ELSEVIER

Journal of Chromatography A, 906 (2001) 51–71

JOURNAL OF
CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Review

Chiral chromatographic separations based on ligand exchange

Alexander Kurganov'

Institute of Inorganic Chemistry and Analytical Chemistry, J. Gutenberg University, VFE, Obere Zahlbacher Str. 63, 55101 Mainz, Germany

Abstract

Taking ligand-exchange chromatographic systems as an example, the effect of the stoichiometry of the solute–chiral selector interaction on the efficiency, selectivity and solute peak profile is discussed. Recent achievements and practical applications of chiral ligand-exchange chromatography are also briefly reviewed. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Ligand exchange; Secondary equilibria; Band profile; Chiral ligand-exchange chromatography

Contents

1. Introduction 51
2. Theoretical background of chiral ligand-exchange chromatography 52
   2.1. Secondary equilibria as fundamentals of chiral separations 52
      2.1.1. Secondary equilibria in chiral ligand-exchange chromatographic systems 53
   2.2. Dimerization type of secondary equilibria in chromatographic systems 55
   2.3. Effect of secondary equilibria of the dimerization type on the selectivity of enantiomer separations in CLEC 58
3. Recent achievements in chiral ligand-exchange chromatography 62
4. Conclusions 70
References 70

1. Introduction

Chiral ligand-exchange chromatography (CLEC), invented by Davankov in the early seventies [1], was the first liquid chromatographic technique successfully applied for the complete and reliable separation of optical isomers of amino acids and other solutes which are able to form coordination compounds with metal ions. Impressive results of the first separations gave rise to intensive investigations in the field and numerous publications appeared in the literature, which have been reviewed [2–11]. The investigations were concentrated on the fundamental principles of CLEC [2–5], synthesis of stationary phases [7,8,12], application of CLEC in chiral separations [5,8–10] and particular attention was paid to fundamentals of chiral recognition [13–15]. The scope of CLEC was extended to the complexes formed by polydentate ligands and to outer-sphere complexation [3].

Meanwhile, chromatographic techniques utilizing other mechanisms of chiral recognition were developed and applied for the resolution of optical isomers, e.g. charge-transfer stationary phases by

0021-9673/01/$ – see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(00)00502-1

Pirkle [16], cyclodextrin stationary phases by Armstrong [17], protein-containing stationary phases [18], polysaccharide stationary phases by Okamoto [19], etc. Many of these techniques are more simple experimentally than CLEC. They use simpler eluents and can be applied to a broad range of analytes. The practical importance of CLEC was significantly reduced with the establishment of these techniques. Nevertheless, CLEC remains the best investigated technique from the theoretical point of view. Many theoretical concepts developed in CLEC are of general interest for the explanation and prediction of chiral recognition in all chiral chromatographic systems.

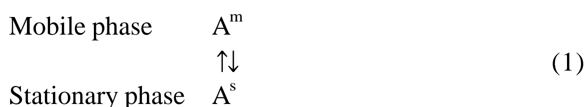
This review concentrates on the new theoretical problems of CLEC and on the more recent practical usage of the technique in LC covering the literature during the last 5–6 years. The earlier publications discussed in the reviews cited above will be considered only when necessary for clear presentation of the material.

2. Theoretical background of chiral ligand-exchange chromatography

2.1. Secondary equilibria as fundamentals of chiral separations

Enantiomers, as is well known, have identical physico-chemical properties until placed in an environment which is itself chiral. Therefore, they must have equal retention on interaction with any achiral stationary phase and cannot be separated in achiral chromatographic systems. To create a chiral environment, one has to add an additional chiral component to the chromatographic system, the so-called chiral selector. The chiral selector may be present in the mobile phase (chiral mobile-phase mode, CMP) or in the stationary phase (chiral stationary-phase mode, CSP). However, in the general case, the chiral selector is present in both phases and chiral recognition occurs simultaneously in the mobile and stationary phases. When considering the theoretical models we will always assume this general case because the corresponding relationships for the CMP and CSP modes can easily be derived from the general model.

Chromatography commonly deals with rather simple types of adsorption–desorption processes:



Mobile phase A^m
$\uparrow\downarrow$ (1)
Stationary phase A^s

As early as 1943, DeVault [20] derived a relationship between the dynamic properties of the chromatographic system, namely the retention volume of the solute V_R, and the fundamental thermodynamic characteristics of the system:

$$V_R = V_M(1 + \phi k_A) \quad (2)$$

where $\phi = V_S/V_M$ is the phase ratio, V_M and V_S are the volume of mobile and stationary phase in the column, respectively, and k_A is the Henry's adsorption constant of solute A. Eq. (2) assumes that the solute concentration in the stationary phase A^s is related linearly to the solute concentration in the mobile phase A^m, i.e. $A^s = k_A A^m$. Dividing the left- and right-hand sides of Eq. (2) by the flow-rate of the mobile phase in the column, one obtains Eq. (2) in a form more common to HPLC:

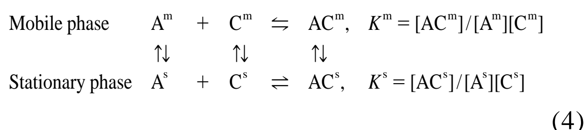
$$t_R = t_0(1 + \phi k_A) \quad (3)$$

or written in terms of the solute retention factor k'_A:

$$k'_A = \phi k_A \quad (3a)$$

where t_R and t_0 are the retention times of the retained and unretained solutes, respectively.

Eqs. (3) and (3a) are widely used in liquid chromatography, but they cannot be applied directly to chiral chromatographic systems. In chiral chromatographic systems, adsorption equilibrium (1) is always accompanied by the interaction of the solute A with the chiral selector C:



Mobile phase $A^m + C^m \leftrightharpoons AC^m$, $K^m = [AC^m]/[A^m][C^m]$
$\uparrow\downarrow \quad \uparrow\downarrow \quad \uparrow\downarrow$
Stationary phase $A^s + C^s \rightleftharpoons AC^s$, $K^s = [AC^s]/[A^s][C^s]$

(4)

Eqs. (3) and (3a) do not account for any secondary equilibria and have to be modified to describe correctly the result of chromatographic separation in chiral chromatography. Unfortunately, the general solution for the system presented by equilibria (4) is rather complex and has not been given yet.

Significant simplification of the system is achieved

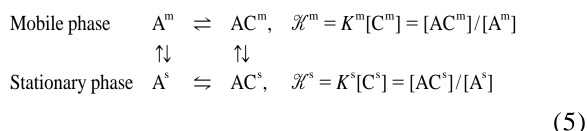
when one, for practical purposes, assumes that the concentration of the chiral selector C always remains constant during the chromatographic run. Instead of real equilibrium constants describing the equilibria in the mobile and stationary phases, one can apply, under these circumstances, their apparent counterparts, which are the products of the real constant and the corresponding concentration of the chiral selector:

$$\mathcal{K}^{m} = K^{m}[C^{m}] = [AC^{m}]/[A^{m}] \quad (4a)$$

and

$$\mathcal{K}^{s} = K^{s}[C^{s}] = [AC^{s}]/[A^{s}] \quad (4b)$$

The apparent equilibrium constants (4a) and (4b) describe more simple types of equilibria than given by equilibria (4), namely:



$$\begin{array}{llll} \text{Mobile phase} & A^{m} & \rightleftharpoons & AC^{m}, \quad \mathcal{K}^{m} = K^{m}[C^{m}] = [AC^{m}]/[A^{m}] \\ & \upharpoonleft\downharpoonright & & \upharpoonleft\downharpoonright \\ \text{Stationary phase} & A^{s} & \leftrightharpoons & AC^{s}, \quad \mathcal{K}^{s} = K^{s}[C^{s}] = [AC^{s}]/[A^{s}] \end{array} \quad (5)$$

This is an isomerization type of secondary equilibria. The basic relationship for this type of chromatographic system was derived by Martin [21] as early as 1949:

$$t_{R} = t_{0}\left(1 + \phi\frac{k_{A} + k_{AC}\mathcal{K}^{m}}{1 + \mathcal{K}^{m}}\right) = t_{0}(1 + \phi k_{A}^{*}) \quad (6)$$

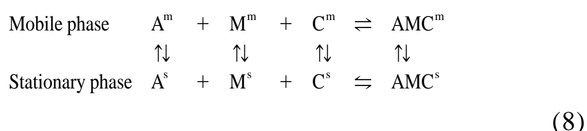
or in a form written in terms of the apparent retention factor k'^{*}:

$$k_{A}'^{*} = \phi k_{A}^{*} \quad (7)$$

where k_{A}^{*} is the apparent Henry's constant, and k_{A} and k_{AC} are the real Henry's constants of the solute A and its adduct with the chiral selector AC. Eq. (7) is similar to the basic Eq. (3a) providing the additional equilibrium of the solute–chiral selector interaction does not cause any changes in the system description when the apparent Henry's constant is used instead of its real counterparts. The deduction of Eqs. (6) and (7) is repeated many times in the literature for both chiral [22,29] and non-chiral [23] separations, adapting the equation to the needs of particular investigations, i.e. determination of the equilibrium constants K, investigation into the effect of selector concentration on solute retention, etc.

2.1.1. Secondary equilibria in chiral ligand-exchange chromatographic systems

In chiral ligand-exchange chromatography, in contrast to the other chiral separation techniques, the interaction between the chiral selector and the enantiomers does not occur in direct contact. The interaction is mediated by a metal ion which coordinates simultaneously to the chiral selector and the enantiomers to be separated with the formation of ternary mixed-ligand complexes. The corresponding equilibria are commonly presented in the form [2]:



$$\begin{array}{llllllll} \text{Mobile phase} & A^{m} & + & M^{m} & + & C^{m} & \rightleftharpoons & AMC^{m} \\ & \upharpoonleft\downharpoonright & & \upharpoonleft\downharpoonright & & \upharpoonleft\downharpoonright & & \upharpoonleft\downharpoonright \\ \text{Stationary phase} & A^{s} & + & M^{s} & + & C^{s} & \leftrightharpoons & AMC^{s} \end{array} \quad (8)$$

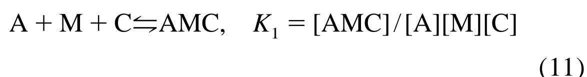
Equilibria (8) reveal the formation of ternary mixed-ligand complexes between the chiral selector and the enantiomers to be separated as a mandatory step in chiral recognition. When the concentrations of the metal ion and the chiral selector remain constant, Eq. (6) can be applied to CLEC with the only difference being that the apparent equilibrium constants are now given by the relationships:

$$\mathcal{K}^{m} = K^{m}[M^{m}][C^{m}] \quad (9)$$

and

$$\mathcal{K}^{s} = K^{s}[M^{s}][C^{s}] \quad (10)$$

However, equilibria (8) alone cannot correctly represent the participants of chiral recognition in the chromatographic column. For typical solutes in CLEC, namely amines and amino acids, the complexation with transition metals has been investigated in depth [2,25,26,46,47]. Considering complexation of these solutes with bivalent metal ions (Cu^{2+} is most frequently used in CLEC), the following equilibria result in the formation of a mixed-ligand ternary complex (charges at the metal atom and ligands are omitted, all constants relate to the mobile phase):



$$A + M + C \leftrightharpoons AMC, \quad K_{1} = [AMC]/[A][M][C] \quad (11)$$

$$AM + C \leftrightharpoons AMC, \quad K_{2} = [AMC]/[AM][C] \quad (12)$$

$$\mathrm{A} + \mathrm{CM} \leftrightarrows \mathrm{AMC}, \quad K_3 = [\mathrm{AMC}]/[\mathrm{A}][\mathrm{CM}] \tag{13}$$

$$\mathrm{AM} + \mathrm{CM} \leftrightarrows \mathrm{AMC} + \mathrm{M}, \quad K_4 = [\mathrm{AMC}][\mathrm{M}]/[\mathrm{AM}][\mathrm{CM}] \tag{14}$$

$$\mathrm{AM} + \mathrm{C_2M} \leftrightarrows \mathrm{AMC} + \mathrm{CM}, \quad K_5 = [\mathrm{AMC}][\mathrm{CM}]/[\mathrm{AM}][\mathrm{C_2M}] \tag{15}$$

$$\mathrm{A_2M} + \mathrm{CM} \leftrightarrows \mathrm{AMC} + \mathrm{AM}, \quad K_6 = [\mathrm{AMC}][\mathrm{AM}]/[\mathrm{A_2M}][\mathrm{CM}] \tag{16}$$

$$\mathrm{A_2M} + \mathrm{C_2M} \leftrightarrows 2\mathrm{AMC}, \quad K_7 = [\mathrm{AMC}]^2/[\mathrm{A_2M}][\mathrm{C_2M}] \tag{17}$$

These equilibria do not account for the formation of protonated and hydroxo complexes and it was assumed that the stability of the complexes formed by the chiral selector and the enantiomers are approximately equal. When the stabilities of the complexes are markedly different, additional equilibria have to be taken into account, e.g. a displacement of the chiral selector by the solute $\mathrm{A} + \mathrm{C_2M} \leftrightarrows \mathrm{AMC} + \mathrm{C}$ as reported by Galaverna et al. [27], etc. Accounting for these equilibria may be important in practical work. In theoretical modeling, however, such systems are too specific and have not yet been elaborated to a significant extent. Again, the theoretical model discussed below assumes that the chiral selector and the solute are added to the system in the form of their complexes, i.e. $\mathrm{C_2M}$ and $\mathrm{A_2M}$, and not as free ligands. Free metal ions and free solute and chiral selector are present in the chromatographic column due to the dissociation of the corresponding complexes only. This condition is not very common for CLEC, where at least the solute is commonly added as a free ligand. However, the requirement allows us to reduce the number of equilibria which have to be considered in the modeling system.

Nevertheless, the situation in CLEC appears to be rather complex even with the approximations given above. The numerous equilibria still contribute to the formation of the ternary mixed-ligand complexes and may affect the result of separations. A further simplification can be achieved when the distribution of complexes is considered depending on the pH of the eluent. The complex distribution in the mobile phase is exemplified in Fig. 1 for the system containing copper (2+) ions and two α-amino acids, namely valine and serine. One of the amino acids can be considered as a solute and the other as a chiral selector. The concentration distribution of the complexes formed was calculated based on the stability constants reported in the literature [24,25]. It can be seen that the concentrations of the free solute and the free chiral selector remain negligible over the whole pH range applied in HPLC. The solute and the chiral selector exist in the mobile phase as protonated and/or complexed species. Therefore, Eqs. (11)–(13) do not contribute significantly to enantiomer separation. The equilibrium constant of equilibrium (14) was found to be close to the statistical values [24,25], i.e. $\log K_4 \sim 0 \pm 0.5$. This equilibrium may be important for the pH range where both the free metal ions and the mixed ternary complexes are present simultaneously at sufficient concentrations. In general, however, such a pH range does not exist. A significant amount of free metal ions is present in the chromatographic column at acidic pH below 4, but the concentration of the mixed-ligand ternary complexes is very low in this pH range. No separation can commonly be observed in CLEC with eluents having pH lower than 3.5 because no complex formation occurs. A remarkable amount of the mixed-ligand ternary complex is formed in the chromatographic column at pH>5. Under these

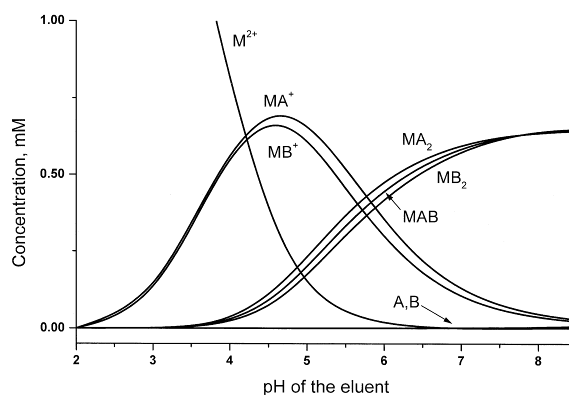


Fig. 1. Distribution of complexes formed in a solution containing (*S*)-valine (A, 2 m*M*), (*S*)-serine (B, 2 m*M*) and copper (2+) ions (4 m*M*) depending on pH. The distribution is calculated based on the stability constants of the complexes reported in Refs. [24,25].

conditions the processes described by equilibria (15)–(17) should dominate in the column. Taking into account that the concentrations of the chiral selector and the metal ions in CLEC are always much higher than the solute concentration, equilibria (15) and (16) involving the bis-complexes of the solute should be more important at higher pH than equilibrium (14) involving the mono complex of the solute. Equilibrium (14) should be more pronounced in the more acidic pH range.

Consideration of the complexation equilibria in CLEC is important to elucidate the dominating processes in the column depending on the chromatographic conditions. The concentrations of the chiral selector, the metal ions and the pH of the eluent are commonly kept constant during the chromatographic run. As a result, the concentrations of mono- and bis-complexes of the chiral selector also remain constant and equilibria (11)–(17) can be reduced to a more simple type when the corresponding apparent equilibrium constants are applied:

$$\text{Eq. (11)} \Rightarrow \mathcal{K}_1 = K_1[\text{M}][\text{C}], \quad \text{A} \leftrightarrows \text{AMC} \tag{18}$$

$$\text{Eq. (12)} \Rightarrow \mathcal{K}_2 = K_2[\text{C}], \quad \text{AM} \leftrightarrows \text{AMC} \tag{19}$$

$$\text{Eq. (13)} \Rightarrow \mathcal{K}_3 = K_3[\text{CM}], \quad \text{A} \leftrightarrows \text{AMC} \tag{20}$$

$$\text{Eq. (14)} \Rightarrow \mathcal{K}_4 = K_4[\text{CM}]/[\text{M}], \quad \text{AM} \leftrightarrows \text{AMC} \tag{21}$$

$$\text{Eq. (15)} \Rightarrow \mathcal{K}_5 = K_5[\text{C}_2\text{M}]/[\text{CM}], \quad \text{AM} \leftrightarrows \text{AMC} \tag{22}$$

$$\text{Eq. (16)} \Rightarrow \mathcal{K}_6 = K_6[\text{CM}], \quad \text{A}_2\text{M} \leftrightarrows \text{AMC} + \text{AM} \tag{23}$$

$$\text{Eq. (17)} \Rightarrow \mathcal{K}_7 = K_7[\text{C}_2\text{M}], \quad \text{A}_2\text{M} \leftrightarrows 2\text{AMC} \tag{24}$$

Equilibria (11)–(15) have been reduced to the conventional isomerization type. Therefore, Eqs. (6) and (7) can be used together with these equilibria to describe the retention of the solute. Equilibria (16) and (17) cannot be reduced to the isomerization type even when the concentrations of the chiral selector and the metal ions are kept constant. Equilibrium (24) is of the dimerization type, while equilibrium (23) is of an association type. The latter, however, can be treated mathematically as an equation of the dimerization type taking into account that an equal amount of the mixed ternary complex AMC and the mono-complex AM are always formed according to equilibrium (23).

Secondary equilibria of the dimerization type in chromatographic systems have been discussed recently [28]. The relationships derived were applied for the explanation of separations of non-racemic mixtures of enantiomers on achiral stationary phases [28,29] and the peak profiles of ionic analytes upon elution with non-buffered eluents [30]. The effect which the secondary equilibria of the dimerization type may have on the chromatographic behavior of the enantiomers in CLEC is more minutely considered in the following section.

2.2. Dimerization type of secondary equilibria in chromatographic systems

Eqs. (6) and (7) derived by Martin [21] for isomerization type of secondary equilibria are used extensively for chiral and other types of liquid chromatography, i.e ion-pairing chromatography, inclusion complex chromatography, etc. Chromatographers used to believe that, at least in analytical linear chromatography, the solute retention factor k' was always proportional to the product of the phase ratio ϕ multiplied by the real or apparent Henry's constant, which is the ratio of the total solute concentration in the stationary phase to those in the mobile phase. Recently, Ringo and Evans [31] generalized Eq. (6) for multiple interactions between solute A and chiral selector C:

$$p\text{A} + q\text{C} \leftrightarrows \text{A}_p\text{C}_q, \quad K = [\text{A}_p\text{C}_q]/[\text{A}]^p[\text{C}]^q \tag{21}$$

The general expression was derived by substituting the apparent equilibrium constant in Eq. (7) by the real equilibrium constant taken from Eq. (21):

$$\mathcal{K} = K[\text{C}]^q = [\text{A}_p\text{C}_q]/[\text{A}]^p$$

This results in the relationship:

$$\frac{1}{k^{*\prime}_{\text{A}}} = \frac{1 + pK[\text{C}]^q[\text{A}]^{p-1}}{k'_0 + k'_\infty[\text{C}]^q[\text{A}]^{p-1}} \tag{22}$$

where $k^{*\prime}$ is the measured apparent retention factor, and k'_0 and k'_∞ are the retention factors of the free solute A and its highest adduct with chiral selector

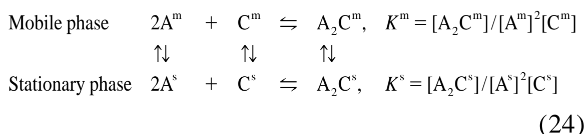
A_pC_q, respectively. Therefore, deriving Eq. (22) the authors have assumed that Eq. (7) is valid for any type of solute–selector interaction independent of the stoichiometry. However, Eq. (7) is valid only when a linear relationship exists between the total solute concentrations in the stationary and mobile phases.

When a single solute molecule interacts with q chiral selector molecules the linear relation between these concentrations is preserved because the equilibrium is reduced to the isomerization type when the chiral selector concentration is kept constant and the apparent equilibrium constant can be applied instead of the real counterpart:

$$A \leftrightharpoons AC_q, \quad \mathcal{K} = K[C]^q = [A]/[AC_q] \tag{23}$$

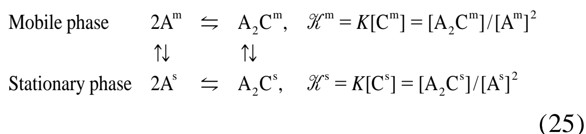
When more than one solute molecule interacts with q chiral selector molecules the linear relation between the total concentrations of the solute in the stationary and the mobile phases no longer holds. The relationship has a different form depending on the number of solute molecules involved in the interaction.

Let us consider the next most simple type of multiple solute–chiral selector interaction, namely an interaction of two solute molecules with one molecule of the chiral selector. The equilibria representing the processes in the chromatographic column are:



(24)

The concentration of the chiral selector in chiral separations commonly remains constant during the chromatographic run. Therefore, equilibria (24) can be reduced to secondary equilibria of the dimerization type applying the apparent equilibrium constant $\mathcal{K}$:



(25)

The total concentrations of solute A in the mobile and stationary phases are given by the equations:

$$A^m = [A^m] + 2[A_2C^m] = [A^m] + 2\mathcal{K}^m[A^m]^2 \tag{26}$$

$$A^s = [A^s] + 2[A_2C^s] \tag{27}$$

Considering the analytical range of concentrations, the Henry's isotherms are commonly valid for the adsorption of the individual species A and A_2C:

$$[A^s] = k_A[A^m] \tag{28}$$

$$[A_2C^s] = k_{A_2C}[A_2C^m] \tag{29}$$

and substituting Eqs. (28) and (29) into Eq. (27) one arrives at the relationship:

$$A^s = k_A[A^m] + 2k_{A_2C}\mathcal{K}^m[A^m]^2 \tag{30}$$

Solving Eq. (30) for $[A^m]$, taking into account that only the positive route has a physical meaning, and eliminating $[A^m]$ between Eqs. (26) and (30), one obtains the expression for the apparent adsorption isotherm of the solute involved in a secondary equilibrium of the dimerization type:

$$A^s = k_A \frac{-1 + \sqrt{1 + 8\mathcal{K}^m A^m}}{4k} + k_{A_2C} \frac{(-1 + \sqrt{1 + 8\mathcal{K}^m A^m})^2}{8k} \tag{31}$$

As can be seen from Eq. (31), the apparent adsorption isotherm is not linear in spite of the fact that the linear Henry's isotherms are assumed to be valid for the individual species. The chromatographic peak profile of the solute with a non-linear adsorption isotherm has to be calculated by means of non-linear chromatography, as is described in the literature on preparative separations [32].

The peak profile in non-linear chromatography is considered to consist of two parts (Figs. 2 and 3). The first part is the so-called 'shock' position where the solute concentration changes sharply from (or to) zero to (or from) the maximal value. The second part is the so-called 'diffuse boundary' where the concentration gradually changes from the maximal value to zero. The diffuse and shock parts of the peak in the system discussed are given by the following equations [28]: the diffuse part:

$$A^m = \frac{1}{8\mathcal{K}}\left[\left(\frac{t_A - t_{A_2C}}{t - t_{A_2C}}\right)^2 - 1\right] \tag{32}$$

the shock position:

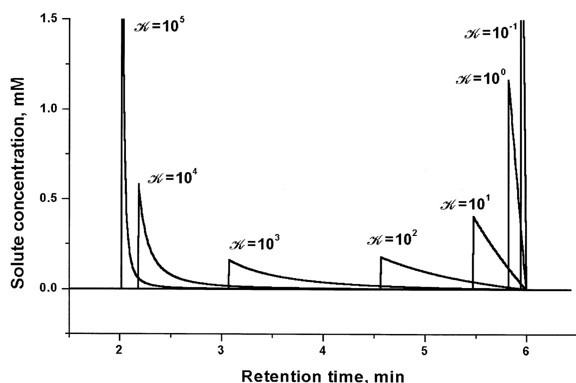


Fig. 2. Simulated peak profile of a solute involved in a secondary equilibrium of the dimerization type in a system where the free solute retains stronger than the adduct with the chiral selector. Parameters used in the simulation: $t_A = 6$ min; $t_{A_2C} = 2$ min; $F_V = 10^{-3}$ l min^{-1}; $q = 10^{-6}$ mol; K values (l mol^{-1}) are given on the corresponding curves.

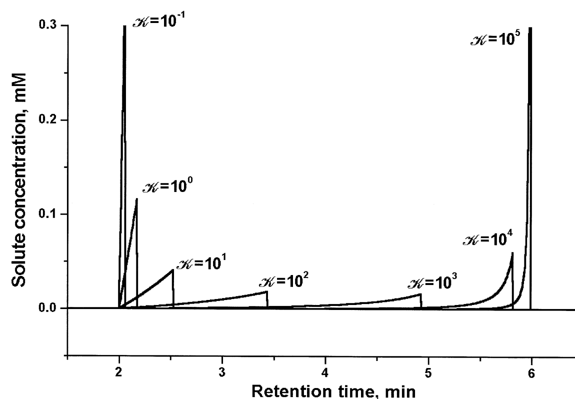


Fig. 3. Simulated peak profile of a solute involved in a secondary equilibrium of the dimerization type in a system where the free solute retains less than the adduct with the chiral selector. Parameters used in the simulation are the same as in Fig. 2 except $t_A = 2$ min; $t_{A_2C} = 6$ min.

$$t_{sh} = t_A - \frac{\mathscr{A}}{2} + \frac{\sqrt{\mathscr{A}^2 + 4\mathscr{A}(t_{A_2C} - t_A)}}{2} \quad \text{when } t_A < t_{A_2C} \tag{33}$$

$$t_{sh} = t_A + \frac{\mathscr{A}}{2} - \frac{\sqrt{\mathscr{A}^2 + 4\mathscr{A}(t_A - t_{A_2C})}}{2} \quad \text{when } t_A > t_{A_2C} \tag{34}$$

with:

$$\mathscr{A} = 8\mathscr{K}^m q / F_V \tag{35}$$

where t_A and t_{A_2C} are the retention times of solute A and its adduct with the chiral selector A_2C, respectively, in the absence of the secondary equilibrium; q is the sample amount (mol), and F_V is the volumetric flow-rate of the mobile phase (l min^{-1}).

The simulated chromatographic peak profiles are displayed in Figs. 2 and 3 for the two cases presented by Eqs. (33) and (34). It can be seen that the chromatographic peaks always stretch between two limiting values, namely, t_A and t_{A_2C}. Peak tailing is observed when the retention of solute A is larger than the retention of the adduct A_2C (Fig. 2). Peak fronting is observed when the retention of solute A is smaller than the retention of the adduct A_2C (Fig. 3). Increasing peak tailing (fronting) is observed in the chromatograms as $\mathscr{K}^m$ values increase. The heights of the peaks are correspondingly reduced as the peak area is maintained constant. At very high $\mathscr{K}^m$ values the peak height again increases, but a very long tail (front) is still present, although in a low concentration range. Quite different situations can, therefore, be expected depending on the ratio of the equilibrium concentrations of solute A and adduct A_2C. Little tailing (fronting) is present in the chromatogram when a large amount of the solute coexists with a small amount of the adduct (low $\mathscr{K}^m$ values). In contrast, intensive tailing (fronting) has to be expected when a large amount of the adduct is in equilibrium with a small amount of the solute (large $\mathscr{K}^m$ values).

The shock position in a chromatogram depends not only on the apparent equilibrium constant $\mathscr{K}^m$ as shown above, but also on the sample amount q (Eqs. (33)–(35)). The effect of q on the shock position is depicted in Fig. 4. With increasing sample the shock position shifts towards the retention time of the adduct A_2C, i.e. to higher retention times when $t_A < t_{A_2C}$ and to lower retention times when $t_A > t_{A_2C}$. Fig. 5 demonstrates the same dependence as Fig. 4, but for the peaks heights normalized to 1. It can be seen that the peak distortion progressively increases with an increase of the sample amount q. This dependence is similar to those known in preparative chromatography [32] where it results

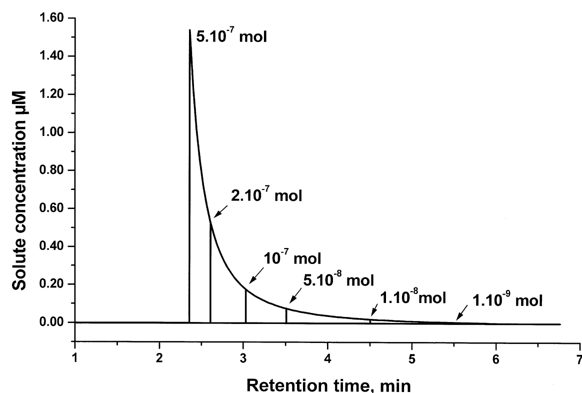


Fig. 4. Dependence of the peak profile of a solute involved in a secondary equilibrium of the dimerization type on the sample amount q. Parameters used in the simulation are the same as in Fig. 2 except $\mathcal{K} = 10^{-10}$ l mol^{-1}. The sample amount q (mol) is shown on the curves.

from column overloading. In chiral chromatographic systems subjected to secondary equilibria of the dimerization type the effect should be observed even in the analytical concentration range.

Summarizing the results of the theoretical consideration one has to conclude that secondary equilibria of the dimerization type differ strongly in appearance from secondary equilibria of the isomerization type. Secondary equilibria of the isomerization type do not disturb the solute peak profile as compared with the system without any secondary equilibrium. They affect the retention of the solute only. The changes caused by secondary equilibria of the dimerization type are more multivariate:

- the solute peak shape is distorted and both fronting and tailing can be observed depending on the relative solute/adduct retention;
- the peak shape (diffuse part of the peak) depends on the apparent equilibrium constant;
- solute retention (shock position) depends both on the sample amount and the apparent equilibrium constant.

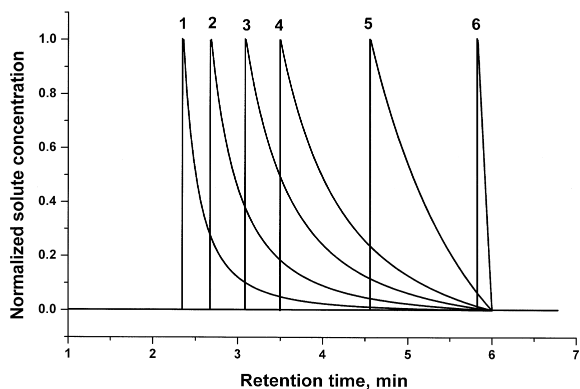


Fig. 5. Dependence of the peak profile of a solute involved in a secondary equilibrium of the dimerization type on the sample amount. All parameters are the same as in Fig. 4, but the heights of all peaks are normalized to 1. The sample amount is: (1) $5 \cdot 10^{-7}$ mol, (2) $2 \cdot 10^{-7}$ mol, (3) $1 \cdot 10^{-7}$ mol, (4) $5 \cdot 10^{-8}$ mol, (5) $1 \cdot 10^{-8}$ mol, (6) $1 \cdot 10^{-9}$ mol.

Taking into account the strong effect of a secondary equilibrium of the dimerization type on the solute peak profile and solute retention one can expect that they may affect the selectivity of separations and the elution order of enantiomers [33]. The characteristic features of this phenomenon are presented in the next section.

2.3. Effect of secondary equilibria of the dimerization type on the selectivity of enantiomer separations in CLEC

The selectivity of enantiomer separation is one of the most important parameters in chiral chromatography. Accounting for the very similar properties of the enantiomers, the development of highly selective stationary phases and chiral chromatographic systems is always a challenging task for chiral separations.

In the case of a secondary equilibrium of the isomerization type, which is most common in chiral chromatography, the selectivity can easily be derived from Eqs. (6) and (7) of Martin:

$$\alpha = \frac{k_R^{*\prime}}{k_S^{*\prime}} = \frac{k_R^*}{k_S^*} = \frac{k_R + k_{RC}\mathcal{K}_R}{1 + \mathcal{K}_R} \times \frac{1 + \mathcal{K}_S}{k_S + k_{SC}\mathcal{K}_S}$$
$$= \frac{k_R + k_{RC}K_R[\mathrm{C^m}]}{k_S + k_{SC}K_S[\mathrm{C^m}]} \times \frac{1 + K_S[\mathrm{C^m}]}{1 + K_R[\mathrm{C^m}]} \quad (36)$$

Eq. (36) was obtained in slightly different form by Davankov et al. [34] for the CLEC system and by Feibush et al. [35] for the CSP mode of chiral chromatography. According to Eq. (36) the selectivity in chiral chromatography is a function of the chiral selector concentration $[\mathrm{C^m}]$, the stability constants of the adducts formed with the chiral selector,

K_R and K_S, and the adsorption properties of the two diastereomeric adducts, k_{RS} and k_{SC}. The adsorption properties of the enantiomers to be separated are identical, i.e. $k_R = k_S$. As is common for linear chromatography the selectivity of enantiomer separation does not depend on the sample amount.

In the case of a secondary equilibrium of the dimerization type the selectivity of separations can be calculated from the shock positions of two enantiomers by means of Eqs. (33) and (34). The retentions of the S- and R-enantiomers when the chiral selector is absent are identical, i.e. $t_S = t_R$. The possible combinations of Eqs. (33) and (34) result in four equations for four different situations with respect to the retention of the solutes and the adducts [36]:

$$\alpha = \frac{t_{\text{sh}_R} - t_0}{t_{\text{sh}_S} - t_0} = \frac{2(t_R - t_0) - A_R + \sqrt{A_R^2 + 4A_R(t_{R_2\text{C}} - t_R)}}{2(t_S - t_0) - A_S + \sqrt{A_S^2 + 4A_S(t_{S_2\text{C}} - t_S)}}$$

when $t_R < t_{R_2\text{C}}, t_S < t_{S_2\text{C}}$ (37)

$$\alpha = \frac{t_{\text{sh}_R} - t_0}{t_{\text{sh}_S} - t_0} = \frac{2(t_R - t_0) + A_R - \sqrt{A_R^2 + 4A_R(t_R - t_{R_2\text{C}})}}{2(t_S - t_0) + A_S - \sqrt{A_S^2 + 4A_S(t_S - t_{S_2\text{C}})}}$$

when $t_R > t_{A_2R}, t_S > t_{S_2\text{C}}$ (38)

$$\alpha = \frac{t_{\text{sh}_R} - t_0}{t_{\text{sh}_S} - t_0} = \frac{2(t_R - t_0) - A_R + \sqrt{A_R^2 + 4A_R(t_{R_2\text{C}} - t_R)}}{2(t_S - t_0) + A_S - \sqrt{A_S^2 + 4A_S(t_S - t_{S_2\text{C}})}}$$

when $t_R < t_{R_2\text{C}}, t_S > t_{S_2\text{C}}$ (39)

$$\alpha = \frac{t_{\text{sh}_R} - t_0}{t_{\text{sh}_S} - t_0} = \frac{2(t_R - t_0) + A_R - \sqrt{A_R^2 + 4A_R(t_R - t_{R_2\text{C}})}}{2(t_S - t_0) - A_S + \sqrt{A_S^2 + 4A_S(t_{S_2\text{C}} - t_S)}}$$

when $t_R > t_{R_2\text{C}}, t_S < t_{S_2\text{C}}$ (40)

All expressions are too bulky and to elucidate enantiomer behavior in systems with a secondary equilibrium of dimerization type the dependencies presented by Eq. (37) are displayed graphically in Figs. 6–8.

Fig. 6 demonstrates the changes in the retention and selectivity of enantiomer separations caused by changes in the values of the apparent equilibrium constant $\mathcal{K}$. With increasing apparent equilibrium constant the retention of both enantiomers decreases, but to different extents. Under the conditions taken in the example the corresponding curves intersect each other at the point where $\mathcal{K} \sim 10^3$ l mol^{-1}. Therefore, the elution order of the enantiomers is inverted with increasing apparent equilibrium constant above this point.

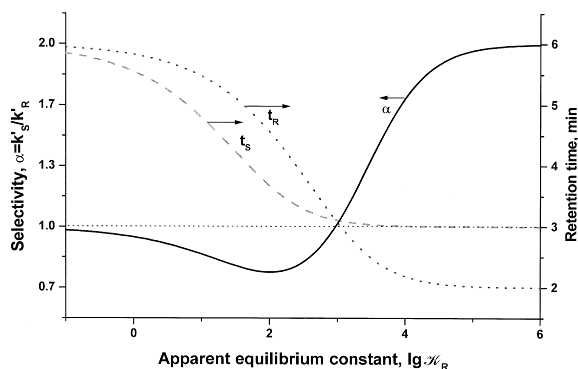


Fig. 6. Dependence of enantiomer retention and selectivity of enantiomer separations on the apparent equilibrium constant $\mathcal{K}$ (l mol^{-1}). Parameters used in the calculations: $t_R = t_S = 6$ min; $t_{R_2\text{C}} = 2$ min; $t_{S_2\text{C}} = 3$ min; $\mathcal{K}_S/\mathcal{K}_S = 10$; $F_\text{V} = 10^{-3}$ l min^{-1}; $q = 10^{-6}$ mol.

As can be seen from Eq. (35) the sample amount of solute should have an effect on enantiomer retention and the selectivity of separation which is similar to those of the apparent equilibrium constant. The corresponding dependencies are shown in Fig. 7. An increase in the sample amount causes a decrease in the retention of the enantiomers and an inversion of the elution order of the enantiomers in the system presented, in the same way as described for the apparent equilibrium constant.

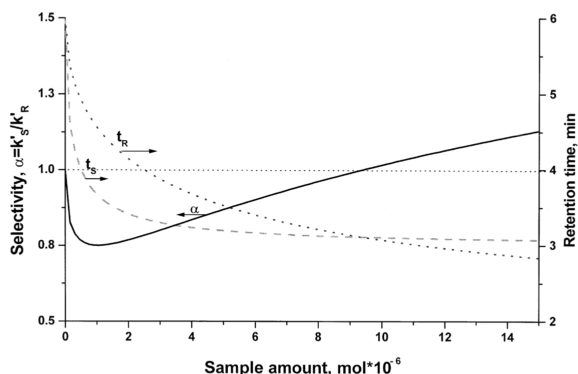


Fig. 7. Dependence of enantiomer retention and selectivity of enantiomer separation on the sample amount q (mol). Parameters used in calculations are the same as in Fig. 6 except $\mathcal{K}_R = 10^3$ l mol, $\mathcal{K}_S = 10^4$ l mol.

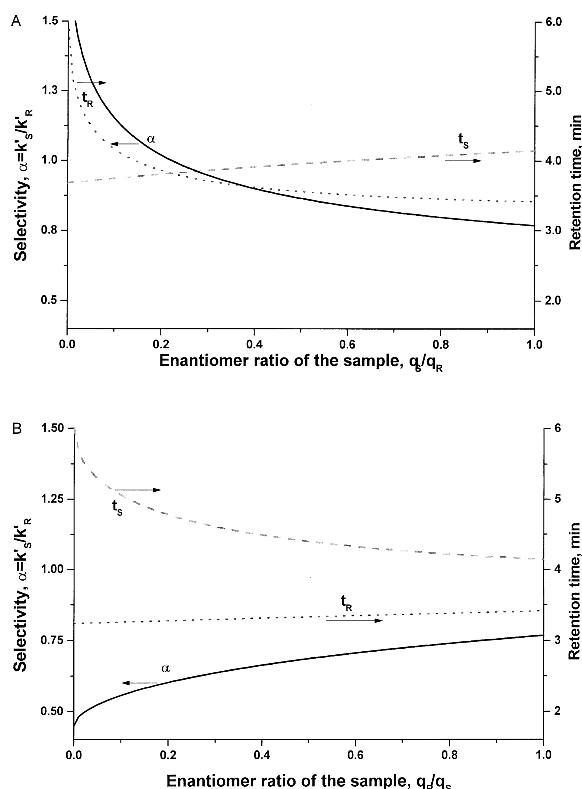


Fig. 8. Dependence of enantiomer retention and selectivity of enantiomer separation in a chiral chromatographic system involving a secondary equilibrium of dimerization type on the optical purity (enantiomer ratio) of the sample (A — the enantiomer ratio changes from 100% (*R*)-enantiomer to a racemic mixture; B — the enantiomer ratio changes from 100% (*S*)-enantiomer to a racemic mixture). Parameters used in calculations are the same as in Fig. 7 except $q = q_S + q_R = 4 \cdot 10^{-7}$ mol.

Taken into account that the optical purity of a sample is determined by the amount of enantiomers in the sample, the retention of the optical isomers and the selectivity of separation should also be affected by the optical purity of the sample. These relationships are shown graphically in Fig. 8. Depending on which of the two enantiomers is present in excess, the appearance of the relationship is different. Fig. 8A demonstrates the changes in the retention and the selectivity of separation when the optical purity of the sample changes from pure *R*-enantiomer to the racemic mixture, i.e to the enantiomer ratio 1:1. Because the total sample amount remains constant a decrease in the amount of the *R*-enantiomer means at the same time an increase in the amount of the *S*-enantiomer. As shown above, an increase in the sample amount causes, in the system under discussion, a decrease in the retention time, while a decrease in the sample amount causes an increase in the retention time. As a result, the *R*-enantiomer is eluted before the *S*-enantiomer for samples of high optical purity (i.e. high content of *R*-enantiomer) and later than the *S*-enantiomer for samples the composition of which is close to the racemic mixture (Fig. 8A). The selectivity of separation changes correspondingly from values >1 for small values of the enantiomer ratio to values <1 when the sample composition approaches that of the racemic mixture. At the enantiomer ratio $q_S/q_R \approx 0.2$ the selectivity of separation is equal to 1 and no separation occurs.

Fig. 8B displays the corresponding changes in the selectivity and retention when the optical purity of the sample changes from pure *S*-enantiomer to the racemic mixture. Because the amount of *S*-enantiomer in the sample decreases, the retention of the *S*-enantiomer increases. The inverse changes are observed for the *R*-enantiomer. Accounting for the higher value of the apparent equilibrium constant for the *S*-enantiomer than for the *R*-enantiomer assumed in the simulation procedure, the changes in enantiomer retention are not expressed so strongly as in the previous example. In the same range of enantiomer ratio from 0 to 1, the *S*-enantiomer always retains stronger than the *R*-enantiomer (Fig. 8B). The selectivity of separation decreases, but the elution order of the enantiomers is preserved. Fig. 9 demonstrates the simulated separations of two samples having the same enantiomer ratio of 10:1. The first sample (Fig. 9A) contains an excess of the *S*-enantiomer, while the second sample (Fig. 9B) has an excess of the *R*-enantiomer. As can be seen the elution order of the enantiomers is inverted for these two samples.

Depending on which of Eqs. (36)–(40) describes the actual equilibrium in the system, the dependencies can be even more complex than those presented above. In the general case, a system with secondary equilibria of the dimerization type should exhibit a dependence of the selectivity of the separation and the elution order of the enantiomers on the sample amount and the enantiomer ratio in the sample. These dependencies cannot be observed in chiral

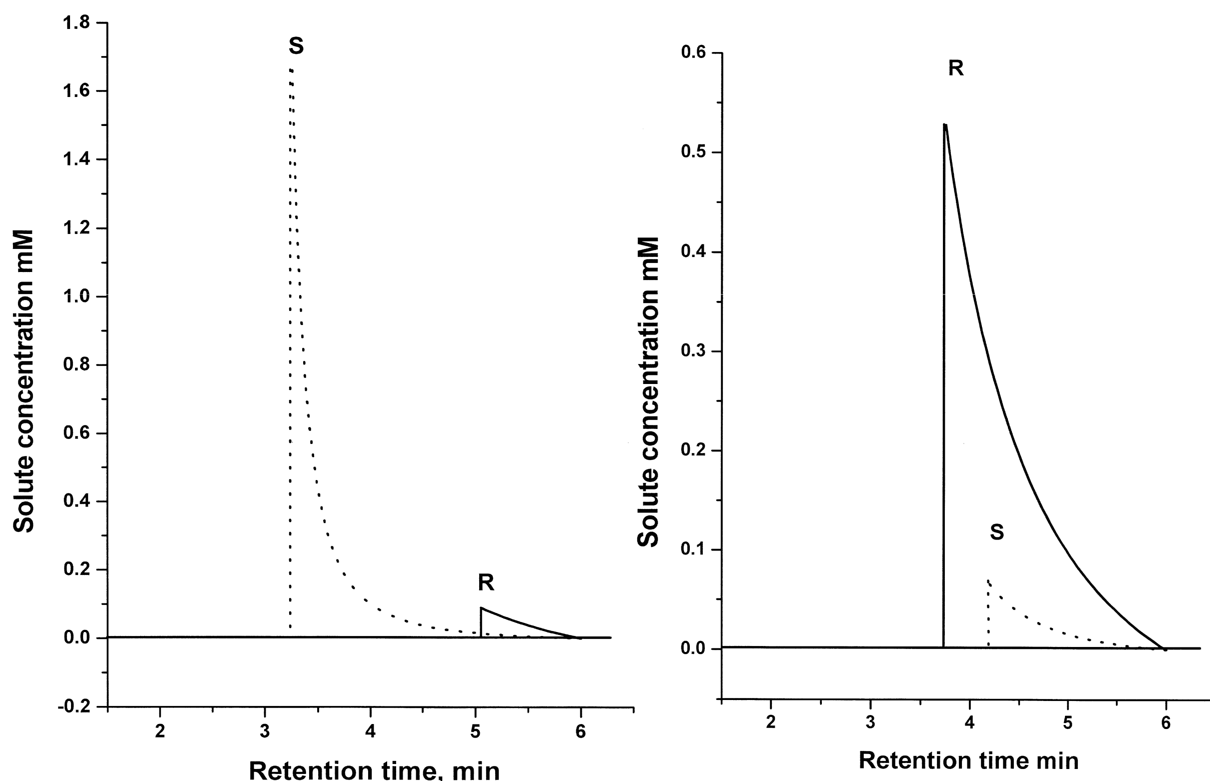


Fig. 9. Simulated separation of enantiomer mixtures in a chiral chromatographic system involving a secondary equilibrium of the dimerization type. The enantiomer ratio is (A) $q_R/q_S = 0.1$ and (B) $q_S/q_R = 0.1$. Parameters used for simulation are the same as in Fig. 6.

chromatographic systems involving secondary equilibria of the simple isomerization type and demonstrate significant differences in solute behavior in the two systems [36].

Considering the enantioselectivity of enantiomer separations in CLEC, one commonly distinguishes two main contributions arising from the enantioselectivity of adduct formation between the solute and the chiral selector in the mobile phase and the stereoselectivity of adsorption of two diastereomeric adducts on the stationary phase [11,34]. The enantioselectivity of adduct formation is characterized by a difference in stability of the corresponding adducts. This difference is accounted for in Eqs. (37)–(40) by the corresponding apparent equilibrium constants $\mathcal{K}$. The difference in adsorption properties of the two diastereomeric species S_2C and R_2C is accounted for by the retention times t_{S_2C} and t_{R_2C}. The differences $t_{S_2C} - t_0$ and $t_{R_2C} - t_0$ are directly proportional to the Henry's adsorption constants of the solutes:

$$t_{S_2C} - t_0 = \phi t_0 k_{S_2C} \quad (41)$$

$$t_{R_2C} - t_0 = \phi t_0 k_{R_2C} \quad (42)$$

When developing new stationary phases and/or new chiral chromatographic systems it is generally of interest to know which of these two contributions is more important for successful enantiomer separation. Is it better to have a higher enantioselectivity of complex formation or it is better to obtain a greater difference in the adsorption properties of the diastereomeric complexes? And, in the extreme case, is separation still possible when either the enantioselectivity of complex formation or the stereoselectivity of diastereoisomer adsorption is absent? All these questions can be answered by analyzing Eqs. (37)–(40) and accounting for the properties of the given chromatographic system. As an example we consider the same system as shown in Figs. 6–8.

Fig. 10 displays the dependencies of the selectivity

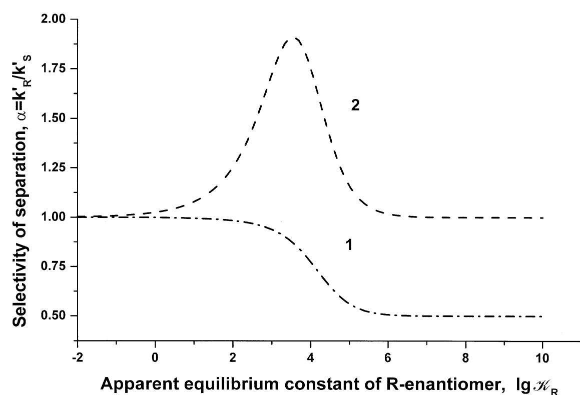


Fig. 10. Dependence of the selectivity of enantiomer separation in a chiral chromatographic system involving a secondary equilibrium of the dimerization type on the apparent equilibrium constant: when the stabilities of the adducts formed by the enantiomers are identical (curve 1) or when the adsorption properties of the diastereomeric adducts are identical (curve 2). Parameters used in calculations are the same as in Fig. 6 except $\mathcal{K}_R = \mathcal{K}_S$, $q = 10^{-7}$ mol (curve 1) and $t_{R_2C} = t_{S_2C} = 2$ min, $q = 10^{-7}$ mol (curve 2).

of separation on the values of the apparent equilibrium constants when (a) no enantioselectivity of complex formation is present (curve 1, $\mathcal{K}_{S_2C} = \mathcal{K}_{R_2C}$) and (b) the adsorption properties of the two diastereomeric complexes are identical (curve 2, $t_{S_2C} = t_{R_2C}$). When the apparent equilibrium constants $\mathcal{K}$ are small, the interaction of the solutes with the chiral selector is negligible and, as a result, separation is absent in both systems ($\alpha \approx 1$). With increasing $\mathcal{K}$ values the selectivity of separation α decreases in the first system and increases in the second system. Therefore, separation becomes possible in both systems, with the only difference that, in the first system, the *S*-enantiomer is eluted before the *R*-enantiomer while the inverted elution order is observed in the second system. With further increase of $\mathcal{K}$ values the selectivity of separation levels off and remains almost constant in the first system (Fig. 10, curve 1). In the second system the selectivity of separation reaches the maximum at $\mathcal{K} \sim 10^4$ and then decreases again to $\alpha = 1$ with a further increase of the apparent equilibrium constant (Fig. 10, curve 2). The dependencies described are elucidated in Figs. 11 and 12, where simulated chromatograms are shown for selected values of the equilibrium constants. As can be seen, neither the absence of selectivity of complex formation, nor the identity of the adsorption properties of the two diastereomeric complexes alone, prevent successful separation of the enantiomers when the chromatographic conditions are chosen correctly. At the same time, analysis of Eqs. (37)–(40) shows that the parameter which can completely prevent enantiomer separation is the difference in the adsorption properties of the free solute and its adducts with the chiral selector. When the adsorption properties of the solutes and the adducts are identical, the retentions of both diastereomeric complexes and enantiomers are also identical, i.e. $t_R = t_S = t_{R_2C} = t_{S_2C}$. In that case, only a single peak will appear in the chromatogram and no separation can be observed, independent of how large the enantioselectivity of complex formation, i.e. how large the difference between $\mathcal{K}_S$ and $\mathcal{K}_R$.

In Ref. [36] it was shown that the same relationships are valid not only for CLEC, but for any other chiral chromatographic system involving a secondary equilibrium of the dimerization type.

3. Recent achievements in chiral ligand-exchange chromatography

As mentioned in the Introduction, CLEC has been reviewed extensively, mainly by Davankov. While the earlier papers [2–6,8] contained a description of the achievements and developments published in the literature, the last review [11] by Davankov discussed the problem of the efficiency of separations performed by CLEC. The article covers publications up to 1994. Based on the results of kinetic and thermodynamic measurements reported in the literature for copper(II) complexes of amino acids and amines, Davankov [11] concluded that the stability of diastereomeric complexes formed in CLEC is higher than the stability of the diastereomeric adducts formed by many other chiral selectors. The relatively high stability of the diastereomeric complexes may result in slow ligand exchange and reduced plate count of CLEC columns [11]. However, the thermodynamic stability of the adduct formed by the solute and the chiral selector does not relate directly to its kinetic properties. The stability constant is the ratio of the kinetic formation and dissociation constants. The absolute values of the

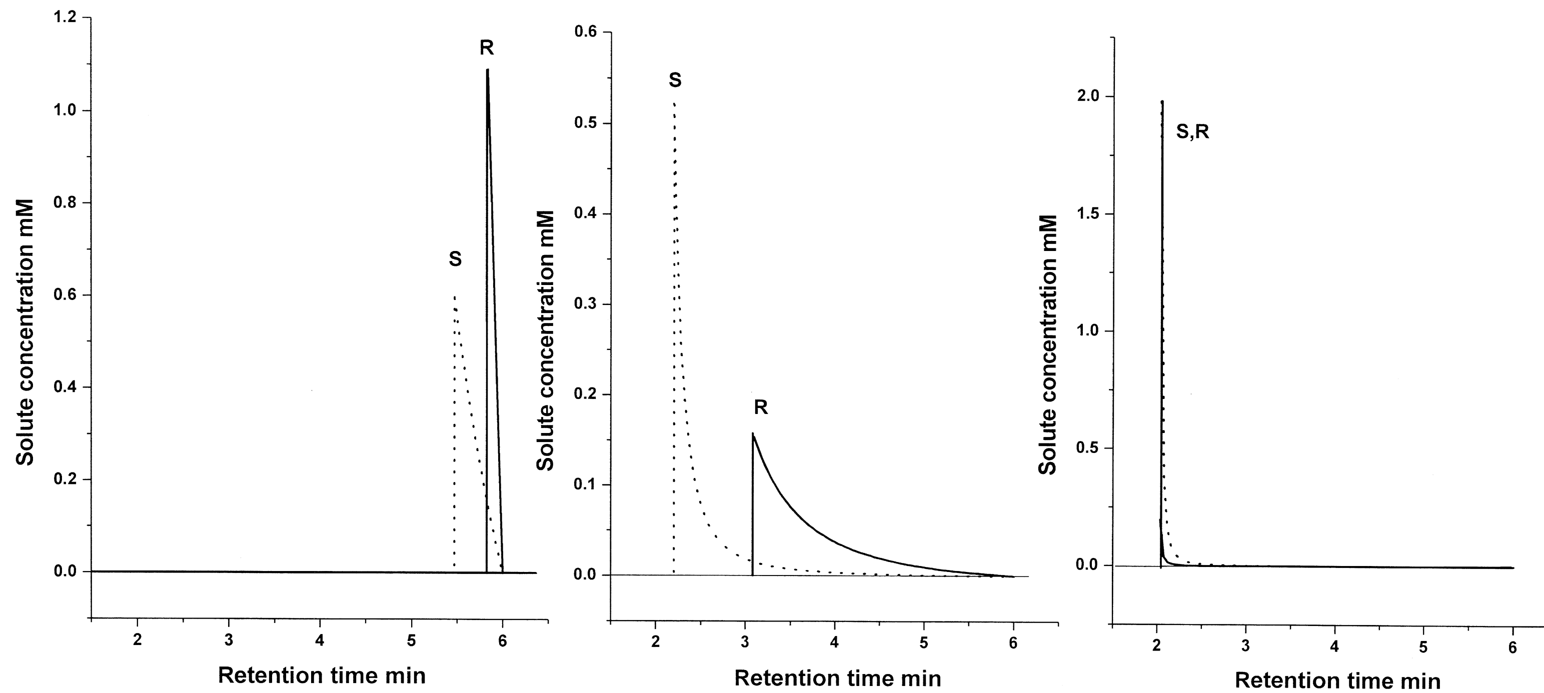


Fig. 11. Simulated chromatograms of enantiomer separation in a chiral chromatographic system involving a secondary equilibrium of the dimerization type. Parameters used in the simulation are the same as in Fig. 10, curve 1, with $K_R = 10^1$ (A), 10^4 (B) and 10^6 (C) l mol^{-1}.

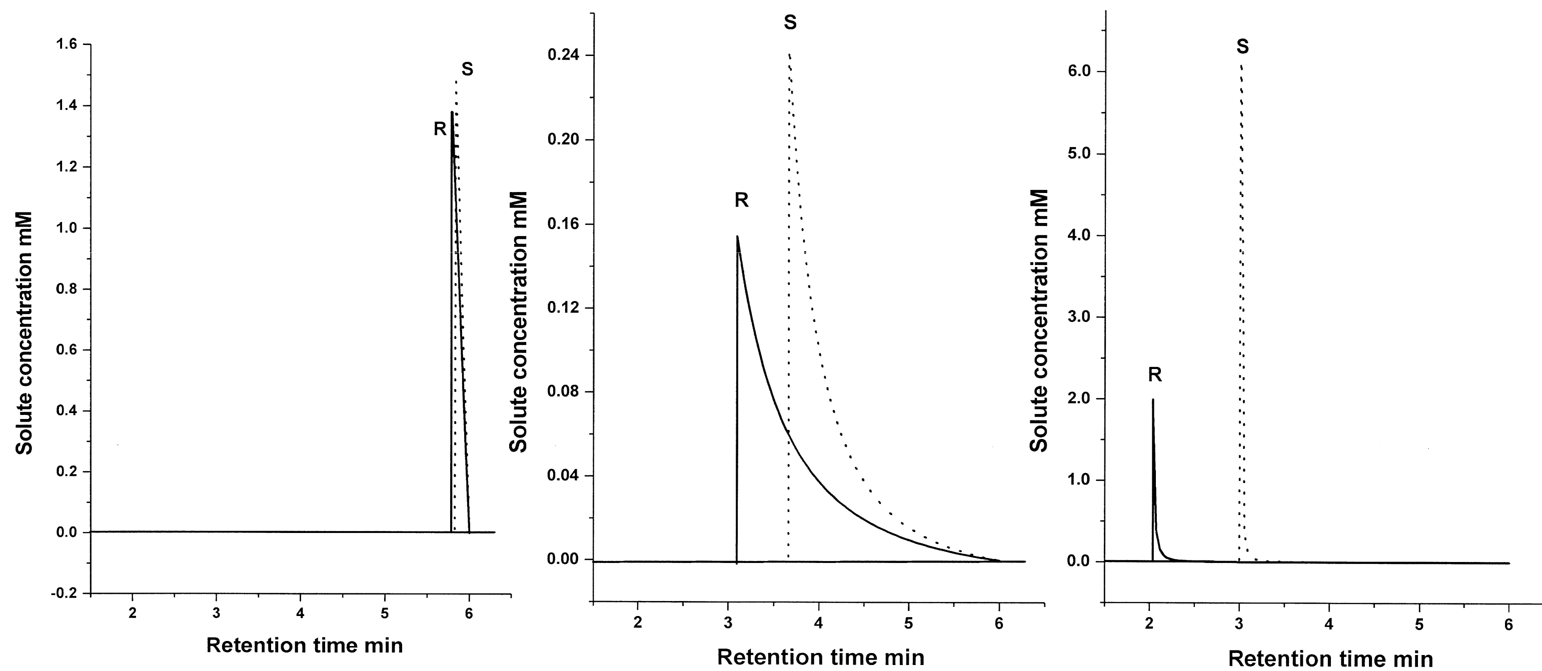


Fig. 12. Simulated chromatograms of enantiomer separation in a chiral chromatographic system involving a secondary equilibrium of the dimerization type. Parameters used in the simulation are the same as in Fig. 10, curve 2, with $K_R = 10^1$ (A), 10^4 (B) and 10^6 (C) l mol^{-1}.

kinetic constants of the formation and dissociation processes are not necessarily reflected in the stability constants. One can observe high stability constants for both very slow reactions, e.g. formation of Cr(III) complexes, and very fast reactions, e.g. formation of Cu(II) complexes [37]. A possible explanation of the slow mass-transfer kinetics in CLEC may be the large size of the transition state of coordination compounds during ligand exchange [37]. This problem appears to be even more critical when the complex is bonded onto the sorbent surface. Unfortunately, this point was not elucidated to a sufficient extent in the literature.

A detailed investigation of the chiral ligand-exchange chromatographic system using chiral selector Cu(II)–N^{τ}-*n*-decyl-L-histidine was performed by Remelli et al. [38,39]. The chiral selector was deposited on Backerbond ODS silica and more than 20 different racemic mixtures of underivatized amino acids were resolved using a simple water–Cu(II) acetate eluent with a small additive (up to 15%) of organic modifier. Histidine and its derivatives are frequently used in CLEC as a chiral selector in physically adsorbed [40] and chemically bonded [41–43] forms. However, in contrast to the previous publications, the modification of *S*-histidine was performed in such a way that α-carboxy and α-amino functional groups of *S*-histidine remain unchanged and alkylation took place at the N^{τ}-atom of the imidazole ring [44]. It was assumed that retaining the free α-carboxy and α-amino functions of histidine is important for the chiral recognition by the chiral selector. In support of this point the authors recall good resolving ability of the selector observed in TLC [43]. The selectivity of enantiomer separations and the retention of solutes were investigated with respect to various parameters: the mobile-phase flow-rate, Cu(II) concentration in the eluent, type and amount of organic modifier in the eluent, pH of the mobile phase, temperature, type of counter-ion used with Cu(II) salt, and buffer concentration. The efficiency of the separation is markedly improved when reducing the mobile-phase linear velocity. However, a minimum of the Van-Deemter's plot was not reached even at a flow-rate as low as 0.2 ml/min. The authors interpreted this observation as a clear indication of the slow ligand-exchange kinetics. The enantioselectivity and also the peak asymmetry appeared almost unaffected by the mobile-phase velocity. An increase in Cu(II) ion concentration in the eluent caused a continuous decrease in the retention of the solute with negative consequences on the selectivity of separations [38]. Particularly strong reduction of solute retention was caused by the addition of an organic modifier to the eluent. Unfortunately, the presence of an organic modifier also affects column stability, probably due to leaching of the chiral selector from the column. Therefore, this parameter should be used and considered with care.

The pH of the eluent was recognized to be one of the most important parameters governing solute retention [39]. The retention of all analytes increases as pH increases. The selectivity of separation was only affected moderately by pH changes, while the efficiency of the column demonstrated a different trend depending on the relative solute retention. The efficiency of the column increases for the least-retained solutes and decreases for the most retained ones. A temperature increase reduces the retention of all samples, except basic amino acids [39]. The selectivity of separation is invariably reduced by an increase in the temperature, while the column efficiency is always increased by a higher temperature.

An increase in buffer concentration when both pH and Cu(II) concentration are kept constant causes a decrease in solute retention and selectivity for all amino acids besides the basic ones. As a result, the separation of basic analytes was possible only with the most concentrated buffers.

The elution order observed for the majority of analytes was *S*-enantiomer before *R*-enantiomer [39]. An exception is presented by the basic amino acids, for which an inverted elution order was observed. Using the stability constants of Cu(II)–histidine complexes determined in batch aqueous solutions [45] and the model of adsorption proposed by Davankov et al. [40] the authors presented a theoretical explanation of the elution order. They underlined that knowledge of the structures of model complexes and the possible additional intermolecular interactions are very useful when interpreting the chromatographic behavior of the various samples.

In a general project aimed at studying the nature of the interactions responsible for chiral discrimination in HPLC, Galli et al. [46] investigated new

chiral stationary phases prepared by covalent bonding *S*- and *R*-phenylalaninamide onto a silica surface modified with γ-glycidoxypropyltrimethoxysilane. The content of chiral selector of 0.35 mmol g^{-1} calculated from elemental analysis proved that only half of the total amount of epoxide groups (ca. 0.62 mmol g^{-1}) was involved in the interaction with the chiral selector. Nevertheless, the dansyl (Dns) and dabsyl (Dbs) derivatives of amino acids were successfully separated on the synthesized CPS. The elution order of all investigated amino acid derivatives was *R*-enantiomer before *S*-enantiomer for CSP containing *S*-phenylalaninamide. The selectivity of the separation of Dns derivatives of polar amino acids (Ser, Thr, Asp, Glu) was significantly higher than those of non-polar, hydrophobic amino acids (Val, Leu, Phe, Trp). Similar results were obtained for Dbs derivatives, although the selectivity of the separation was lower than in the case of Dns derivatives. An acetonitrile–ammonium acetate buffer mixture containing 0.25 m*M* Cu(acetate)$_2$ was used as the mobile phase throughout the work. The buffer concentration has only a moderate effect on solute retention and selectivity, while a decrease of pH from 7.52 to 6.0 caused a remarkable increase in retention and a slight decrease in selectivity.

The opposite effect was observed on the addition of an organic modifier to the eluent. The retention of both enantiomers was reduced and the selectivity of separation was enhanced with an increase of the content of the organic modifier in the eluent for all analyzed Dns derivatives.

Temperature has a strong effect on retention and selectivity. Both parameters decreased with an increase in temperature. Nevertheless, the resolution of enantiomers was improved with a temperature increase because of the increase of column efficiency at higher temperatures. The thermodynamic parameters of chiral separations were calculated from the dependencies of the retention factor on the temperature (Table 1). The enthalpic and entropic terms have an opposite effect on the recognition process. The unfavorable entropic term essentially arises from the loss of degree of freedom experienced by the most-retained enantiomer. For some solutes (Dns-Ser, Dns-Thr) this contribution is compensated by interaction with the solvent and/or reorganization of ligand coordination around the Cu(II) atom, resulting in $\Delta\Delta S°$ close to zero. In order to discuss the interactions responsible for chiral recognition, the stability of the model Cu(II) complexes of phenyalaninamide was measured by potentiometric and spectroscopic techniques [47,48]. Complexes CuL^{2+}, $CuLH_{-1}^{+}$, $CuL_2H_{-1}^{+}$, CuL_2H_{-2} and CuL_2 were recognized as the dominating species in the pH range used in chromatographic separations. Accounting for the concentration of chiral selector in the stationary phase, the authors concluded that the species $CuLH_{-1}^{+}$ is present at pH 7.0–7.5 and responsible for the enantioselectivity of chiral recognition. However, the stability of the ternary complexes containing ligands of opposite optical configuration was found to be higher than those of complexes containing ligands of the same optical configuration. Therefore, the relative stability of the diastereomeric complexes does not support the elution order observed.

Table 1
Thermodynamic data for the enantioselective interaction of solutes with chiral stationary phase based on (*S*)-phenylalaninamide. From Ref. [46]

Solute	Eluent[a]	$-\Delta\Delta H°$ (cal mol^{-1})	$-\Delta\Delta S°$ (cal mol^{-1})	$-\Delta\Delta G$ (25°C) (cal mol^{-1})
Dns-Ser	A	1039	0.03	1030
Dbs-Ser	B	1139	0.95	856
Dns-Met	A	649	0.51	497
Dbs-Met	A	572	0.82	328
Dns-Thr	A	804	0.05	789
Dbs-Thr	A	1080	1.71	570

[a] A: CH_3CN/0.05 *M* NH_4OAc–0.25 *M* $Cu(OAc)_2$ (70:30), pH 7.52, flow-rate 1 ml/min. B: CH_3CN/0.10 *M* NH_4OAc–0.25 *M* $Cu(OAc)_2$ (70:30), pH 7.52, flow-rate 1 ml/min.

The same chiral selector was applied successfully for the separation of enantiomers of α-hydroxy acids [49]. The selectivity of separation was investigated with respect to pH and polarity of the eluent, chiral selector concentration, etc. and regularities similar to those observed for amino acid analytes were detected.

To obtain an improved model of the stationary phase, the ligands (*S*,*R*)- and (*S*,*S*)-N^2-(2-hydroxypropyl)-phenylalaninamide were synthesized and their complexes with Cu(II) investigated [50]. The same set of complexes was identified in solution and similar relations for the stability of complexes were found as described above for the non-substituted

phenylalaninamide ligand. However, the elution order of enantiomers measured in both CMP and CSP modes was found not to coincide with those predicted from the stability constants [51].

The authors concluded that it is misleading to account for the stability of the ternary complexes formed in solution for the explanation of the enantioselectivity of enantiomer separations in CLEC [46]. The reason is that the complexes preferentially formed in solution are not necessarily the same as those formed in the stationary phase [51]. For example, the enantioselectivity of enantiomer separation with the selectors discussed above is determined in CMP mode by the relative affinities of the ternary complexes for the column stationary phase. In CSP mode it is mainly accounted for by the relative stabilities of the ternary complexes formed on the stationary phase, which are dependent on the allowed geometry of the complex and on the steric repulsion of the amino acid side chain with the spacer [48].

Gübitz et al. [52,53] prepared two new chiral stationary phases with covalently bonded *S*-proline. This chiral selector is particularly popular in CLEC because of its ability for chiral recognition [2–5]. The new synthetic procedures commonly resulted in a different type of bonding of the chiral selector to the support surface. Accounting for the active role of achiral supports in chiral recognition [11], the new phases may provide better selectivity for particular types of analytes. The structures of the new chiral adsorbents are shown in Fig. 13. It can be seen that they differ by the spacer bonding of the chiral selector to the silica surface. The first sorbent contained the chiral selector bonded through (2-hydroxycyclohexyl)ethylene spacer (Fig. 13, structure CSP I) and succeeded in separating amino acids, dipeptides and hydroxy acids. The second adsorbent containing *S*-proline bonded to the silica surface through 6-hydroxy-4-oxa-8-aza-*n*-decen spacer (Fig. 13, structure CSP II) was found to be applicable for the separation not only of amino acids and their derivatives, but also for the separation of racemic barbiturates. Table 2 displays the selectivities observed in the separation of non-derivatized amino acids with the two adsorbents. It can be seen that the spacer type affects not only the selectivity of separations, but also the elution order of enantiomers.

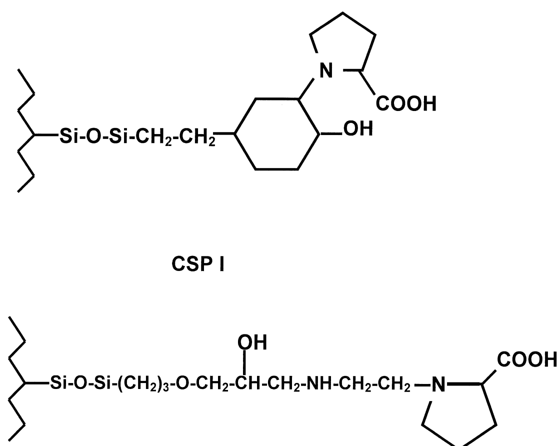


Fig. 13. Structures of the chiral stationary phases prepared by Gübitz et al. From Ref. [52].

Wachsmann and Brückner [54] synthesized chiral stationary phases for CLEC by binding (*S*)-proline and (*S*)-lysine derivatives onto the surface of γ-aminopropylsilica through a triazine spacer. The CSP containing (*S*)-proline was able to separate underivatized α-amino acids and their *N*-(2,4-dinitrophenyl) derivatives, while the CSP containing (*S*)-lysine resolved Dns derivatives of amino acids. A similar chiral stationary phase containing (*S*)-alanine as chiral moiety was synthesized by Yang et al. [55]. The chiral selector was connected to the silica surface through a single *S*-triazine spacer and the CSP prepared was compared in resolving ability with earlier investigated CSP having two spacer groups between the chiral moiety and the silica surface [55]. Both stationary phases were effective in the separation of underivatized amino acids, amino alcohols and diamines. Therefore, the authors concluded that chiral recognition occurs on each single connected arm rather than on both double arms.

A number of publications facilitated with CSP obtained by adsorption of the chiral selector onto the surface of a support. Miyazawa et al. [56] deposited *N*,*S*-dioctyl-D-penicillamine onto the surface of RP-18 silica and observed excellent-to-good selectivity of separation for non-protein amino acids, such as 2-fluorophenylalanine, 2-furfurylmethylalanine, 1-pentylalanine, etc. The same chiral selector and (*R*,*R*)-tartaric acid mono-(*R*)-1-(α-naphthyl)-ethylamide were applied by Oi et al. [57] for

Table 2
Retention factor k' and selectivity of separation α for α-amino acids on CSP I and II (for the structures of CSP, see Fig. 13). From Ref. [52][a]

Amino acid	CSP I				CSP II			
	Eluent	k'_R	k'_S	α	Eluent	k'_R	k'_S	α
Alanine	A	0.9	0.9	1.0	B	3.0	1.83	0.61
Arginine	A	1.2	1.5	1.3	–	–	–	–
Asparagine	A	2.8	5.2	1.9	A	3.17	4.33	1.37
Aspartic acid	A	2.2	2.9	1.3	A	1.87	2.25	1.20
Dihydroxyphenylalanine	–	–	–	–	A	5.20	16.0	3.08
Ethionine	–	–	–	–	A	5.20	5.60	1.08
Glutamic acid	–	–	–	–	B	2.90	2.10	0.72
Histidine	A	1.9	5.5	2.8	A	6.83	12.2	1.78
Leucine	A	2.5	2.5	1.0	B	5.00	2.50	0.50
Methionine	A				A	1.88	2.35	1.25
Norleucine	A	1.5	1.8	1.23	B	10.0	6.50	0.65
Norvaline	A	1.35	1.4	1.04	B	9.00	4.50	0.50
Proline	A	2.4	1.4	0.6	A	2.17	1.17	0.54
Phenylalanine	A	1.5	4.5	3.0	A	5.4	11.0	2.04
Phenylserine	A	8.0	14.8	1.85	–	–	–	–
Serine	A	2.1	3.6	1.7	A	2.17	3.85	1.61
Threonine	A	0.9	1.4	1.5	A	3.33	5.0	1.50
Tryptophan	A	3.7	13.7	3.7	A	12.7	38.0	3.0
Tyrosine					A	5.0	13.2	2.64
Valine	A	1.8	3.3	1.9	A	2.67	3.83	1.44

[a] Conditions: flow-rate, 1 ml min^{-1}; temperature, 50°C. Eluent A: 0.05 M potassium dihydrogenphosphate (pH 4.6)–0.1 mM $CuSO_4$ in water. Eluent B: 0.1 mM $CuSO_4$ in water.

modification of Sumipax ODS columns. Different types of analytes could be separated successfully with the chiral columns, among them 1-aminoethylphosphonic acid, 3-aminopyrrolidine, *trans*-1,2-diaminocyclohexane, tetrahydro-2-furoic acid, etc. [57].

Slivka et al. [58] synthesized *N*-alkylphenoxyglycinols (alkyl chains C_7–C_{10}) and loaded them onto the surface of C-18 silica. The efficiency of the column and the selectivity of separation of racemic amino acids and amino alcohols were considered with respect to the pH of the mobile phase, temperature, the content of organic modifier, etc.

Kurganov et al. [59] prepared CSP by adsorption of Cu[*N*-octadecyl-(*S*)-proline]$_2$ onto the surface of Superspher-100 RP-18 silica as described earlier by Davankov et al. [60]. The effect of the copper(II) ion concentration in the eluent and the sample amount on the selectivity of separation and the peak shape was investigated. The sample amounts injected onto the column were varied from less than 1 μg to almost 2 mg for a column of 125×4 mm in size. The peak shape of the solute changed from slightly fronting or almost symmetrical to a strongly distorted fronting peak with increasing sample amount. The intensity of the second-eluted enantiomer increases with increasing sample amount more strongly than that of the first-eluted enantiomer and after particular mass loads the second-eluted peak becomes more intensive than the first. With a further increase in sample amount, strong peak compression was observed for both enantiomers and the peak shape changed from fronting to tailing. These unusual changes in the peak profiles were not explained in Ref. [59] and the results were presented as experimental observations only. An explanation of the effects may be given based on the theoretical models reviewed in Sections 2.1–2.3. Many of the simulated chromatograms reproduce the unusual peak profiles described in Ref. [59].

A non-traditional support for the preparation of CSP for CLEC was used by Wan et al. [61,62]. The authors synthesized chiral selectors by alkylation and arylation of (*S*)-proline and (*S*)-phenylalanine (alkyl,

C_7, C_9, C_{12}; aryl, methoxybenzyl, naphthylmethyl, anthrylmethyl). *N*-Alkyl- and *N*-aryl-substituted derivatives of amino acids were adsorbed onto the surface of porous graphitic carbon resulting in CSP with capacities varying from 0.55 to 1.26 μmol m^{-2}. The separation of 36 racemic amino acids was demonstrated on the prepared CSPs. The elution order of non-polar amino acids on CSPs containing (*S*)-proline was always (*S*)-enantiomer before (*R*)-enantiomer and independent of the type of alkyl or aryl substituent [62]. The highest enantioselectivity was observed for CSP with *N*-(9-naphthylmethyl)-(*S*)-proline chiral selector adsorbed onto the surface of the porous graphite. An inverted elution order was found for non-polar amino acids on the CSP containing *N*-alkyl-substituted (*S*)-phenylalanine [61], i.e. the (*R*)-enantiomer of the solute eluted before the (*S*)-enantiomer. The elution order is again reversed, however, when the anchor chain changes from an alkyl to an aryl group. The difference in the retention order of amino acid enantiomers observed with alkyl- and aryl-(*S*)-phenylalanine was explained in line with structural analysis of the sorption complexes. The proposed structures of the complexes are shown in Fig. 14. On alkyl-(*S*)-phenylalanine CSPs, the side chain of an amino acid analyte can interact with the phenyl group and with the alkyl chain of the selector, but the interaction with the phenyl group is most likely to be dominant. Thus, the elution order on this phase is *S*-enantiomer before *R*-enantiomer. On the aryl-(*S*)-phenylalanine CSP, the side chain of the amino acid analyte interacts more strongly with the aryl group of the selector and the elution order is inverted (Fig. 14). According to the authors, coated CSPs based on porous graphitic carbon have enabled a more effective investigation of the mechanism of enantioselectivity, without the undesirable interference from secondary interactions observed with silica-based CSPs.

The CMP mode of CLEC is easier for practical realization than the CSP mode. It does not require a special synthetic procedure for preparation of CSPs

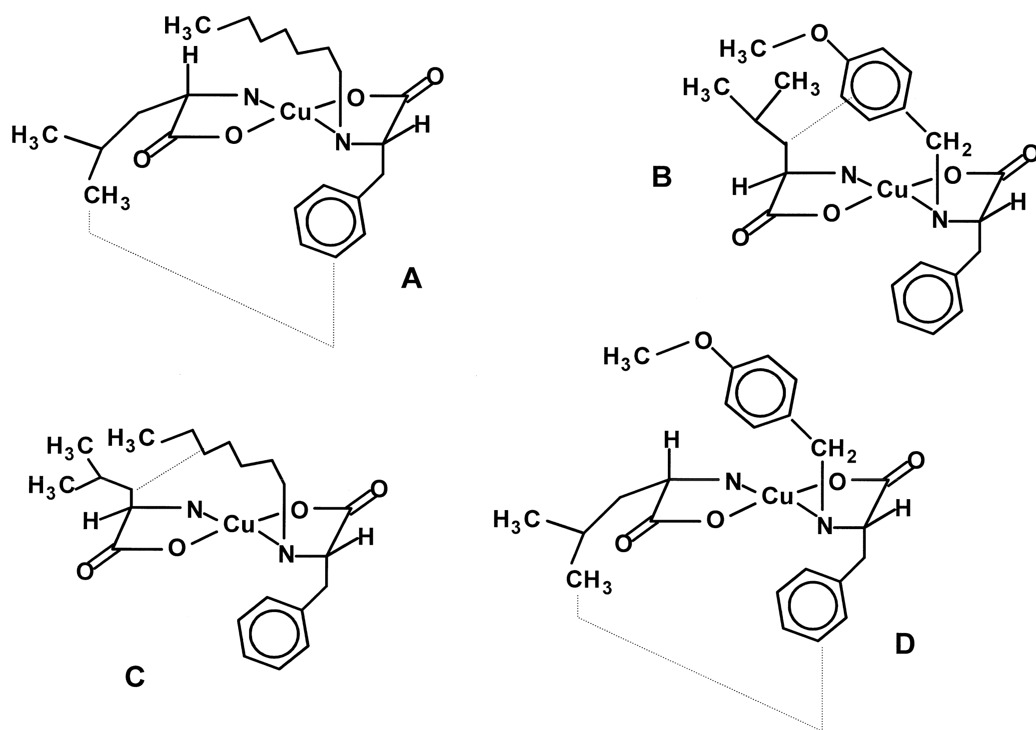


Fig. 14. Proposed structure of the mixed-ligand ternary complex formed between (A) (*S*)-leucine and *N*-alkyl-(*S*)-phenylalanine; (B) (*R*)-leucine and *N*-aryl-(*S*)-phenylalanine; (C) (*R*)-leucine and *N*-alkyl-(*S*)-phenylalanine and (D) (*S*)-leucine and *N*-aryl-(*S*)-phenylalanine. (· · ·) Potential hydrophobic interactions which stabilize the complex and thereby influence the enantioselectivity. From Ref. [61].

and is commonly used in analytical applications and/or to perform a quick test of chiral selectors. The theoretical and practical fundamentals of the technique were reviewed recently [63].

Two research groups used (*S*)-phenylalaninamide [49] and (*S*,*S*)-N^2-(2-hydroxypropyl)-phenylalaninamide [51] in CMP mode for the separation of enantiomers of amino acids and amino alcohols and compared the result of separations in CMP mode with those obtained in CSP mode as mentioned above.

Tanaka et al. [64] applied CMP mode with additiion of the Cu(II) complex of (*S*)-proline to the eluent for the separation of enantiomers of amino and hydroxy acids and β-amino alcohols. Amino and hydroxy acids were readily resolved, while no separation was observed for β-amino alcohols. Addition of achiral barbiturates to the mobile phase significantly improved the selectivity of separation and resulted in complete separation of many analytes. To explain this unusual effect the authors proposed that barbiturates participate in the formation of the mixed-ligand ternary complexes responsible for chiral recognition and in such a way affect the selectivity of separation.

4. Conclusions

The chiral ligand-exchange chromatographic technique invented more than 30 years ago was the first liquid chromatographic method which succeeded at a complete separation of optical isomers. Chiral recognition in CLEC was explained as a result of the formation of mixed-ligand ternary complexes between the chiral selector and the enantiomers to be separated. The mixed ternary complexes resulted from numerous equilibria existing in the chromatographic column. The relative importance of a particular equilibrium depends on the conditions of the separation (pH, temperature, etc.). As long as these equilibria are of the most simple isomerization type or can be reduced to them, they do not affect the linear behavior of the system. However, a significant amount of the ternary complex is present in the column only under conditions where dominating equilibria are of the dimerization type. This results in a deviation from linear behavior. The most unpleasant effect of non-linearity is distortion of the peak shape and the corresponding reduction of column efficiency. This effect has a purely thermodynamic nature and exists independent on dynamic peak broadening in CLEC described by Davankov [11].

The stoichiometry of the interaction between the solute and the chiral selector affects the chromatographic peak profile and the selectivity of separation not only in CLEC. The relationships derived for CLEC should be valid for any other chromatographic system involving solute–selector interactions of a more complex type than the simple 1:1 type. However, CLEC gives an example of a chromatographic system where the complex secondary equilibria have not only a theoretical interest, but directly determine the chiral recognition and the selectivity of separations.

References

[1] S. Rogozhin, V. Davankov, German patent 1 932 190 (1970); C.A. 72 (1970) 90875c.
[2] V. Davankov, Adv. Chromatogr. 18 (1980) 139.
[3] V. Davankov, A. Kurganov, A. Bochkov, Adv. Chromatogr. 22 (1983) 71.
[4] V. Davankov, J. Navratil, H. Walton, Ligand Exchange Chromatography, CRC Press, Boca Raton, FL, 1988.
[5] V. Davankov, in: A. Krstulovic (Ed.), Chiral Separations by HPLC, Applications to Pharmaceutical Compounds, Ellis Horwood, Chichester, 1989, pp. 175, 446.
[6] V. Davankov, in: D. Cagniant (Ed.), Complexation Chromatography, Marcel Dekker, New York, 1992, p. 197.
[7] V. Davankov, Pure Appl. Chem. 54 (1982) 2159.
[8] V. Davankov, in: K. Unger (Ed.), Packings and Stationary Phases in Chromatographic Techniques, Marcel Dekker, New York, 1990, p. 541.
[9] K. Günter, in: J. Sherman, B. Fried (Eds.), Handbook of Thin-Layer Chromatography, Marcel Dekker, New York, 1991, p. 541.
[10] E. Francotte, A. Junker-Buchheit, J. Chromatogr. 576 (1992) 1.
[11] V. Davankov, J. Chromatogr. A 666 (1994) 55.
[12] R. Corradini, A. Dossena, G. Galaverna, R. Marchelli, G. Palla, Semin. Org. Synth. Summer Sch. 'A Corbella', 19th, 1994, p. 79; C.A. 123 (1995) 350439.
[13] V. Davankov, A. Kurganov, Chromatographia 17 (1983) 686.
[14] V. Davankov, Chromatographia 27 (1990) 391.
[15] V. Davankov, V. Mayer, M. Raiss, Chirality 2 (1990) 208.
[16] C. Welch, J. Chromatogr. A 666 (1994) 3.
[17] D. Armstrong, W. DeMond, J. Chromatogr. Sci. 22 (1984) 411.
[18] S. Allenmark, S. Andersson, J. Chromatogr. A 666 (1994) 167.

[19] Y. Okamoto, Y. Kaida, J. Chromatogr. A 666 (1994) 403.
[20] D. DeVault, J. Am. Chem. Soc. 65 (1943) 532.
[21] A. Martin, Biochem. Soc. Symp. 3 (1949) 4.
[22] D. Armstrong, J. Liq. Chromatogr. 3 (1980) 895.
[23] R. Mohseni, R. Hurtubise, J. Chromatogr. 499 (1990) 395.
[24] G. Brooks, L. Pettit, J. Chem. Soc., Dalton Trans. (1977) 1918.
[25] D. Perrin (Ed.), Stability Constants of Metal–Ion Complexes, Part B, Pergamon Press, Oxford, 1982.
[26] G. Borghesani, F. Pulidori, M. Remelli, R. Purello, E. Rizarelli, J. Chem. Soc., Dalton Trans. (1990) 2095, and references therein.
[27] G. Galaverna, R. Corradini, E. de Munari, A. Dossena, R. Marchelli, J. Chromatogr. A 657 (1993) 43.
[28] A. Kurganov, Chromatographia 43 (1996) 17.
[29] A. Kurganov, GIT Z. (1997) 23.
[30] A. Kurganov, K. Unger, J. Chromatogr. A 797 (1998) 57.
[31] M. Ringo, C. Evans, Anal. Chem. 70 (1998) 315A.
[32] S. Golshan-Shirazi, G. Guiochon, J. Chromatogr. A 658 (1994) 495.
[33] A. Kurganov, in: Presented at the 12 International Symposium on Chiral Discrimination, Chicago, IL, USA, July, 1999.
[34] V. Davankov, A. Kurganov, T. Ponomareva, J. Chromatogr. 452 (1988) 309.
[35] B. Feibush, M. Cohen, B. Karger, J. Chromatogr. 282 (1983) 3.
[36] A. Kurganov, in: Presented at the 21st International Symposium on Chromatography, Rome, Italy, September, 1998.
[37] F. Basolo, R. Pearson, Mechanisms of Inorganic Reactions. A Study of Metal Complexes in Solution, 2nd ed., Wiley, New York, 1967.
[38] M. Remelli, P. Fornasari, F. Dondi, F. Pulidori, Chromatographia 37 (1993) 23.
[39] M. Remelli, P. Fornasari, F. Pulidori, J. Chromatogr. 761 (1997) 79.
[40] V. Davankov, S. Bochkov, Y. Belov, J. Chromatogr. 218 (1981) 547.
[41] N. Watanabe, H. Ohzeki, E. Niki, J. Chromatogr. 216 (1981) 406.
[42] N. Watanabe, J. Chromatogr. 260 (1983) 75.
[43] S. Hu, D. Do, J. Chromatogr. 646 (1993) 31.
[44] M. Remelli, R. Piazza, F. Pulidori, Chromatographia 32 (1991) 278.
[45] G. Borghesani, F. Pulidori, M. Remelli, R. Purello, E. Rizzarelli, J. Chem. Soc., Dalton Trans. (1990) 2095.
[46] B. Galli, F. Gasparini, D. Misiti, C. Villani, R. Corradini, A. Dossena, R. Marchelli, J. Chromatogr. A 666 (1994) 77.
[47] F. Dallavalle, E. Fisiucaro, R. Corradini, R. Marchelli, Helv. Chim. Acta 72 (1989) 1479.
[48] F. Dallavalle, G. Folesani, R. Marchelli, G. Galaverna, Helv. Chim. Acta 77 (1994) 1623.
[49] G. Galaverna, F. Panto, A. Dossena, R. Marchelli, F. Bigii, Chirality 7 (1995) 331.
[50] F. Dallavalle, G. Folesani, T. Bertuzzi, R. Corradini, R. Marchelli, Helv. Chim. Acta 78 (1995) 1785.
[51] R. Marchelli, R. Corradini, T. Bertuzzi, G. Galaverna, A. Dossena, F. Gasparini, B. Galli, C. Villani, D. Misiti, Chirality 8 (1996) 452.
[52] G. Gübitz, S. Mihellyes, G. Kobinger, A. Wutte, J. Chromatogr. A 666 (1994) 91.
[53] G. Gübitz, B. Vollmann, G. Cannazza, H. Schmitt, J. Liq. Chromatogr. Relat. Technol. 19 (1996) 2933.
[54] M. Wachsmann, H. Brückner, Chromatographia 47 (1998) 637.
[55] M. Yang, M. Hsieh, J. Lin, Taiwan Kexue 50 (1997) 17; C.A. 128 (1998) 265545.
[56] T. Miyazawa, H. Minowa, K. Jamagawa, T. Yamada, Anal. Lett. 30 (1997) 867.
[57] N. Oi, H. Kitahara, F. Aoki, J. Chromatogr. A 707 (1995) 380.
[58] M. Slivka, M. Slebioda, A. Kolodziejczyk, Chem. Anal. (Warsaw) 42 (1998) 895; C.A. 128 (1998) 225372.
[59] A. Kurganov, V. Davankov, K. Unger, F. Eisenbeiss, J. Kinkel, J. Chromatogr. A 666 (1994) 99.
[60] V. Davankov, A. Bochkov, A. Kurganov, P. Roumeliotis, K. Unger, Chromatographia 13 (1980) 677.
[61] Q.-H. Wan, P. Shaw, M. Davies, D. Barrett, J. Chromatogr. A 765 (1997) 187.
[62] Q.-W. Wan, P. Shaw, M. Davies, D. Barrett, J. Chromatogr. A 786 (1997) 249.
[63] X. Li, S. Zeng, Sepu 14 (1996) 354; C.A. 126 (1997) 14133.
[64] H. Tanaka, K. Nakogoni, T. Tanimura, Chromatography 18 (1997) 308; C.A. 128 (1998) 97114.