Separation of Racemic Mixtures of Amino Acids Using Chiral Eluents

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A commercially available non-chiral RP-C8 chromatographic column is used with an aqueous chiral mobile phase containing N,N-dimethyl-L-phenylalanine and copper in a ratio 20 : 1 for the enantiomeric resolution of the following amino acids: DL-valine (Val), DL-methionine (Met), DL-leucine (Leu), DL-phenylalanine (Phe) and DL-tyrosine (Tyr). The influence of organic modifier, temperature and flow-rate on the capacity factors and on the selectivity is experimentally determined and the separation behavior is discussed. The capacity factors of some of the amino acids remained constant with changing the composition of the mobile phase, i.e. increasing methanol amount. With increase of the temperature the retention times decreased and the selectivities were slightly reduced. In a study of the effect on retention and selectivity, linear van't Hoff plots were obtained giving ΔH and ΔS values of the solute transfer from the mobile to the stationary phase. These were found to be within the range obtained for hydrophobic compounds in RP-HPLC systems. Using van Deemter plots high separation efficiency and low apparent axial dispersion coefficients for all amino acids could be determined.

Key words:
Amino acid, chiral mobile phase, ligand exchange chromatography, van Deemter plot, van't Hoff plot

Introduction

The increasing demand of optically active substances in respect of high purities and amounts in the pharmaceutical, food and chemical industries necessitates the development of new separation and production techniques. Such substances are amino acids, amino alcohols, amines, alcohols and epoxides. Amino acids are used as products and intermediates for pharmaceuticals as well as in the food industry for production of sweetening agents and flavor enhancers.[1,2] All amino acids, with the exception of glycin, are chiral substances; however, only the single enantiomeric forms are used. Modern technologies for production of amino acids single enantiomers are based on chemical or biochemical synthesis and fermentation methods.[3–7] The asymmetric synthesis is associated with high costs, complexity, small yields, etc. Due to this, the separation of enantiomers from racemic mixtures is quite often an alternative. For these separations the most commonly used methods are fractioning crystallization and chiral chromatography.[8,9]

Furthermore, the analysis and purification of amino acids and peptides is an important step in the pharmaceutical industry, where one of the enantiomeric forms can be even toxic and cause fatal consequences in the human body. The synthesis and the use of chiral amino acids are particularly important in bioorganic and medical chemistry. Therefore, reproducible methods to analyse enantiomeric mixtures of these compounds are needed to be used routinely.[10]

Separation of the enantiomers is generally conducted by resolution of a racemic modification using chiral reagents. The separation method used then makes use of the different physical properties of the resulting diastereomers, such as solubility in the case of fractional crystallization. Here the enantiomeric separation of amino acid racemates is investigated using the ligand exchange chromatography (LEC). In this process, the diastereomeric complexes formed differ in their stability constants and distribute in a different manner between the stationary and the mobile phase, resulting in a different chromatographic retention.

High performance liquid chromatography (HPLC) is one of the most frequently used methods for analysis of enantiomeric mixtures. However, the commercially available chiral stationary phases have some disadvantages, e.g. high costs and short lifetimes. Some of the phases can be even seldom available because of the complexity of their synthesis. These disadvantages have made the chiral chelate addition in the mobile phase a more attractive alternative.[11]

The LEC developed by Davankov in the early 1970's[12–14] is a more cost effective analytical

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method, as chiral mobile phases combined with a commercial RP C18 column can be used. In order to achieve a LEC process, a chiral mobile phase additive is used, which forms different stable complexes with the L- and D-amino acid enantiomers in the presence of copper. The addition of chiral metal complexes to the eluent is particularly useful in analytical applications, being very easy to handle, flexible and efficient.

In the literature[15–18] very often free or modified amino acids are used as chiral selectors for the separation of α-amino acids and Dns-α-amino acids, but mostly a post column derivatization of the solutes is required. However, this additional procedure elongates the analysis time and causes higher costs because of the supplementary equipment and reagents.

In the present work, the separation of racemic mixtures of amino acids (phenylalanine, tyrosine, valine, methionine and leucine) applying LEC was investigated. The used chiral selector was N,N-dimethyl-L-phenylalanine dissolved in water as a copper complex at copper:selector ratio 1 : 20. The stationary phase used was a commercially available non-chiral RP-C8 column. The goal of the present paper was to investigate the influence of selector:copper ratio, flow-rate, temperature and organic modifier on the separation of amino acids in the LEC. Reducing the amount of copper in the mobile phase enables direct application of the eluent without adjustment of the pH. It could be shown that the higher ratio saves time and chemicals; furthermore, the absorbance is less intensive. Under these conditions, in a very short time good separation was achieved with pure aqueous mobile phase containing just the selector and the copper.

Materials and methods

Materials

All amino acids, L-phenylalanine, D-phenylalanine, DL-phenylalanine, DL-valine, L-valine, DL-tyrosine, DL-leucine, L-leucine, DL-methionine and L-methionine were supplied by Fluka (Buchs, Switzerland) in analytical grade quality and were used with no further purification. Bi-distilled water was used for preparing the stock and working solutions. Copper(II) acetate and N,N-dimethyl-L-phenylalanine were purchased by Sigma (St. Louis, MO, USA). The methanol used was of HPLC grade from Merck (Darmstadt, Germany).

HPLC analysis

The experiments were carried out with a high performance liquid chromatographic unit equipped with an UV/VIS photodiode array detector SPD-M10Avp (Shimadzu), solvent delivery module LC-10ADvp (Shimadzu), an on-line vacuum degasser DGU-20A3 Prominence (Shimadzu), and an autosampler SIL-20A Prominence (Shimadzu). The chromatographic column was placed in a glass water jacket connected to a thermostat (Julabo F12) to gain temperature control. A non-chiral alkyl silica RP-C8 chromatographic column (Zorbax-Eclipse XDB-C8, 150 x 4 mm, 5 μm) from Agilent Technologies (Waldbronn, Germany) was used. In all the experiments 0.1 mmol L^{-1} copper(II) acetate and 2 mmol L^{-1} N,N-dimethyl-L-phenylalanine in water or in methanol/water was used as eluent. The resulting pH of the mobile phase was between 4.95 and 5.05.

Flow rates of 1 mL min^{-1} at room temperature were used unless indicated otherwise. Generally, UV detection was performed at 254 nm. The injected sample volume was 20 μL and the amino acids concentrations were 0.5 mg mL^{-1}, and for tyrosine 0.25 mg mL^{-1}, all the samples were prepared in water.

Data analysis

The volume of the liquid in the chromatographic column was determined using water as non-adsorbable component. It is equal to 1.37 mL. Knowing the retention time of the non-adsorbable component, the total volume of liquid in the chromatographic column and the column volume, the total column porosity was calculated using the following formula:

$$\varepsilon_t = \frac{t_0 \cdot F}{V_{column}} \tag{1}$$

where t_0 is the retention time of non-adsorbed component [min], F is the flow rate [mL min^{-1}] and V_{column} is the column volume [mL]. The total porosity was calculated to 0.55. From the porosity, the phase ratio was calculating using eq. (2) being equal to 0.82:

$$\phi = \frac{1 - \varepsilon_t}{\varepsilon_t} \tag{2}$$

The separation factor α was determined as the ratio between the capacity factors of the enantiomers:

$$\alpha = \frac{k'_L}{k'_D} \tag{3}$$

and the capacity factors were determined as follows:

$$k' = \frac{t_{R,i} - t_0}{t_0} \quad (4)$$

where $t_{R,i}$ is the retention time of any enantiomer (i = D or L).

The capacity factors were average values of at least triplicate determinations, whereas the standard deviation was between 1 and 2 %.

For the determination of the apparent dispersion coefficient, D_{ai} injections of low concentrated samples were performed to generate analytical peaks. Through the retention times of these peaks $t_{R,i}$ and the width at half peak height $w_{i,1/2}$, calculation of the number of theoretical plates for each component N_i could be performed:

$$N_i = 5.54 \frac{t_{R,i}^2}{w_{i,1/2}^2} \quad (5)$$

The apparent dispersion coefficient $D_{a,i}$ was calculated from the well known relation:[19]

$$D_{a,i} = u \cdot L/2N \quad (6)$$

where u is the interstitial velocity [cm s^{-1}] and L is the length of the column [cm].

With the known number of theoretical plates and column length, the height of one theoretical plate HETP$_i$ could also be correlated for each component:

$$\mathrm{HETP}_i = \frac{L}{N_i} \quad (7)$$

The number of theoretical plates and respectively the HETP values are different for each component, and depends on the interstitial velocity in the column. This dependency is expressed through the van Deemter equation:

$$\mathrm{HETP}_i = A_i + B_i u + \frac{C_i}{u} \quad (8)$$

The three terms in this equation describe the different broadening effects in the chromatographic column. The A-term is governing with the eddy-diffusion and almost constant through the column. The B-term is connected to the mass transfer resistance and the C-term is with the axial dispersion.

For determining the thermodynamic parameters, the van't Hoff plot was used:

$$\ln k' = -\Delta H/RT + \Delta S/R + \ln \phi \quad (9)$$

where k' is the capacity factor, R is the gas constant [J mol^{-1} K^{-1}], T is the absolute temperature [K], ϕ is the phase ratio in the column (the volume of stationary to that of mobile phase), ΔH and ΔS are respectively the enthalpy [J mol^{-1}] and the entropy [J mol^{-1} K^{-1}] differences of transfer of a solute from the mobile to the stationary phase. If the diagram ln k' versus T^{-1}, the van't Hoff plot, is linear, then the enthalpy and entropy are constant and the mechanism of the retention process is invariant over the whole temperature range under investigation.[20] Thus, ΔH can be determined from the slope and ΔS from the intercept of the regression line, provided the column phase ratio is known. All the measurements were done under isocratic conditions at a constant flow rate of 1 mL min^{-1}. The retention times necessary for the calculation of the thermodynamic parameters were obtained from at least three individual determinations. The relative standard deviations of the slopes were between 2 and 8 %. The correlation coefficients were 0.991 for D-Phe; 0.982 for D-Leu; 0.987 for D-Met and 0.998 for D-Tyr.

Results and discussion

The capacity factors for the amino acids were very low in the aqueous eluent (between 0.02 and 4.03 for the D-enantiomers and between 0.38 and 6.03 for the L-enantiomers) decreasing in the following sequence for the D-enantiomers: D-Phe > D-Tyr > D-Met > D-Leu > D-Val and for the L-enantiomers: L-Phe > L-Tyr > L-Leu > L-Met > L-Val. For all amino acids, the D-form always eluted first. The peak identity was controlled by injecting the L-enantiomers. At the experimental pH (below the isoelectrical point) the capacity factors are very low due to the increased concentration of ligand (NH_3) and partial protonation of the ligands competing for coordination with copper(II) ions.[21] Compared to LEC with chiral stationary phase, the retention times here were much shorter at a lower flow rate,[22,23] which saves time and mobile phase.

The hydrophobicity of the amino acids is directly linked to the retention of the diastereomeric complexes on the RP-C8 solid support in the column. Amino acids with high hydrophobicity are retained stronger by the RP-C8 support and have therefore higher capacity factors. Thus, the following order should be observed with a stoichiometry of copper:selelector of 1 : 2: Phe < Leu < Tyr < Val < Met. However, with a copper:selector ratio of 1 : 20, a different order for the D and L enantiomers was found, e.g. not according to the hydrophobicity of the amino acids.[24] Obviously, besides the hydrophobic interaction, the equilibrium of the ligand-exchange as well as steric hindrance cannot be neglected.[15] The separation of amino acid enantiomers with straight-chain alkyl substituents at the amino group gave also highest selectivity. The selectivity

for Leu and Tyr is 2.5, for Met and Phe it is 1.5 for all flow rates investigated. It seems that the hydroxyl group of Tyr favours the steric formation of the complex because of additional hydrogen bond compared to Phe. In the case of Met, the presence of sulphur could somehow disturb the complex formation thus resulting in a lower selectivity.

Influence of organic modifier

Usually, increasing the concentration of organic modifier in the eluent in the reversed phase chromatography leads to reduction of the retention times of the solutes because of an increasing solvent strength. The influence of methanol as modifier was investigated with the aliphatic amino acids Val, Leu, and Met. The results are summarized in Table 1. Even though the three amino acid diastereomeric complexes differ in their hydrophobicity, almost no influence was observed on enantiomer retention times. One explanation for this could be, that hydrophobic interactions markedly contribute to retention (what could also be seen from the capacity factors of the L-enantiomers) and these hydrophobic interactions appear to be important enough to cause a slightly increase in the enatioselectivity with a pure aqueous phase (see Table 1).

In general, the amino acids having large aliphatic or aromatic substituents at the alpha-carbon atom, display the highest *k*' values with pure water as eluent. This indicates that hydrophobic interactions with the sorbent surface are mainly responsible for the retention of these solutes.[10] Normally, retention decreases with increasing content of organic component in the eluent. These regularities do not hold for hydrophilic amino acids where the contribution from hydrophobic interactions to the total retention is small compared with the contribution from coordination interactions,[14] which is also confirmed from the obtained results (see Table 1). Under slightly different chromatographic conditions (with copper concentration 1 mmol L^{-1} and at pH 4.5 and RP-C18 column), the capacity factors of D-enantiomers were reduced by 40 to 60 % and for the L-enantiomers by 50 to 70 % with a mobile phase containing 10 % methanol. However, the selectivity decreased with increased modifier content, so pure water remained the optimal eluent phase.[15]

Influence of temperature

It is known that separating enantiomers at reduced temperatures is less applicable to LEC since ligand-exchange complexation is a relatively slow process. That is why increasing temperature leads to higher efficiency.[25] With increased temperature, the solute−sorbent interaction rates and the diffusion coefficients improved, resulting in higher column efficiency. However, in respect to enantioselectivity, there is a point called T_{inv} (not measured here since the maximum allowed column temperature is 60 °C), which is the temperature when $\ln \alpha = 0$ and both enantiomers elute with the same k' values.[26] Changing this point allows control of the enantiomers' elution order.[27] In general, with increasing the temperature the selectivity decreases, but also the opposite effect is possible as entropic contributions dominate over the enthalpic contributions.

In order to clarify all these facts, the effect of temperature on the retention and separation of amino acid enantiomers between 10 °C and 50 °C, was investigated. Fig. 1 depicts the decrease of the

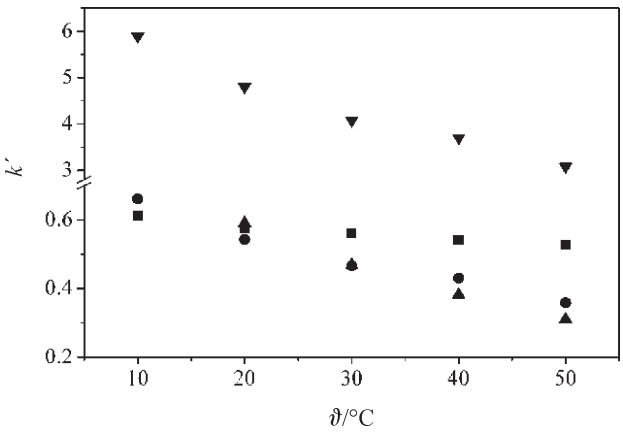
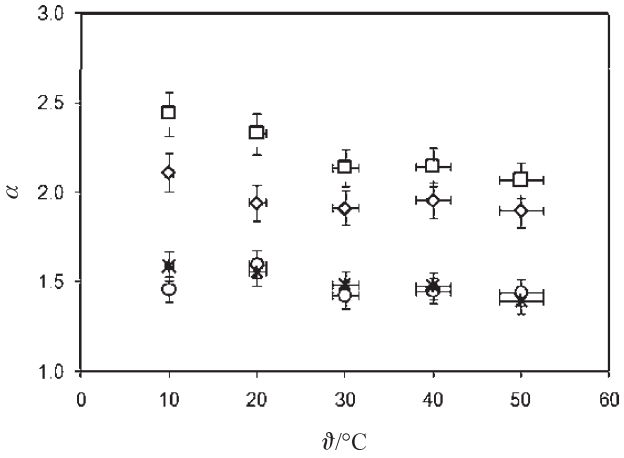


Fig. 1 – *Capacity factors as a function of temperature for the D-enantiomers, F = 1 mL min^{-1}:* ▼ *– k' D-Phe;* ■ *– k' D-Leu;* ▲ *– k' D-Met and* ● *– k' D-Tyr*

Table 1 – *Influence of organic modifier on the capacity factors*

	Water			5 % Methanol			10 % Methanol			15 % Methanol		
Amino acid	k'_D	k'_L	α	k'_D	k'_L	α	k'_D	k'_L	α	k'_D	k'_L	α
Valine	0.08	0.74	–	0.05	0.65	–	0.08	0.65	–	0.05	0.62	–
Methionine	0.91	1.39	1.52	0.82	1.22	1.50	0.77	1.13	1.47	0.82	1.2	1.47
Leucine	0.86	2.03	2.36	0.77	1.77	2.31	0.74	1.68	2.25	0.77	1.75	2.28

capacity factors of the D-enantiomers with increasing the temperature. As expected, there was a linear correlation between the capacity factors and the temperature. For the L-enantiomers, similar results were obtained (not depicted here). The selectivity for Phe and Met (see Fig. 2) remain constant and equals 1.5 in the whole temperature range investigated. In the case of Tyr and Leu, the selectivity slightly decreased from 2.5 to 2.2.



F i g . 2– *Selectivity as a function of temperature, F = 1 mL min^{-1}: * – D,L-Phe; ◇ – D,L-Leu; ○ – D,L-Met and □ – D,L-Tyr*

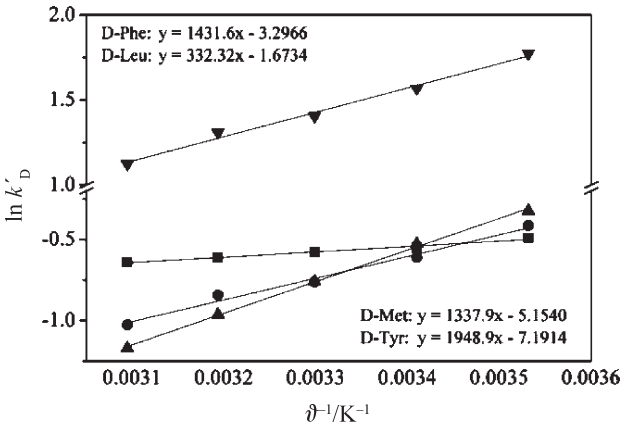
Chromatographic retention can be used to calculate the partial molar enthalpy of the transfer of a solute from mobile to stationary phase. Using the van't Hoff plot, the authors could determine the enthalpy and entropy of the analytes transfer from mobile to stationary phase. The calculated thermodynamic properties are summarized in Table 2.

T a b l e 2 – *Thermodynamic parameters*

	ΔH/J mol^{-1}	ΔS/J mol^{-1} K^{-1}
D-Leu	−2763	−12.20
D-Met	−11124	−41.14
D-Phe	−11903	−25.69
D-Tyr	−16204	−58.08

In the literature, three effects are discussed as enthalpic factors governing the solute distribution between the mobile and stationary phase − specific solute-solvent interaction effect, cavity formation effect and the hydrophobic (iceberg formation) effect. The specific solute-solvent interactions are based on their physical properties. So for instance, if a solute is polar, the specific solute-solvent interaction in the mobile phase should be stronger in a more polar solvent. As to the cavity formation effect, a hole should be formed in the phase to accommodate the solute and the cavity formation is enthalpically endothermic. Finally, the hydrophobic effect is caused by restricting and reinforcing the surrounding water molecules (iceberg formation) when a solute is introduced in an aqueous liquid. Water molecules around the solute tend to hold one another more tightly to compensate for the molecular interactions broken by insertion of the solute and such structural reinforcement is strong enough to cancel out the enthalpic energy loss of cavity formation to a large extent, especially when the solute is nonpolar and/or the solvent mixture is mainly aqueous.[28,29]

In order to estimate which effect dominates in the investigated system, the van't Hoff plots were determined only for the pure aqueous mobile phase. A typical example of the plots is shown in Fig. 3. The ΔH and ΔS values of the D-amino acid are summarized in Table 2.



F i g . 3 – *Van't Hoff plots for the D-enantiomers, F = 1 mL min^{-1}: ▼ – k′ D-Phe; ■ – k′ D-Leu; ▲ – k′ D-Met; ● – k′ D-Tyr and D-Tyr*

The ΔH values obtained for the D-enantiomers of the amino acids are within the ΔH range obtained for hydrophobic compounds in RP-HPLC systems, e.g. between −11 and −29 kJ mol^{-1}, which is within the limits previously reported for the transfer of nonpolar compounds from hydro-organic mobile phases to alkyl-bonded stationary phases.[20]

Bearing in mind the three effects described above, it is expected in respect of the interaction enthalpy between a solute and a solvent, that a polar solute would prefer the mobile phase and a non-polar one will have no particular preference to any phase. Lee and Cheong[29] have estimated in their work, that the cavity formation effect controls the solute distribution between the phases. The

same effect was observed here, e.g. the stationary phase was enthalpically favoured by all the solutes, and ΔH for all amino acids has negative values (see Table 2), changing in the following order: D-Leu > D-Met > D-Phe > D-Tyr. The interesting effect here is that Tyr is the only one amino acid that is polar, and following the cavity formation effect has the highest negative value instead of the solute-solvent interaction as the mobile phase is polar. The enthalpies for D-Phe and D-Met have comparable values; they interact with the stationary phase in a similar manner.

The entropies of solute transfer from the mobile to the stationary phase are negative for all amino acids. The negative sign of entropies can be explained by the following argument. Achieving the surface of the C8 phase the solutes lose a portion of their freedom (entropy) compared to the solutes in the mobile phase. From Table 2, one can see that D-Phe has a higher entropy value than D-Met. In this case, the entropically endothermic iceberg formation is more significant. Tyr has the lowest ΔS value while Leu has the highest.

Finally, it is noteworthy that the ΔH and ΔS obtained can rarely be ascribed to a single process but are rather a complex combination of various contributions such as solute-site solvation and desolvation, the unsolvated solute-site interaction, solvation of bound solute, conformational changes, multiple equilibria and mixed retention mechanism.[30]

Influence of the flow rate

The only process that is responsible for the chiral recognition of two enantiomers of the analyte is their difference in complex formation with the transition metal ion and the chiral selector of the system. Generally, the rate of ligand-exchange in complex formation with double charged cations of transition metals such as Cu, Ni, Co, Zn, etc. is high and therefore difficult to measure. The rate of coordinating amine molecules to Ni^{2+} ions was estimated to be of the order of 10^3 to 10^5 mol^{-1} s^{-1} and that to Cu^{2+} ions is even higher.[31]

In the literature,[32] relatively high stability of copper(II) amino acids complexes are reported (the stability constants of mono complexes ranged between 10^7 and 10^9 and those of bis-complexes between 10^{12} and 10^{14}). The formed diastereomeric complexes with copper are less stable[33] and can readily dissociate and distribute between the two phases. The separation with LEC is attributed to this effect.

Compared to other methods where the selector is bound to the stationary phase, the chiral mobile phase approach has the advantage that the mass transfer in the stationary phase is improved, which results in a higher column efficiency.[15,23] Rapid mass transfer provides high column efficiency and the chiral resolution can be achieved very fast.

Strong interaction between solutes and stationary phases has always been a reason for extensive peak broadening, especially if secondary equilibria are involved, as is the case of LEC. This is, of course, one of the main drawbacks of this separation system, because it is not always possible to separate a mixture of amino acids in their enantiomeric form in a single chromatographic run. Engelhardt *et al.*[34] using chiral stationary phase have shown that within 70 min analysis time only four amino acids could be determined in their enantiomers, due to the extensive peak broadening. However, with the author's system no peak tailing for any of the amino acids was observed (see Fig. 4).

It is worth mentioning that, when trying to separate serine, threonine and alanine under the same experimental conditions, no enantiomeric resolution was observed. Nazareth *et al.*[10] have observed that a polar hydroxy group in the chiral selector structure decreased the hydrophobic interaction of the diastereomeric complexes and the stationary phase, therefore no separation could be observed for less hydrophobic amino acids. Here, due to the excess of the chiral selector (copper:selector ratio 1 : 20) in

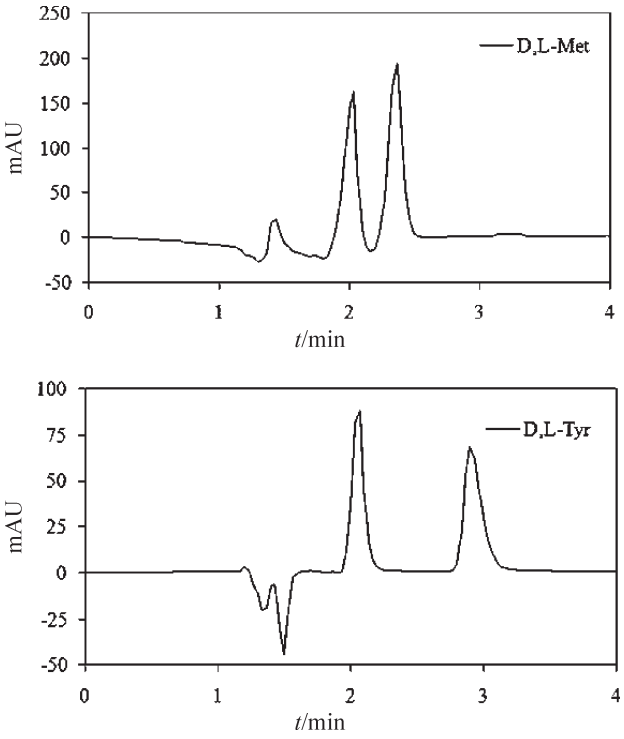


Fig. 4 – *Chromatograms of D,L-Met and D,L-Tyr, $F = 1$ mL min^{-1}, 25 °C mobile phase- 2 mmol L^{-1} N,N-dimethyl-L-phenylalanine and 0.1 mmol L^{-1} Cu^{2+} in water*

the mobile phase, the stationary phase is covered not only with its copper complex but also with single molecules. They in turn impede the hydrophobic interaction between the diastereomeric complexes and the C_8 alkyl chains, resulting in no enantiomeric separation of hydrophilic amino acids. Furthermore, when trying to separate all the amino acids investigated in a single run, only Phe as the longest retained amino acid was successfully spitted in the two enantiomers and simultaneously separated from the other amino acids. The D-complexes of the other amino acids were overlapped due to the equal retention, whereas the L-complexes of Tyr, Met and Leu were resolved.

The dependence of HETP on the flow-rate for the D-enantiomers is shown in Fig. 5 and for the L-enantiomers in Fig. 6, which corresponds to the N-values given in Table 3. The better adsorbable components (L-enantiomers) have higher N-values than those low adsorbable, as expected. The achieved values are between 800 theoretical plates for low adsorbable D-Val, and 2200 for the stronger retainable L-Tyr. Generally, amino acids with high capacity factors have lower HETP values, similar to reports in the literature.[14,15,22,35]

The performance in terms of theoretical plate per meter was found to increase with the capacity factor of the solutes, which can also be found in the literature.[14] While the number of plates calculated from the peak of a scarcely retained compound, e.g. D-Val, is ca. 5 000/m and the D-Phe peak ($k' = 4.03$) results in more than 20 000/m. Nevertheless, very good base line separation without peak tailing for all amino acids was achieved. Obviously, extra column effects play an additional role, which becomes smaller at increasing peak widths.[15]

The dispersion coefficient $D_{a,i}$ increases with increasing flow-rate and the L-enantiomers as longer absorbed have lower dispersion coefficients (Table 3).

For D-Leu, the lowest velocity in the column corresponds to the lowest numbers of theoretical plates, indicating that the main influence on the apparent dispersion coefficient is the mass transfer resistance. For all other D- enantiomers and all the L-enantiomers, the lower velocities in the column are connected with higher N-values, indicating the increasing influence of the axial dispersion and decreasing importance of the mass transfer.

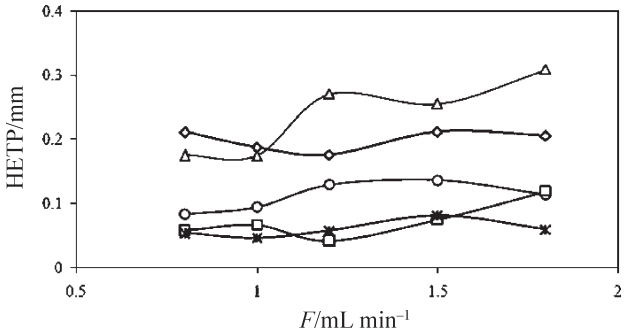


Fig. 5 – *Van Deemter plot for the D-enantiomers at 25 °C: * – D,L-Phe; ◇ – D,L-Leu; ○ – D,L-Met; □ – D,L-Tyr and Δ – D,L-Val*

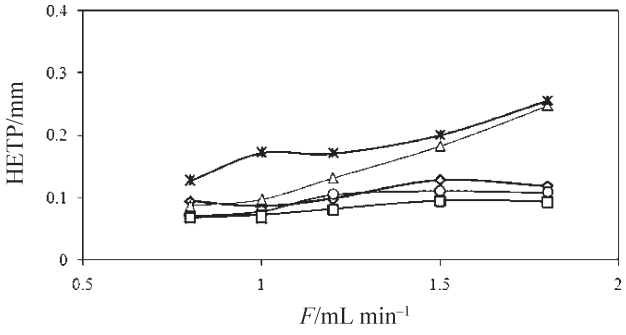


Fig. 6 – *Van Deemter plot for the L-enantiomers at 25 °C: * – D,L-Phe; ◇ – D,L-Leu; ○ – D,L-Met; □ – D,L-Tyr and Δ – D,L-Val*

Conclusions

Using an aqueous solution of N,N-dimethyl--L-phenylalanine dissolved as a copper complex (selector:copper ratio 1 : 20) as a chiral mobile phase and an achiral RP-C8 column it was possible to separate the racemic mixtures of five amino acids in their enantiomeric forms under isocratic conditions at 25 °C. A very good separation in a short time could be achieved, whereas no peak broadening was observed. Using van Deemter plots, high separation efficiency and low apparent axial dispersion coefficients for all amino acids could be determined.

Compared to the traditional RP-HPLC, where an addition of organic modifiers usually reduces the retention times of the solutes, here with methanol as organic modifier, no effect was observed. It could be shown that an in-situ adsorption layer (selector-copper complex) is formed, which modifies the silica surface and influences in this way the retention and interaction between the complexes and the stationary phase.

From the van't Hoff plots, the change of the enthalpy and entropy of transfer from the mobile phase to the stationary phase for all amino acids could be determined. The solute transfer is enthalpically favorable and entropically unfavorable. For the investigated system it was shown that the cavity formation effect is the one that governs the solute distribution between the mobile and stationary phase. One can conclude, that the chiral mobile phase used offers very fast and easy enantiomeric separation of racemic mixtures of

Table 3 – *Values for the number of theoretical plates (N_D, N_L) and apparent dispersion coefficients ($D_{a,D}$, $D_{a,L}$) determined at different flow rates*

Amino acid	Flow-rate/mL min^{-1}	N_D	N_L	$D_{a,D}/\cdot 10^{-6}$ m^2 s^{-1}	$D_{a,L}/\cdot 10^{-6}$ m^2 s^{-1}
	0.8	710	1580	0.15	0.07
	1.0	800	1740	0.17	0.08
DL-Leu	1.2	850	1520	0.19	0.11
	1.5	710	1170	0.29	0.18
	1.8	730	1260	0.34	0.19
	0.8	1800	2140	0.06	0.05
	1.0	1590	1920	0.09	0.07
DL-Met	1.2	1160	1430	0.14	0.11
	1.5	1100	1350	0.19	0.15
	1.8	1300	1390	0.19	0.18
	0.8	860	1740	0.13	0.06
	1.0	860	1560	0.16	0.09
DL-Val	1.2	560	1150	0.30	0.14
	1.5	590	830	0.35	0.25
	1.8	490	610	0.51	0.40
	0.8	2580	2210	0.04	0.05
	1.0	2240	2100	0.06	0.07
DL-Tyr	1.2	3560	1850	0.05	0.09
	1.5	1990	1590	0.10	0.13
	1.8	1260	1610	0.20	0.15
	0.8	2790	1180	0.04	0.09
	1.0	3260	870	0.04	0.16
DL-Phe	1.2	2600	880	0.06	0.19
	1.5	1840	750	0.11	0.27
	1.8	2520	590	0.10	0.42

amino acids under favorable conditions (no adjustment of the pH, no derivatisation, no heating, etc. is necessary) compared to other chromatographic techniques for the separation of such substances. Furthermore, the resolution is achieved in a very short time at relatively low flow rate, which makes this chromatographic process quite beneficial and time-saving.

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List of symbols

k' – capacity factor, –
R – universal gas constant, J K^{-1} mol^{-1}
T – temperature, K
ΔH – standard enthalpy, J mol^{-1}
ΔS – standard entropy, J mol^{-1} K^{-1}
t_0 – retention time of a non-adsorbable component, min
F – flow rate, mL min^{-1}
V_{column} – column volume, mL
L – column length, cm
$t_{R,i}$ – retention time of any enantiomer, min
HETP – height of a theoretical plate, cm

N_i – number of theoretical plate for each enantiomer, –

$D_{ax,i}$ – apparent axial dispersion coefficient, $cm^2\ s^{-1}$

u – interstitial velocity, $cm\ s^{-1}$

α – selectivity, –

ϕ – phase ratio, –

ε_t – total porosity, –

ϑ – temperature, °C

References

1. *Kusumoto, I.,* J. Nutr. **131** (2001) 2552S.
2. http://www.holisticmed.net/aspartame/aminoacid.pdf (accessed 12. 12. 2009).
3. *Rayapati, P. J., Crafton, C. M.,* (Archer-Daniels-Midland Company), US Patent 733321 B2, 29 Jan 2008.
4. *Behling, J. R., Boys, M. L., Cain-Janicki, K. J., Colson, P.-J., Doubleday, W. W., Duran, J. E., Farid, P. N., Knable, C. M., Muellner, F. W., Nugent, S. T., Topgi, R. S.,* (G. D. Serale & Co., Chicago, US), US Patent 5840961, 24 Nov. 1998.
5. *Kreft, A. F., Antane, M. M., Cole, D. C., Kubrak, D. M., Resnick, L., Stock, J. R., Wang, Z.,* (Wyeth, Madison, NJ, US), US Patent 6800764 B2, 5. Oct. 2004.
6. *Vrlijc, M., Eggeling, L., Sahm, H.,* (Forschungszentrum Jülich GmbH, Jülich, DE), US Patent 6858406 B1, 22. Feb. 2005.
7. *Marx, A., Mockel, B., Pfefferle, W., Sahm, H., De Graaf, A., Eggeling, L.,* (Degussa Huls AG, Hanau, DE), US Patent 6355454, 12. Mr. 2002.
8. *Davankov, V. A,* Ligand-exchange chromatography of chiral compounds, in: *Cagniant*, *D.* (Ed.), Complexation Chromatography, M. Dekker, New York, 1992, pp 197–245.
9. *Tatsumi, S., Sasaji, I., Ohno, K.,* (Ajinomo Company, Inc.), US Patent 3182079, 4 May 1965.
10. *Nazareth, P. M. P., Antunes, O. A. C.,* J. Braz. Chem. Soc. **13** (2002) 658.
11. *Lee, S. H., Ryu, J. W., Park, K. S.*, Bull. Korean Chem. Soc. **7** (1986) 45.
12. *Rogoshin, S. W., Dawankow, W. A.,* (Institut für Element-organische Verbindigen der Akademie der Wissenschaften, Moskau), Deutsches Patent DE 1932190, 8. Jan. 1970.
13. *Rogozhin, S. V., Davankov, V. A.*, Chem. Commun. **10** (1971) 490.
14. *Davankov, V. A., Bochkov, A. S., Kurganov, A. A.,* Chromatographia **13** (11) (1980) 677.
15. *Wernicke, R.,* J. Chromatogr. **23** (1985) 39.
16. *Hare, P. E.,* Chromatogr. Chiral. Sep. **40** (1988) 165.
17. *Hancock, W. S., Harding, D. R. K.,* CRC Handbook of HPLC for Separation of Amino Acids, Peptides and Proteins, Vol. 1, CRC Press, Boca Raton, Fla. 1982, pp. 429–442.
18. *Lee, S. H., Oh, T. S., Lee, H. W.,* Bull. Korean Chem. Soc. **13** (1992) 280.
19. *Seidel-Morgenstern, A.,* Mathematische Modellierung der präparativen Flüssigchromatographie, Deutscher Universitäts-Verlag GmbH, Wiesbaden, 1995.
20. *Balcan, M., Anghel, D.-F., Voicu, A., Balkan, D.-C.,* Colloids Surf. A **204** (2002) 141.
21. *Davankov, V. A., Bochkov, A. S., Belov, Yu. P.,* J. Chromatogr. **218** (1981) 547.
22. *Kostova, A., Bart, H.-J.,* Sep. Purif. Technol. **54** (2007) 340.
23. *Rizzi, M. A.,* J. Chromatogr. **542** (1999) 221.
24. *Hoppe, B., Martens, J.,* Aminosäuren – Bausteine des Lebens, Folienserie des Fonds der chemischen Industrie Nr. 11, Frankfurt am Main (1981).
25. *Davankov, V. A., Kurganov, A. A., Ponomareva, T. M.,* J. Chromatogr. **452** (1988) 309.
26. *Davankov, V. A.*, in: *Cagniant, D.* (Ed.), Complexation Chromatography (1992) 197.
27. *Watabe, K., Charles, R., Gil-Av, E.,* Angew. Chem. **101** (1989) 195.
28. *Tran, J. V., Molander, P., Greibrokk, T., Lundanes, E.*, J. Sep. Sci. **24** (2001) 930.
29. *Lee, C. S., Cheong, W. J.,* J. Chromatogr. A. **848** (1999) 9.
30. *Sellergen, B., Shea, K. J.,* J. Chromatorg. A. **690** (1995) 29.
31. *Davankov, V. A.,* Methods Mol. Biol. **243** (2004) 207.
32. *Kurganov, A., Davankov, A., Unger, K., Eisenbeiss, F., Kinkel, J.,* J. Chromatogr. **666** (1994) 99.
33. *Davankov, V. A.,* J. Chromatogr. **666** (1994) 55.
34. *Engelhardt, H., Koenig, Th., Kromidas, St.,* Chromatographia **21** (1986) 205.
35. *Kostova, A., Bart, H.-J.,* Sep. Purif. Technol. **54** (2007) 315.