino acids				
Serine	0.21(D)	0.25(L)	1.19	A
Allothreonine	0.32(D)	0.55(L)	1.72	A
Proline	0.69(L)	0.84(D)	1.22	A
Valine	1.40(L)	1.81(D)	1.29	A
Methionine	3.04(D)	3.95(L)	1.30	A
Allo-isoleucine	3.19(L)	3.82(D)	1.20	A
Histidine	3.82(D)	4.51(L)	1.18	A
tert.-Leucine	5.45(L)	7.30(D)	1.34	A
Leucine	5.66(L)	6.17(D)	1.09	A
Aspartic acid	5.87(L)	6.52(D)	1.11	A
Isoleucine	6.39(L)	7.34(D)	1.15	A
Phenylalanine	2.18(D)	3.79(L)	1.74	B
Tryptophan	3.21(D)	6.58(L)	2.05	B
3-Aminobutyric acid	1.91	2.29	1.20	A
3-Amino-2-methylpropionic acid	2.87	3.09	1.08	A
Hydroxy acids				
Lactic acid	17.36	27.06	1.56	B
Glyceric acid	13.61	15.40	1.13	B
2-Hydroxybutyric acid[a]	41.86	91.59	2.19	B
3-Hydroxybutyric acid[b]	53.53	62.07	1.16	B

[a] Column 10 × 4 mm I.D.
[b] Column 50 × 4.6 mm I.D.

antiomers in many instances. Durability of the chiral system was demonstrated by repeating an amino acid analysis 600 times with water or water–methanol (70:30, v/v) as the eluent, the retention parameters and enantioselectivity of the column remaining unchanged.

Fukuhara and Yuasa [72] saturated an RP-18 column with a cyanocobalamin, Co_α-[α-(5,6-dimethylbenzimidazolyl)] - Co_β - cyanocobalamide, by pumping about 3 l of its 0.1 m*M* solution in 50 m*M* sodium acetate buffer (pH 4.5) containing 0.1 m*M* Cu(II). The ligand exchanger obtained resolved most amino acids.

Thus, the initial idea [50] of preparing chiral LEC packings by simple adsorption of hydrophobic chiral ligands on reversed-phase columns has

developed successfully by involving new chiral selectors and new classes of racemic selectands. It is also worth mentioning the great success of using the same idea in the field of chiral thin-layer chromatography [11, 73–81].

4.3. Use of chiral mobile phases

Also productive proved to be the idea of using achiral column packing materials in combination with eluents doped with chiral metal complexes. Bis(L-prolinato)copper is one of most popular mobile phase additives [82,83]. It was used [84] at a concentration of 4 m*M* at pH 5.5 with a μBondapak C_{18} column (300 × 7.8 mm I.D., $d_p = 10$ μm) to resolve, in amounts sufficient for testing biological activity (100–500-mg scale), racemates of the ornithine analogues α-methylornithine, α-fluoromethylornithine, α-difluoromethylornithine, α-fluoromethyldehydroornithine and α-chlorofluoromethylornithine (which contains two asymmetric atoms). Copper ions were then precipitated from the effluent fractions of interest with Na_2S and the ornithine derivatives were finally separated from the excess of the eluent-derived L-Pro on Dowex AG 50W-X8 cation-exchange resin. In the chiral chromatographic process, retention and enantioselectivity were found to decrease with increase in the ionic strength of the eluent and the temperature of the column. In contrast, increasing pH and concentration of the chiral additive in the mobile phase caused a clear increase in retention and a less marked increase in enantiomeric resolution. These regularities are common in the chiral eluent LEC technique. Interestingly, ornithine itself was not resolved, whereas all α-substituted analogues were well resolved with α values steadily increasing with icreasing size of the substituent, in the order CH_3, CH_2F, CHF_2, CH_2Cl. Other substitutents such as CH_2OH, CH_2OCH_3, $CH{=}CH_2$ and $C{\equiv}CH$ also provided good resolution. Chlorofluoromethylornithine, which contains two asymmetric atoms, was separated into four peaks although the separation factor between the β-stereoisomers was fairly small. Similarly well resolved were α-substituted analogues of dehydroornithine and lysine. It is of interest that the methyl ester of fluoromethyldehydroornithine could not be resolved, which indicates the importance of the carboxy group of the amino acid for the process of formation of a ternary complex with copper and L-proline.

Another popular mobile phase additive is the copper(II) complex of L- or D-phenylalanine. The latter served recently in the identification and configuration assignment of several natural pterins in protozoans [85]. Copper(II) complexes with L-phenylalaninamide, L-valinamide and L-prolinamide as chiral selectors in the CMP-mode resolution of racemic dansylamino acids have been the subject of detailed investigations [86,87]. Interestingly, for a series of DNS-amino acids these selectors show higher enantioselectivity than that obtained with the corresponding non-derivatized amino acid–copper complexes. Chromatographic parameters were optimized to arrive at an analytical procedure sensitive enough to permit determinations at the picomole level of the compounds injected. Using Cu–L-$PheNH_2$, it was shown that some D-amino acids, such as D-Ala and D-Glu, are surprisingly present not only in harshly treated foods, such as roasted coffee, but also in fermentation products, such as cheese and yoghurt [88].

A more hydrophobic chiral selector, L-prolineoctylamide, in combination with Ni(II) ions was used to determine *p*-hydroxyphenylphenylhydantoin enantiomers in biological fluids during an examination of the stereospecificity of the metabolism of the antiepileptic drug phenytoin, which is a prochiral compound [89,90]. Methanol–water (50:50) at pH 9 was found to be the optimum eluent, containing 0.375–1.5 m*M* L-Pro-NHC_8H_{17}, 1.5 m*M* $Ni(OAc)_2$ and 0.1 m*M* NH_4OAc. A temperature of 35°C was selected as the optimum, as heating was found to enhance the efficiency of the column at the expense of the selectivity of separation.

A combination of a 1.5 m*M* copper bis(L-tartrate) solution, pH 4.8, with a TSK gel IC-Anion-PW anion-exchange column (Tosoh), was suggested [91,92] for the selective determination of malic acid enantiomers in adulterated fruit juice. In the photometric ion chromatograms obtained, L-malic acid eluted before the D-isomer and the D-isomer gave a negative peak.

Nevertheless, linear calibration graphs were obtained in the range 3–100 nmol.

Copper(II) complexes of the nucleotides adenosine diphosphate, β-nicotinamide adenine dinucleotide and, in particular, flavine adenine dinucleotide were shown [72] to provide the resolution of many amino acid racemates on reversed-phase columns at pH 4.5 with sufficient selectivity.

When evaluating the advantages and drawbacks of the chiral mobile phase LEC technique [7], we emphasized the fact that "the two enantiomers separated in the column enter the detector cell in the form of ternary complexes. These complexes are diastereomeric and can significantly differ in molar absorptivities". A first case of enantioselective fluorescence quenching of dansylamino acids by a chiral Cu(II) complex of L-phenylalanylamide in aqueous solutions was reported recently [51], showing the necessity for using two calibration graphs for the determination of the enantiomers.

5. CONCLUSIONS

Twenty-five years have passed since first complete enantiomeric resolutions were observed in liquid chromatography. The achievements of the LEC technique were possible at that time because of the extremely high enantioselectivity of formation of ternary complexes by chiral multidentate ligands which assemble around a transition metal cation. Compared with the many other selector–selectand associates successfully employed nowadays in chiral chromatographic separations, diastereomeric ternary complexes are relatively stable. This property was beneficial in that a large difference, *i.e.*, enantioselectivity, is only feasible between two large values, which are free energies of formation of two diastereomeric adducts. In addition, many physico-chemical techniques could be applied to investigate the structural peculiarities of the relatively stable ternary complexes, which gave unique and versatile generally important information on the mechanism of chiral recognition of enantiomers by a chiral selector. In this respect, only chiral charge-transfer complexation chromatography, due to the efforts of Pirkle and his group, can be compared with LEC in terms of the depth of understanding of the intimate interactions between a chiral selector and its complementary enantiomers.

On the other hand, the relatively high stability of sorption complexes leads to the disadvantage of slow ligand exchange and reduced plate count of chiral LEC columns. This again provides the unique opportunity to examine in more detail the role of the association kinetics in the peak broadening in a chromatographic column. This information also should be valuable for the further development of other chiral and achiral chromatographic systems.

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