Journal of Chromatography A, 666 (1994) 99–110
Elsevier Science B.V., Amsterdam

CHROM. 25 597

Unusual peak distortion in ligand-exchange chromatography of enantiomers under overloaded conditions

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ABSTRACT

The separation of amino acid enantiomers by means of ligand-exchange chromatography on an adsorbed chiral stationary phase was investigated under overloaded conditions. The selectivity of separation, the limit of mass loading and the peak shapes of enantiomers are strongly affected by the copper(II) concentration in the eluent. Under the optimum conditions it was possible to separate up to 2000 μg of amino acid on a 125 × 4 mm I.D. column. With increase in mass loading the peak shape of the enantiomers changed from symmetrical Gaussian into fronting and subsequently into trapezoidal. During this transformation, peak compression was observed. The complex changes in solute peak shape observed in ligand-exchange chromatography of amino acid enantiomers imply a complex form of adsorption isotherm in these systems.

INTRODUCTION

Ligand-exchange chromatography (LEC) of enantiomers was introduced in the early 1970s [1]. There are now many publications devoted to the analytical applications of this method, which have been intensively reviewed [2–4]. All three theoretically possible variants of chiral chromatographic systems have been described in LEC: chiral eluent method [5], stationary phases

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SSDI 0021-9673(93)E1032-U

modified by adsorption of chiral ligands [6] and numerous covalently bonded chiral stationary phases [4]. A higher column efficiency in the enantiomeric separations is mainly characteristic of the methods with chiral eluents and adsorbed stationary phases [7,8]. Covalently bonded stationary phases should be operated at elevated temperatures to give a reasonable efficiency [9,10].

Less attention has been paid to preparative or micropreparative separations by means of LEC. Davankov *et al.* [11] reported an overloading phenomenon in a system where a microbore column packed with RP-silica and modified by adsorption of N-(*n*-octadecyl)-(*S*)-hydroxyproline was used for the micropreparative separation of valine enantiomers. Interestingly, the peak distortions were different for the two enantiomers. The first-eluting enantiomer displayed a fronting peak with an increase in loading, whereas the second displayed tailing. As a result, the two peaks in the chromatogram looked like mirror reflections. The situation observed was favourable for preparative separations, because at the moment when with mass loading peak overlapping was expected, their shape changed and baseline separation was observed again.

Kinkel *et al.* [12] reported a similar observation of different peak distortions for the enantiomeric separation of Tröger base on microcrystalline triacetylcellulose. In contrast to the above system, however, the first-eluted enantiomer displayed tailing and the second fronting. The different behaviours of two enantiomers with increase in loading was correlated with a difference in the adsorption isotherms of the two Tröger base enantiomers [13].

In this paper, we describe the impact of overloading on the peak profiles of various amino acids separated by chiral LEC. Volume and concentration overloading were investigated and the general amino acid peak profile distortion was evaluated from the experimental data. The influence of different chromatographic parameters on the peak shape, the selectivity of enantiomer separations and mass loading of the column was studied.

EXPERIMENTAL

Chromatographic system

An L-6200 intelligent pump, L-4250 variable-wavelength detector, T-3100 thermostat and D-2500 integrator (all from Merck, Darmstadt, Germany) were used. All experiments were performed at a constant temperature of 25°C. A Superspher-100 RP-18 column (125 × 4 mm I.D.) was also obtained from Merck.

Reagents

Water of "for chromatography" grade, all amino acids (proline, valine, phenylalanine, norvaline, alanine, histidine and 3,3,3-trifluoro-2-aminopropionic acid) and malic acid were purchased from Merck.

Synthesis of chiral selector and amino acid copper(II) complexes

N-(*n*-Octadecyl)-(*S*)-proline was synthesized as described previously [6] with the only difference that instead of methanol, 2-propanol was used as a solvent. The copper(II) complex of N-(*n*-octadecyl)-(*S*)-proline was prepared by shaking a solution of the latter in toluene with copper(II) acetate solution. The pH of the solution was adjusted to 8.0 with sodium acetate, the organic layer was separated and dried over sodium sulphate and toluene was evaporated. The residue was crystallized from hexane. The complex was characterized by elemental analysis.

Copper(II) complexes of amino acids were prepared by mixing solutions of copper(II) acetate and amino acid of concentration *ca.* 0.1 *M* in water in a molar ratio of 1:2. The pH was adjusted to 8 by adding sodium hydroxide solution. Usually the copper(II) complex precipitated from the solution was filtered off and recrystallized from water. With proline, the aqueous solution was evaporated to dryness and the residue was extracted with ethanol. The extract was concentrated and the complex was crystallized from ethanol.

Coating procedure

A saturated solution of Cu[N-(*n*-octadecyl)-(*S*)-proline]$_2$ in methanol was pumped through

the Superspher-100 RP-18 column until the coloured eluent appeared at the outlet of the column. The column was then washed with water containing an appropriate amount of copper(II) sulphate. After equilibration the column was used in chromatographic experiments.

Determination of adsorption isotherm

The adsorption isotherm of Cu[N-(*n*-octadecyl)-(*S*)-proline]$_2$ on the Superspher-100 RP-18 column from a solution in methanol was determined by the frontal analysis method as described elsewhere [14]. The equilibrium concentration of the solute adsorbed was calculated from the integral mass balance equation:

$$Q_{i+1} = Q_i + (V_R - V_0)(C_{i+1} - C_i)/V_S$$

where Q_{i+1} and Q_i are the concentrations of the solute in the stationary phase at equilibrium with the mobile phase containing concentrations C_{i+1} and C_i of the solute, respectively, V_0 is the column void volume, V_R is the retention volume of the breakthrough front at step $i + 1$ and V_S is the volume of the packing in the column. V_0 was measured by injection of pure water on to the column and eluting with an eluent containing copper(II) ions. A negative peak was observed at 0.90 ml. The volume of stationary phase in the column calculated from the geometric volume of the column used and the column void volume was found to be 0.67 ml. The amount of packing in the column (1.01 g) was determined by weighing the packing material removed from the column and dried at 75°C and 0.01 Torr (1 Torr = 133.322 Pa).

RESULTS

A slightly modified LEC system described by Davankov *et al.* [6] was used. The Superspher-100 RP-18 column was coated with the synthesized copper(II) complex of N-(*n*-octadecyl)-(*S*)-proline from a solution of the latter in methanol. The adsorption isotherm of the chiral modifier on the Superspher-100 RP-18 column was measured to determine the saturation capacity of the packing. As can be seen from

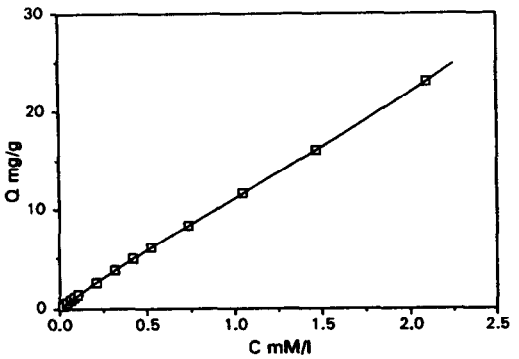

Fig. 1. Adsorption isotherm of Cu[N-(*n*-octadecyl)-(*S*)-proline]$_2$ on a Superspher-100 RP-18 column (125 × 4 mm I.D.) from solutions in methanol at 25°C.

Fig. 1, up to the highest concentration studied (the concentration of the saturated solution is *ca.* 2.1 m*M*) the experimental points are well described by the Henry isotherm and therefore the saturation capacity cannot be calculated from the isotherm observed. In this study, the coating procedure was performed with a saturated solution of the chiral selector and the amount adsorbed was found to be *ca.* 23 mg per column. No bleeding of the adsorbed chiral selector was detected because N-(*n*-octadecyl)-(*S*)-proline and its copper(II) complex are totally insoluble in the aqueous eluent used.

The separation ability of the modified adsorption column was tested with a number of analytes under typical conditions used in LEC with water containing small amounts of copper(II) sulphate as eluent. The chromatograms are displayed in Fig. 2. The column demonstrated a good resolving ability and efficiency on the analytical scale of chiral separations. Mixtures of three or four racemates could be easily resolved into individual enantiomers with this column.

The overloading of the column was investigated for seven amino acids: histidine, alanine, valine, proline, phenylalanine, norvaline and 3,3,3-trifluoro-2-aminopropionic acid. The overloading was caused by injection of different sample volumes ranging from 10 to 700 μl of three different concentrations of the amino acid, 0.34, 3.4 and 34 m*M*. Peak overlapping for amino acids exhibiting a small selectivity of

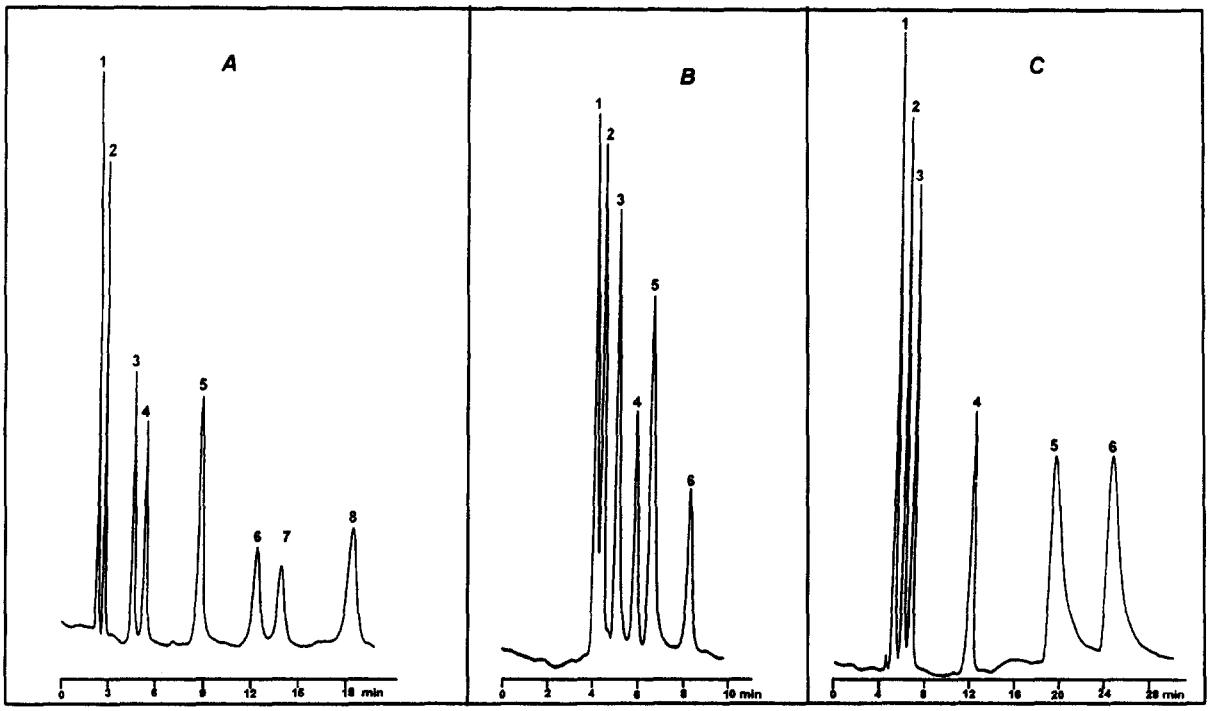

Fig. 2. Separation of amino and hydroxy acid enantiomers on a Superspher-100 RP-18 column (125 × 4 mm I.D.) coated with Cu[N-(*n*-octadecyl)-(*S*)-proline]$_2$. Eluent, water containing 0.05 m*M* $CuSO_4$; flow-rate, (A, C) 0.5 ml/min and (B) 0.25 ml/min. Solutes: (A) 1 = (*S*)-alanine, 2 = (*R*)-alanine, 3 = (*S*)-valine, 4 = (*S*)-norvaline, 5 = (*S*)-methionine, 6 = (*R*)-valine, 7 = (*R*)-norvaline, 8 = (*R*)-methionine; (B) 1 = (*S*)-lysine, 2 = (*R*)-lysine, 3 = (*S*)-arginine, 4 = (*S*)-citrulline, 5 = (*R*)-arginine, 6 = (*R*)-citrulline; (C) 1 = (*R*)-histidine, 2 = (*S*)-histidine, 3,4 = 3,3,3-trifluoro-2-aminopropionic acid, 5,6 = malic acid.

enantiomer separation, *i.e.*, histidine and alanine, was observed, which prevented the evaluation of peak shapes over a wide range of mass loadings. Therefore, subsequent experiments were performed with amino acids showing a high selectivity of separation, *e.g.*, proline and valine.

As a representative example, Fig. 3 shows the changes that occurred in the chromatograms of (*R*,*S*)-valine. The chromatograms were measured at three different concentrations of copper(II) sulphate in the eluent. At a copper(II) concentration of 0.05 m*M* (Fig. 3A) the peak of the first-eluted enantiomer increased in intensity with increase in loading, whereas the peak of the second-eluted enantiomer remained of lower intensity and moved towards the first peak. At loadings above 400 μg there was no longer any separation of the peaks, which coalesced into one tailing peak. The picture described is essentially the same as was observed by Guiochon *et al.* [15] for the resolution of N-benzoylalanine on a bovine serum albumin-coated packing.

With an eluent containing 50 m*M* of copper(II) sulphate, the peaks of both enantiomers became of equal intensity at higher mass loadings and markedly broader than in analytical separations (Fig. 3C). At loadings of 300–400 μg the peaks overlapped with each other and their separation was no longer complete. As can be seen in Fig. 3C, at this high copper(II) concentration in the eluent it is much better to inject a sample in a smaller volume with a higher concentration of the amino acid than to inject a larger volume with a lower concentration. For example, injection of 100 μl at a concentration of 34.14 m*M* allowed almost complete resolution of the loading of 399 μg of valine, whereas for a sample of 700 μl with a concentration of 3.41 m*M* (loading 279.7 μg) only a very poor separa-

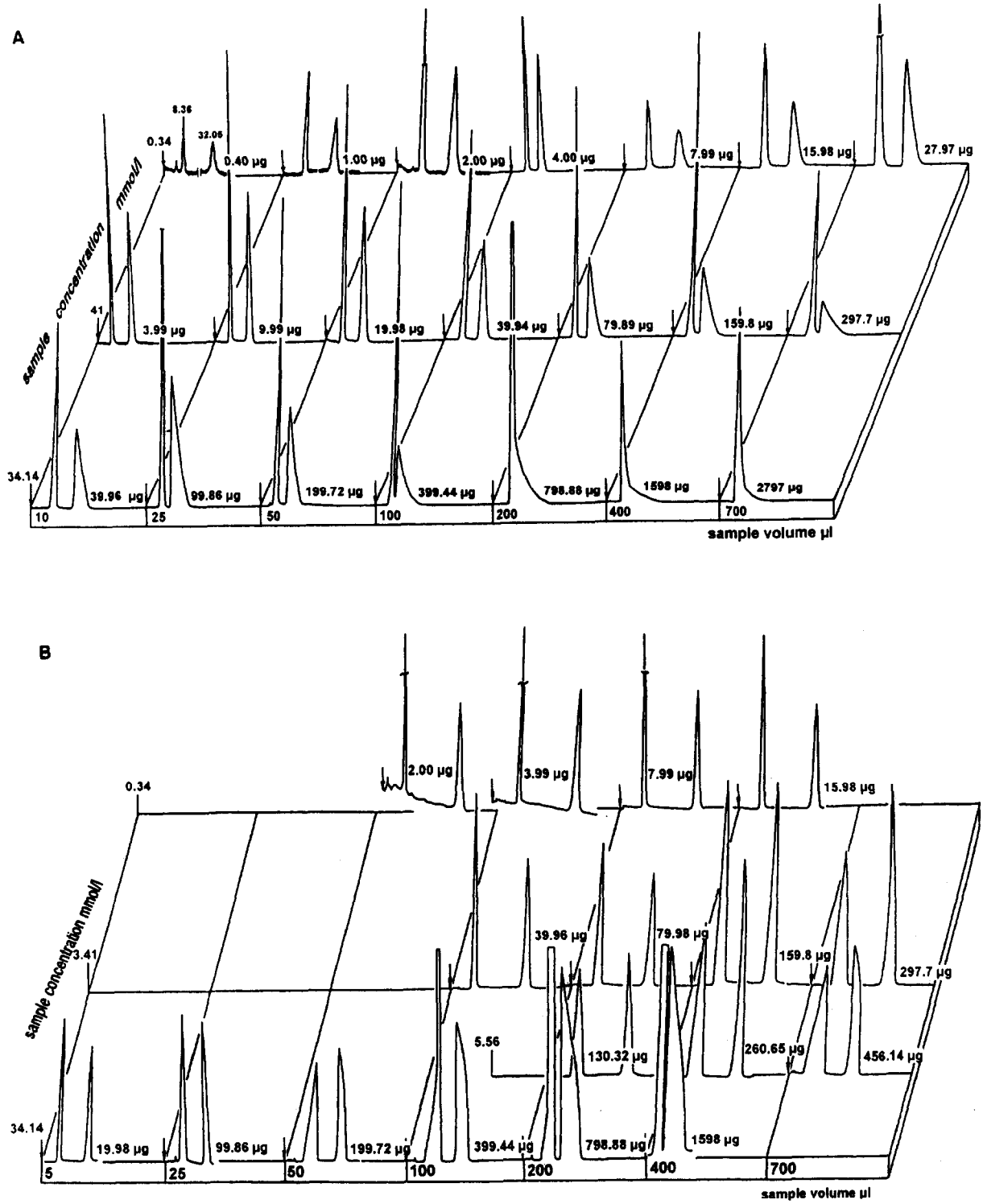

Fig. 3.

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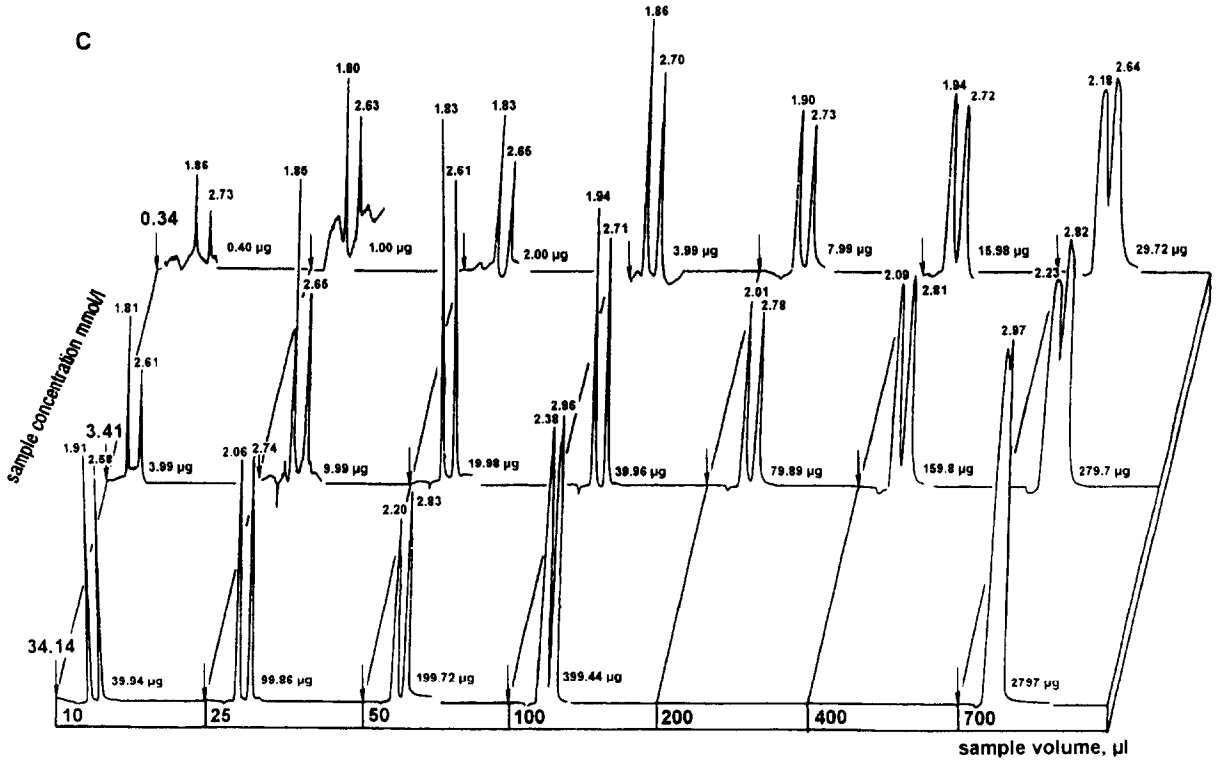

Fig. 3. Resolution of (*R*,*S*)-valine on a Superspher-100 RP-18 column (125 × 4 mm I.D.) coated with Cu[N(*n*-octadecyl)-(*S*)-proline]$_2$ with various sample volumes or sample concentrations. Flow-rate, 1 ml/min. Eluent: water containing (A) 0.05, (B) 0.5 and (C) 50 m*M* $CuSO_4$. Values at peaks indicate retention times in min.

tion was observed. In contrast, approximately the same separations were observed for these samples with an eluent containing 0.05 m*M* copper(II) sulphate (Fig. 3A).

The most unexpected peak shape changes were observed at the intermediate copper(II) concentration of 0.5 m*M* in the eluent (Fig. 3B). An increase in loading caused first an increase in the peak height of the second-eluted enantiomer, with the two peak heights becoming equal. Until this moment, the peak shape of both enantiomers can be described as fronting. A further increase in loading changed the peak shape of the second-eluted enantiomer into a trapezoidal form with a small shoulder at the front of the peak. This second peak was now markedly higher and slimmer than the first peak. With further increase in sample volume injected, compression of the first peak also occurred (see Fig. 3B, loading 399.44 µg). This was much more pronounced than for the second-eluted enantiomer. Further increases in loading did not cause unusual changes: the first peak remained slim and high, whereas the second peak was wider and lower. The second peak moved towards the first peak until both peaks merged. The resolving ability of the column at the intermediate range of copper(II) concentrations is markedly higher than at higher or lower copper(II) concentrations in the eluent. A 1598-µg amount of (*R*,*S*)-valine could be baseline resolved on the 125 × 4 mm I.D. column.

The chromatographic conditions in the above experiments are typical for chromatographic operations in LEC where the analyte is simply added to the eluent used. The results obtained by this common method at high loadings are difficult to interpret, as too many parameters are changed at once. Amino acids (AK) added to the eluent are complexed with copper(II) ions according to the following reactions:

$$AK + Cu^{2+} \rightleftharpoons Cu(AK)^{+} + H^{+} \quad (1)$$

$$Cu(AK)^+ + AK \rightleftharpoons Cu(AK)_2 + H^+ \quad (2)$$

Hence any change in the amino acid concentration in the sample causes certain changes in the concentration of all its copper(II) complexes and also the pH of the eluent. To simplify the system, we examined the use of synthesized complexes of amino acids and studied the above-described regularities with individual complexes. The dissolution of the complexes in the eluent does not cause any changes in its pH, but the dissociation

$$Cu(AK)_2 \rightleftharpoons Cu(AK)^+ + AK^- \rightleftharpoons Cu^{2+} + 2AK^- \quad (3)$$

or disproportion of the complexes

$$Cu(AK)_2 + Cu^{2+} \rightleftharpoons 2Cu(AK)^+ \quad (4)$$

are still possible. Taking into account the relatively high stability of copper(II) amino acid complexes (the stability constants of mono-complexes ranged between 10^7 and 10^9 and those of bis-complexes between 10^{12} and 10^{14} [16]) and the presence of free copper(II) ions in the eluent, the dissociation of complexes (Eq. 3) can be neglected. The disproportion (Eq. 4) can contribute to the distribution of solute species especially at high copper(II) concentrations.

Using the amino acid complexes as solutes, however, caused another problem: their solubility in the eluent was found to be lower than that of uncomplexed amino acids. Therefore, only complexes of valine and proline were subjected to further experiments, as these have the highest solubility among the studied compounds. The complex of phenylalanine is poorly soluble. Interestingly, the solubility of the optically active form of phenylalanine complexes was found to be lower than for the racemic form. The opposite relationship of solubilities of optically active and racemic complexes is more common for amino acids.

Fig. 4 shows the resolution of the copper(II) complex of (*R,S*)-valine with increasing mass loading. The peak changes observed here are

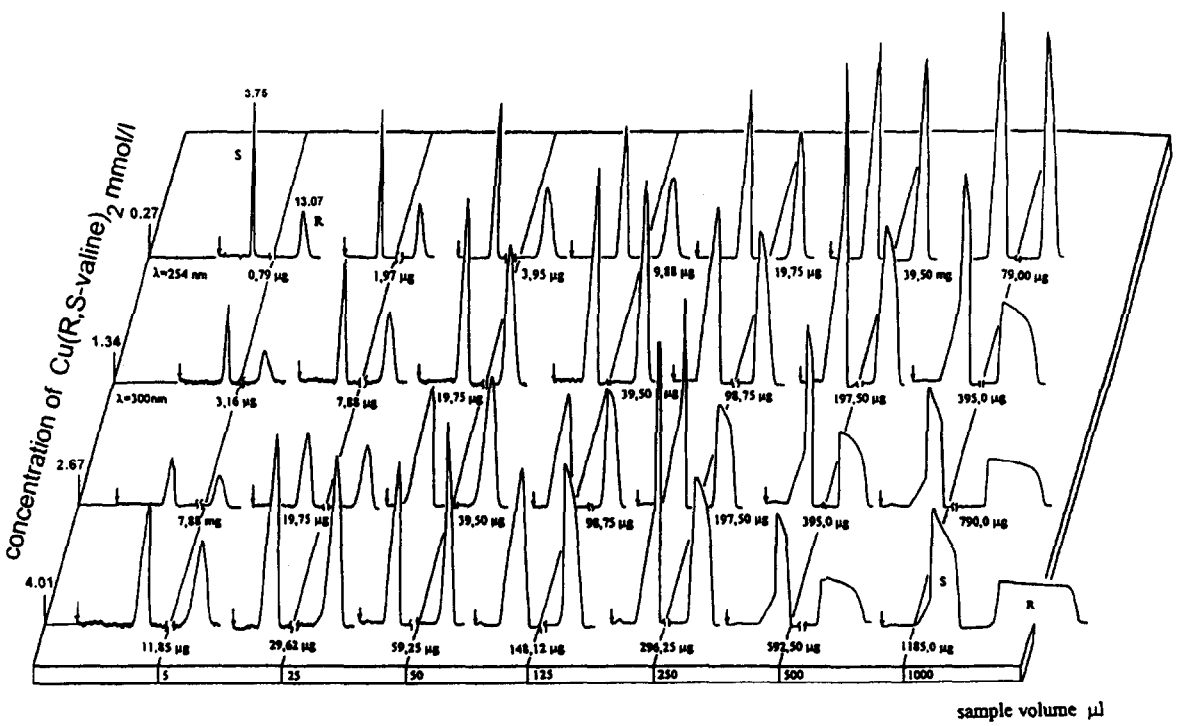

Fig. 4. Resolution of Cu[(*R,S*)-valine]$_2$ on a Superspher-100 RP-18 column (125 × 4 mm I.D.) coated with Cu[N-(*n*-octadecyl)-(*S*)-proline]$_2$ with various sample volumes and sample concentrations. Eluent, water containing 0.5 m*M* $CuSO_4$; flow-rate, 1 ml/min.

essentially the same as described above. The only difference is that, after the peak shape had changed to a trapezoidal form, it did not change any more with further increase in mass loading. The compression of the second peak was not expressed strongly; only the first peak demonstrated compresssion distinctly at a loading of 296 mg. The trapezoidal form of the peaks now looked much sharper than it was in the chromatography of the free amino acids, where the corners were smoothly rounded off. The highest loading in this series of experiments was 1185 μg of valine complex injected as 1 ml of saturated solution (concentration *ca.* 4 m*M*). Complete separation of the peaks was observed, and the loading of the column could be increased even further, as the peaks were still well separated from each other.

An increase in the copper(II) concentration in the eluent has the same effect on the peak shapes of copper(II) complexes of proline and valine enantiomers, and as a representative example this dependence for copper(II) complexes of proline is shown in Fig. 5. Unexpectedly, with pure water as the eluent the efficiency of the column was found to be very low. The second peak was especially broad and could hardly be detected. The presence of copper(II) ions in the eluent improved the peak shape drastically. On the analytical scale, we now observed a highly efficient separation of enantiomers. Injection of progressively increasing amounts of the complex caused changes in the peak shape to trapezoidal, as described above. With further increase in copper(II) concentration in the solution, both enantiomer peaks become slim and independent in their shape of the amount of sample injected. At the same time, the retention of the enantio-

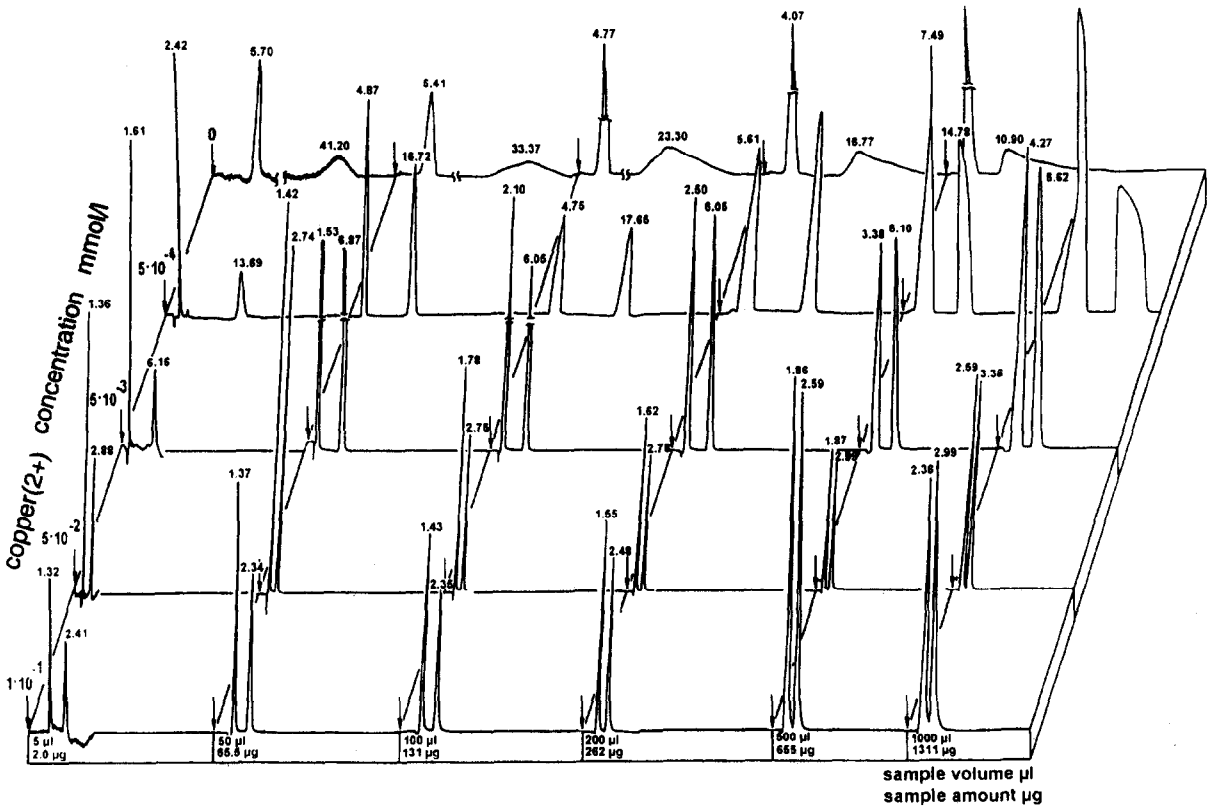

Fig. 5. Resolution of Cu[(*R*,*S*)-proline]$_2$ on a Superspher-100 RP-18 column (125 × 4 mm I.D.) coated with Cu[N-(*n*-octadecyl)-(*S*)-proline]$_2$ with various mass loadings of the complex or copper(II) concentration in the eluent. Eluent: water containing $CuSO_4$; flow-rate, 1 ml/min. Values at peaks indicate retention times in min.

mers and the selectivity of separations were strongly decreased at higher copper(II) concentrations on both analytical and preparative scales. On the analytical scale (sample injected smaller than 1 μg, volume injected 20 μl) the selectivities of separation remained unchanged with increasing copper(II) concentrations in eluent up to 5 m*M* and than decreased quickly with further increase in copper(II) concentration to $\alpha \approx 2$ (Fig. 6B). These decreases in selectivities are the result of decreases in the retentions of both enantiomers. The dependence of log k' on log [copper(II) concentration in the eluent] is almost linear for *R*-enantiomers whereas for *S*-enantiomers it is parabolic (Fig. 6A).

On the preparative scale of separations with

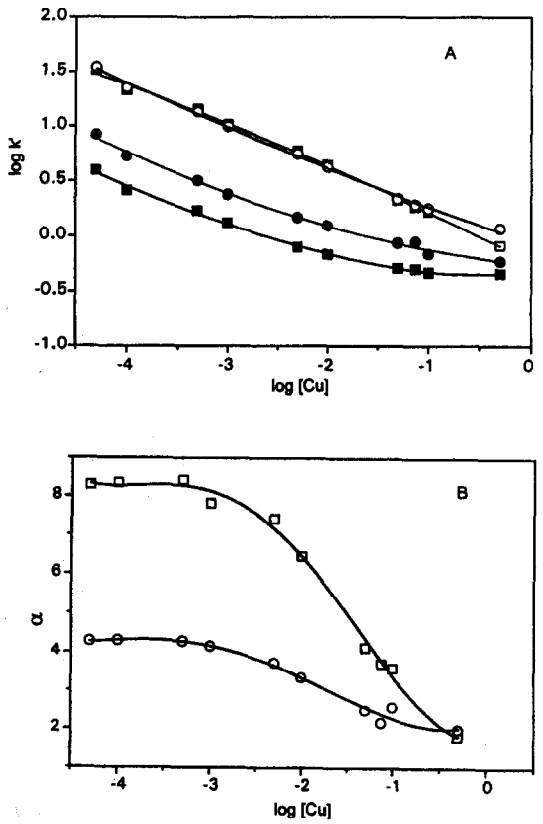

Fig. 6. Dependence of (A) capacity factors, k', and (B) selectivities, α, of the separation of copper(II) complexes of (□, ■) proline and (○, ●) valine on a Superspher-100 RP-18 column (125 × 4 mm I.D.) coated with Cu[N-(*n*-octadecyl)-(*S*)-proline]$_2$ on copper(II) concentration in the eluent. Closed symbols = *S*-enantiomers; open symbols = *R*-enantiomers.

an eluent containing 100 m*M* of copper(II), injection of 1311 μg of the copper(II) complex of proline already produced a small overlap between the peaks (Fig. 5), whereas the same amount of the complex could be completely separated at a moderate copper(II) concentration of 0.5 m*M* and the limit of column loading was not reached (Fig. 5).

DISCUSSION

The results presented above have two different aspects. The first is practical and is related to the problem of the choice of conditions for preparative separations by means of chiral LEC. The second is more theoretical and is related to changes in the peak shape with increase in column loading.

From a practical point of view, the results clearly demonstrate that the separation should be performed at an optimized copper(II) concentration in the eluent. Both a large excess and an insufficient concentration cause a rapid decrease in separation efficiency. It would be preferable to inject the amino acid in the form of its copper(II) complex rather than as the free species. Injection of free amino acids at a high concentration causes distortion of the column equilibrium and requires re-equilibration after each chromatographic run. With a low solubility of the complex the use of the free amino acid may still be preferred, which would allow a higher mass loading to be obtained. The peak shapes under such non-equilibrium conditions can be even more peculiar than those described above. As an example, the peak shapes observed in the separation of free phenylalanine are shown in (Fig. 7). The trapezoidal form of the peaks here is strongly distorted owing to non-equilibrium conditions caused by changes in the pH and copper(II) concentration in the chromatographic zones. Nevertheless, the injected amount of 1600 μg of phenylalanine was almost completely resolved on the 125 × 4 mm I.D. column. If the equilibrium conditions are less distorted, the column loading can be even higher; thus 2200 μg of the Cu(*R*,*S*-valine)$_2$ complex could be completely resolved with the same column.

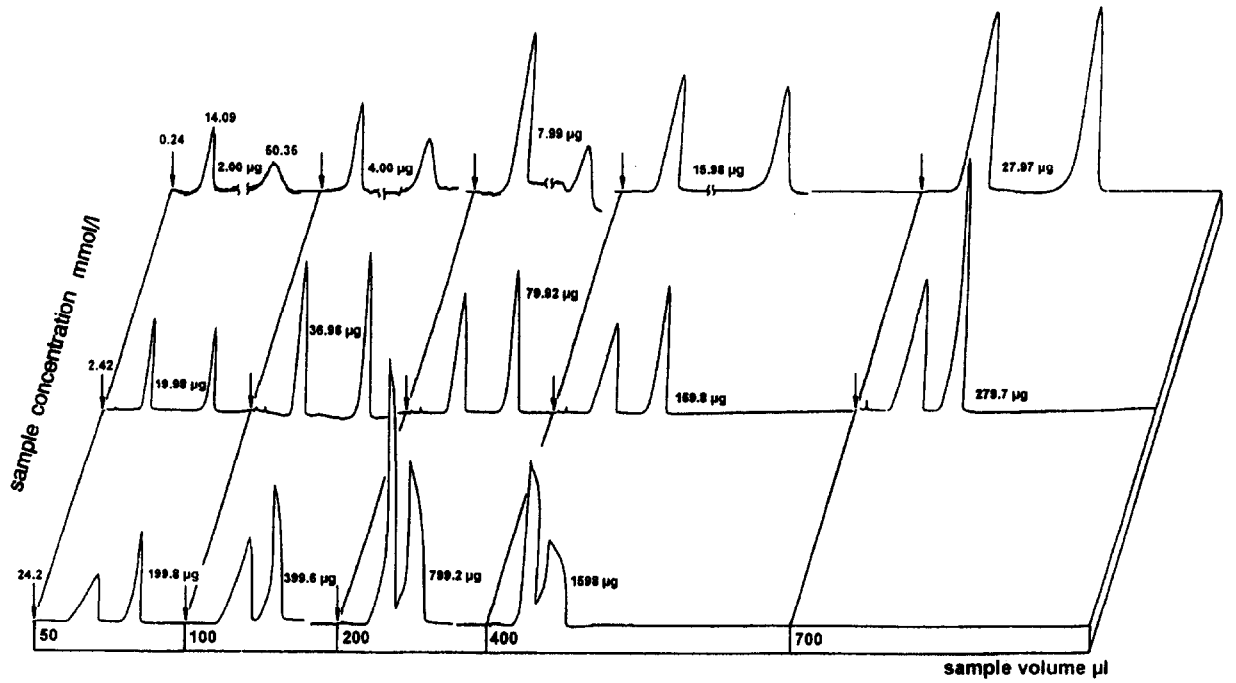

Fig. 7. Resolution of (*R,S*)-phenylalanine on a Superspher-100 RP-18 column (250 × 4 mm I.D.) coated with Cu[N-(*n*-octadecyl)-(*S*)-proline]$_2$. Eluent, water containing 0.5 m*M* $CuSO_4$; flow-rate, 1.5 ml/min.

In addition to the above practical conclusions, some other points of interest arose from the experimental results:

(1) the same mass loading of the column, depending on the copper(II) concentration in the eluent, causes different changes in the chromatograms compared with the analytical separation;

(2) chromatography of amino acid copper(II) complexes with pure water as the eluent demonstrates a very low efficiency of separation, which improves drastically on adding a low small concentration of Cu(II) ions to the eluent;

(3) in the intermediate range of concentrations of Cu(II) ions in the eluent an increase in column loading is possible, revealing complicated changes in the peak shapes of both enantiomers. Strong peak compression at defined mass loadings was detected, which was followed by the transformation of the peak into a trapezoidal shape.

The changes observed on the chromatograms of uncomplexed amino acids with the increasing copper(II) concentration in the eluent can be connected with the complexation equilibria in Eqs. 1 and 2 in the system. The injected amino acid remains mainly uncomplexed in eluents of low copper(II) concentration. At a high copper(II) concentration, the adsorbed chiral selector and injected amino acid mainly exist in the column in the complexed form. Hence with an eluent of low copper(II) concentration the column was overloaded with the uncomplexed amino acid, whereas with an eluent of high copper(II) concentration the column was loaded with the copper(II) complex of the amino acid. The adsorption abilities of a free amino acid and its copper(II) complex on a reversed-phase packing differ strongly and it affect the resulting chromatogram.

The low efficiency of the column observed with pure water as the eluent is difficult to explain. The copper(II) complex of proline was subjected to chromatography (Fig. 5) and, therefore, copper(II) was present in the starting chromatographic zone in the ratio of one copper atom to two solute molecules. One can suspect that the sample injected into the column soon becomes strongly diluted and an irreversible

dissociation process (Eq. 3) becomes of importance. In Fig. 3 the separation of valine as the free amino acid with an eluent containing only 0.05 m*M* Cu(II) is shown, and there no decrease in the column efficiency was observed. Obviously, this amount of copper(II) in the eluent, combined with the copper(II) pool accumulated by the chiral selector of the packing, appears sufficient for the complexation equilibrium to be maintained through the whole length of the column.

In the presence of moderate concentrations of copper(II) in the eluent, the most interesting feature of the systems investigated is the transformation of sharp symmetrical peaks into a trapezoidal shape with increase in mass loading. Trapezoidal peaks are not very common in chromatography. Nevertheless, this peak shape was theoretically predicted by Guiochon *et al.* [17] and observed by them in the separation of phenyldodecane on porous carbon [18]. The appearance of such a peak shape was explained by the highly heterogeneous nature of the surface of porous carbon, where it was not possible to obtain a symmetrical peak even with an analytical concentration of the solute. With the packing used in this work, fairly symmetrical peaks were observed on the analytical scale, hence the surface of the packing at least towards small loadings seems to be homogeneous and additional investigations need to be carried out to elucidate the changes observed.

Another intriguing feature of our systems is the peak compression observed just before the peak acquires a trapezoidal form. Compression was not predicted by the theoretical investigations of Guiochon *et al.* [17] and it is not clear yet what kind of processes may induce this effect. According to Guiochon *et al.*, the key to understanding separations in preparative chromatography is competitive isotherms [14]. Measurements of adsorption isotherms are now in progress.

CONCLUSIONS

The LEC system with an adsorbed chiral selector was investigated under overloaded conditions. Up to 2000 μg of amino acid can be resolved with a 125 × 4 mm I.D. column in one chromatographic run. Higher selectivity and higher mass loadings can be reached when an amino acid is injected into the column in the form of the copper(II) complex. The injection of free amino acids may be preferable for some amino acids if the solubility of their copper(II) complexes is too low.

The selectivity of separation, the limit of mass loading and the peak shape of enantiomers under overloaded conditions are strongly affected by the copper(II) concentration in the eluent. The highest selectivity was observed in the intermediate range of copper(II) concentrations. In this range, the peaks of enantiomers change into a trapezoidal shape with increase in loading. During this transformation peak compression was observed, which first occurred for the second-eluting enantiomer and then for the first-eluting enantiomer. Such a transformation of peak shapes with an increase in loading implies a complex form of adsorption isotherm of enantiomers in the LEC system.

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