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RETENTION BEHAVIOR OF AMINO ACID ENANTIOMERS IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

ELI GRUSHKA* and R. LESHEM
Department of Inorganic and Analytical Chemistry, The Hebrew University, Jerusalem (Israel)
and
C. GILON
Department of Organic Chemistry, The Hebrew University, Jerusalem (Israel)

SUMMARY

The work described in this paper tries to demonstrate that the formation of diastereomers, needed for the resolution of enantiomers of amino acids, takes place in the stationary phase, when aspartylalkylamides are used as chiral reagents. The chromatographic behavior of some amino acid enantiomers are studied under various chromatographic conditions. Resolution of the isomers occurs even under extreme conditions, such as low pH or very low resolving agent concentration. The same elution order was observed whether the chiral reagent was in the mobile phase or only in the stationary phase. The temperature dependence of the resolution was studied. it was found that quite frequently the entropy of the D-diastereomer is larger than that of the L-isomer. The behavior of the capacity ratios as a function of the chiral reagent concentration allowed the calculation of the diastereomer formation constant.

INTRODUCTION

The separation of optical isomers by high-performance liquid chromatography (HPLC) is now a routine task; see for example refs. 1–4. In the case of the separation of enantiomers of amino acids, using reversed-phase liquid chromatography with chiral eluents, there is a question as to pathways of the resolution. The chiral reagent in the mobile phase can form diastereomers with the solutes either in the mobile phase or in the stationary phase. In the former case the diastereomers are distributed between the two phases, whereas in the latter case the chiral reagent is adsorbed on the stationary alkane chain and the diastereomers are formed only in that phase. The situation is similar, if not identical, to that of ion-pair chromatography, where there is an argument in the literature regarding the retention processes (*e.g.* refs. 5 and 6).

In a previous paper[7] we have shown that the chain length of the chiral reagent determines the retention times and the selectivities of the chromatographic system. This seems to indicate that the hydrophobicity of the chiral reagent is of prime importance. The implication is that the chiral reagent is adsorbed on the stationary phase. In recent papers Davankov *et al.*[8,9] as well as Tapuhi *et al.*[10] have shown that

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reversed-phase columns can be loaded with chiral reagents. Such columns can resolve enantiomers even with mobile phases not containing chiral components. It is reasonable to assume, therefore, that when the mobile phase does contain the chiral reagent, the latter is adsorbed on the stationary phase, and the enantiomeric resolution takes place there. In the present paper we shall show that this retention and resolution process is probably the predominant one. A simple mathematical model can be written, which allows the calculation of the formation constants of the diastereomers, from the chromatographic data.

The effect of the temperature on the capacity ratios and the selectivities can also yield important information concerning the thermodynamics of the resolution. It is of interest to investigate the temperature dependence of the retention of enantiomeric pairs in order to learn which diastereomer is more labile. Furthermore, $\Delta\Delta H$ and $\Delta\Delta S$ data can indicate the importance of enthalpic effect *vis à vis* entropic ones.

EXPERIMENTAL

All chromatographic runs were carried out on a Spectra-Physics 8000 (Eldan, Jerusalem, Israel) liquid chromatograph equipped with variable-wavelength UV–VIS detector and an oven. The mobile phase was preheated before entering the column. Detection was at 230 nm[3]. The column was 25 cm × 4.1 mm I.D. It was home packed with ODS (10 μm). The various chiral reagents used were synthesized as described before[3,7]. The concentration of the reagent varied from one experiment to another, as will be discussed shortly.

The columns were loaded with chiral reagents as follows. A stock solution, made up of the chiral reagent and copper ions, in water–methanol, was passed through the column until the detector baseline was constant. Then the mobile phase was changed in a gradient mode to one consisting of an aqueous solution of copper ions, and this was passed through the column until constant capacity ratios (k') were obtained for the injected amino acids.

Other mobile phases, containing the chiral reagents, were prepared as described elsewhere[3,7].

The flow-rate of the mobile phase was 1 ml/min.

RESULTS AND DISCUSSION

Three contact points are thought to be necessary for the resolution of enantiomers. In the present case, where the enantiomers are those of amino acids, and the resolving (chiral) agent is the copper complex of L-aspartylalkylamide, two of these contact points are between the α-NH_3^+ and α-COO^- of the amino acid and the Cu(II) atom of the complex. The third contact point could be either between the side-chain of the amino acid and that of the amide, or between the side-chain of the amino acid and the stationary phase. The former could occur either in the mobile phase or in the stationary phase, while the latter could take place only in the stationary phase. One possible test as to where the third contact point occurs is to run the analysis with very low concentration of the chiral reagent. Table I shows the results of such a study. Even at this low concentration of aspartyl-cyclic(*c*)-hexylamide, the resolution of enantiomers is easily accomplished. However, the resolution is obtained only after a cer-

TABLE I

RESOLUTION OF SOME AMINO ACID ENANTIOMERS AT LOW CONCENTRATION OF THE CHIRAL REAGENT

0.05 m*M* Aspartyl-*c*-hexylamide. 0.025 m*M* $CuCl_2$. Phosphate buffer at pH of 4.8. $T = 35°C$.

Amino acid	k'_L	k'_D	α
Pro	0.32	0.43	1.34
Val	0.62	0.72	1.16
Tyr	2.36	2.71	1.15

tain equilibration time. Injections before that time do not give separation of enantiomers. An explanation for this behavior is the concentration of chiral reagent on the stationary phase. The capacity ratio of aspartyl-*c*-hexylamide is *ca.* 17. This reagent, which is in the mobile phase all the time, will, by solvophobic interactions, favor the alkyl-bonded phase. However, since the concentration in the mobile phase is very low, saturation of the stationary phase will not occur. Hence, resolution can be achieved but not to the same extent as with a mobile phase containing 1 m*M* of the aspartyl derivative. The fact that resolution occurs only after an equilibration time strongly suggests diastereomer formation in, or on, the stationary phase.

To check this hypothesis further, and the arguments which depend on saturation of the stationary phase, the resolution of the enantiomers was studied as a function of the concentration of the chiral reagents. Fig. 1 shows an example of the behavior of k' value of D and L amino acid as a function of the Cu-aspartyl-*c*-hexylamide concentration. Throughout the concentration range, the ratio Cu to aspartyl derivative was maintained at 1 to 2. The concentration of the injected enantiomers was always less than that of the chiral reagent. The graphs provide an excellent way to follow possible retention processes. At very low concentrations of the

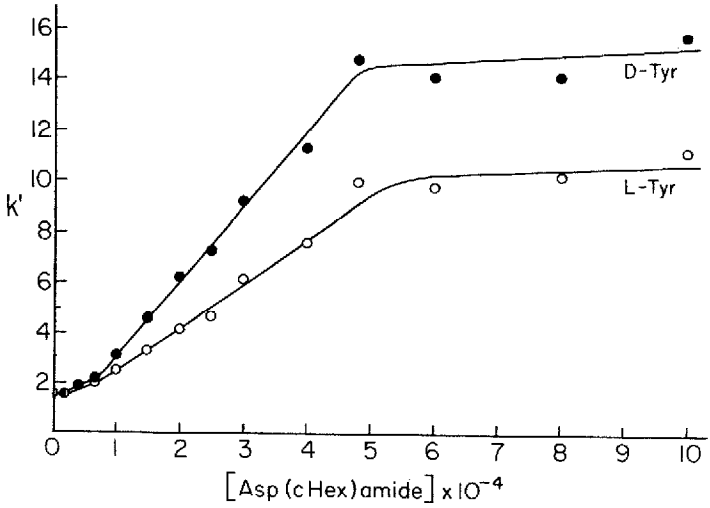


Fig. 1. The behavior of k' as a function of the concentration of aspartyl-*c*-hexylamide in the mobile phase. The concentration of Cu(II) is always twice that of the chiral reagent. $T = 35°C$.

aspartyl derivative ($1 \cdot 10^{-5}$ *M*), the enantio-selectivity of the system is poor; *i.e.* $\alpha \approx 1.0$. As the concentration of the chiral reagent increases to $5 \cdot 10^{-4}$ *M* the capacity ratios of both enantiomers increase as well. The rate of increase is greater for the D-isomer. Further increase in the resolving agent concentration does not alter the magnitude of the capacity ratios. This can be explained, as above, in terms of saturation of the stationary phase with the chiral reagent. If the diastereomers are formed in the stationary phase, then, after that phase is saturated with the resolving agent a further increase in the concentration should not affect the retention time or the selectivity. Such seems to be the case. The selectivity α changes from 1 to 1.5 as the chiral reagent concentration increases to *ca.* $4 \cdot 10^{-4}$ *M*. After that α remains constant at 1.5. The behavior of the tyrosine isomers depicted in Fig. 1 is typical of all the amino acids studied. In all cases, the break in the line where k' becomes constant occurs at about the same concentration of the aspartyl derivative.

At very low concentrations of the chiral reagent, the k' lines are not linear. This is probably due to mixed retention processes; namely, residual silanol affects solvophobic interactions and diastereomer formation. When the concentration of the aspartyl derivative is above a certain level, the second process predominates and k' is then a linear function of that concentration.

The linear portion of the graphs in Fig. 1 allows the calculation of the formation constant of the diastereomers. If it is assumed that the formation of the diastereomers takes place in the stationary phase, then the capacity ratio of the amino acid can be written as:

$$k' = \varphi \frac{[\mathrm{AA}]_s + \mathrm{AA\ Cu[Asp}(c\mathrm{Hex})]_s}{[\mathrm{AA}]_m} \tag{1}$$

In this equation AA is the amino acid, AA Cu[Asp(*c*Hex)] is the diastereomer, subscripts s and m indicate concentrations in the stationary and mobile phase, respectively, and φ is the phase ratio. The capacity ratio of the amino acid alone is

$$k'_0 = \varphi \frac{[\mathrm{AA}]_s}{[\mathrm{AA}]_m} \tag{2}$$

The complex formation constant is given by:

$$K_f = \frac{\{\mathrm{AA\ Cu[Asp}(c\mathrm{Hex})]\}}{[\mathrm{AA}]\ \{\mathrm{Cu[Asp}(c\mathrm{Hex})]\}} \tag{3}$$

The capacity ratio of the aspartyl-*c*-hexylamide copper complex is

$$k_c = \frac{\{\mathrm{Cu[Asp}(c\mathrm{Hex})]\}_s}{\{\mathrm{Cu[Asp}(c\mathrm{Hex})]\}_m} \tag{4}$$

With the aid of eqns. 2–4 k' can be written as

$$k' = k'_0 \left\{1 + \frac{k_c}{\varphi} K_f\ \mathrm{Cu[Asp}(c\mathrm{Hex})]_m\right\} \tag{5}$$

TABLE II

FORMATION CONSTANTS OF SOME AMINO ACID–CU [ASP(*c*HEX)] DIASTEREOMERS, AT 35°C AND pH 4.8

Also given are the slopes and correlation coefficients of k' *versus* concentration of chiral reagent.

Amino acid	*Slope*	*Corr. coeff.*	K_f
L-Tyr	$1.81 \cdot 10^4$	0.996	$1.18 \cdot 10^3$
D-Tyr	$2.87 \cdot 10^4$	0.998	$1.88 \cdot 10^3$
L-Met	$7.28 \cdot 10^3$	0.988	$1.15 \cdot 10^3$
D-Met	$1.13 \cdot 10^4$	0.995	$1.75 \cdot 10^3$
L-Leu	$1.31 \cdot 10^4$	0.998	$1.06 \cdot 10^3$
D-Leu	$1.96 \cdot 10^4$	0.992	$1.57 \cdot 10^3$

Eqn. 5 indicates that k' is a linear function of the concentration of the aspartyl derivative in the mobile phase, provided saturation of the stationary phase does not occur. The slope of the line k' *versus* the concentration of the chiral reagent in the mobile phase yields the formation constant of the diastereomer. For the calculation, the phase ratio φ was estimated at *ca.* 1.5[11]. The effect of pH on the dissociation of the β carboxylate was taken into account. The capacity ratio of the aspartyl-*c*-hexylamide was measured to be 17. Table II shows the slopes, correlation coefficients, and the calculated formation constants for several enantiomers. It should be emphasized that these formation constants are for diastereomers formed on (or in) the alkyl stationary phase. Thus interactions of the amino acids with the stationary phase, if such exist, are included in the calculated K_f values. As expected the formation constants of the D-isomers are larger than those of the L-isomers. This, most likely, is due to the extra interaction between the D-isomer and either the stationary phase or the side-chain of the adsorbed chiral reagent; *i.e.*, the third contact point. This interaction stabilizes the complex, thus causing it to elute after the L-isomer. For the three amino acids shown here, it seems that the formation constants are similar for each enantiomeric conformation, independent of the retention order, which was Tyr > Leu > Met. Whether this observation is true for all the amino acid enantiomers remains to be studied. This is important since it could indicate the role of the chiral reagent not only in optical resolution but also in the general retention of amino acids.

The resolution of the enantiomers at low pH is also of interest. Table III shows that even at pH 3.5 the resolution can be accomplished. At this pH the β-carboxylate

TABLE III

CHROMATOGRAPHIC PARAMETERS OF SOME ENANTIOMERS

Mobile phase is a phosphate buffer at pH 3.5 and 10^{-3} *M* aspartyl-*c*-hexylamide, $5 \cdot 10^{-4}$ *M* $CuCl_2$; T = 30°C.

Amino acid	k'_L	k'_D	α
Tyr	1.05	1.22	1.16
Pro	0.10	0.22	2.20
Leu	0.96	1.15	1.20

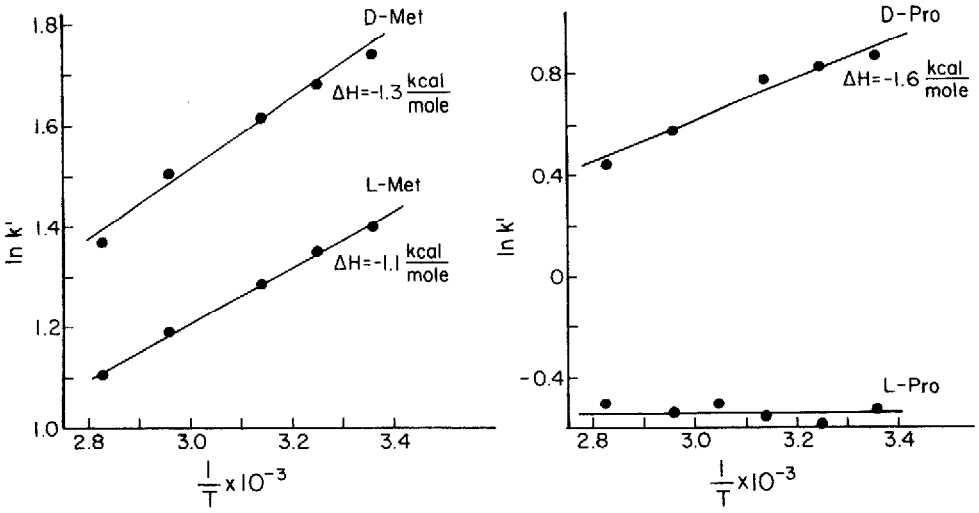


Fig. 2. Plot of ln k *versus* $1/T$ for D-Met and L-Met. The mobile phase contained 0.5 mM aspartyl-c-hexylamide and 0.25 mM $CuCl_2$.

Fig. 3. Plot of ln k' *versus* $1/T$ for D-Pro and L-Pro. Conditions as in Fig. 2.

of the aspartyl derivative is slightly more than 50% protonated. The α-carboxylate of the amino acids is also protonated to some extent. The stability of the (Cu-amino acid) complexes is lower in acidic media. Yet enantioselectivity is still accomplished. This is compatible with the model proposed here of diastereomer formation in the stationary phase. The third contact point could stabilize the complex so that resolution can take place.

The role of the temperature

In general, an increase in the temperature is known to cause a decrease in the retention. Figs. 2 and 3 show that such is the case as well with the amino acid enantiomers. The case of L-Pro, Fig. 3 is noteworthy. It seems that the retention of this solute is temperature independent. This might be a result of experimental errors in measuring the void volume or the actual retention time of the solute. Figs. 2 and 3 clearly indicate that the selectivity decreases with increasing temperature. The decrease is due to enthalpic and to entropic effects. Table IV shows the difference in ΔH and ΔS between the D- and L-isomers for Met and Pro. It is interesting to note that the entropy of the D-isomer is larger than that of the L-isomer. This might be due to the

TABLE IV

DIFFERENTIAL THERMODYNAMIC QUANTITIES FOR TWO AMINO ACIDS

The chiral reagent is Cu-aspartyl-c-hexylamide in the mobile phase (5 mM Asp$c$Hex in TDW).

Amino acid	*ΔΔH (cal/mole)*	*ΔΔS (cal/mole °K)*
Pro	−1600	7.4
Met	−200	1.4

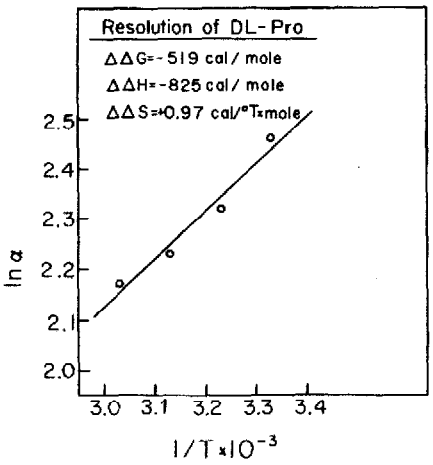


Fig. 4. Plot of ln α *versus* $1/T$ for proline. Support: RP-18. Stationary phase: aspartyldodecylamide + Cu^{2+}. Mobile phase: 0.3 m*M* aqueous $CuCl_2$.

stronger hydrophobic interactions of the diastereomer containing the D-amino acid.

The picture is much the same when the column is loaded with the chiral reagent and the mobile phase contains only Cu(II) ions. For the temperature studies, aspartyldodecylamide had to be used as the resolving agent. Lower alkylamides could not be used, since they were stripped from the column at high temperatures. Fig. 4 shows the dependence of α, in the case of proline, on the temperature. As in the case where the chiral reagent is in the mobile phase the selectivity decreases with an increase in temperature (T). This contradicts the results of Davankov *et al.*[9]. However, since they used a different chiral reagent, N-decyl-L-histidine, and a water–ethanol mobile phase, a direct comparison between these results may not be valid.

The insert in Fig. 4 gives $\Delta\Delta H$ and $\Delta\Delta S$ values for the prolines. Both values are smaller than those obtained with the reagent in the mobile phase. The $\Delta\Delta S$ value is still positive. It should be pointed out that the exact amount of the adsorbed chiral reagent is not shown. Thus a rigorous comparison between the two cases cannot be made. However, the very large α values should be noted. Fig. 5 shows the dependence

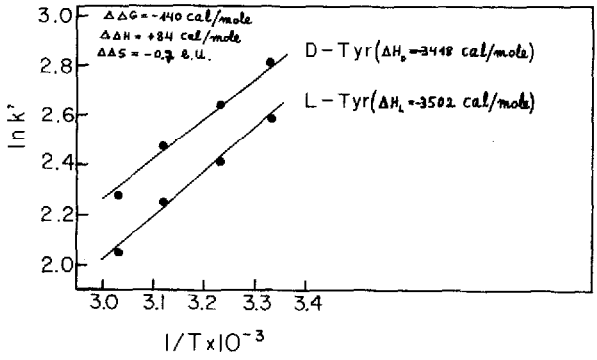


Fig. 5. Plot of ln k' *versus* $1/T$ for the tyrosine enantiomers. Conditions as in Fig. 4.

TABLE V

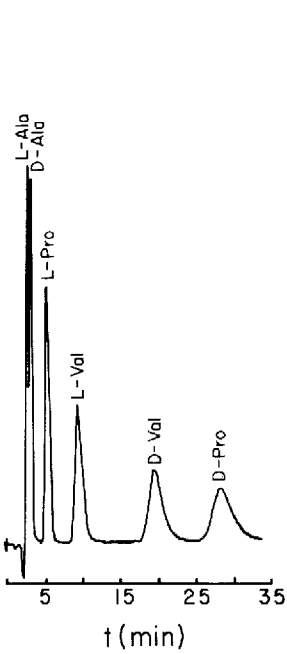
CHROMATOGRAPHIC PARAMETERS OBTAINED ON A COLUMN LOADED WITH L-ASPARTYL-*n*-OCTYLAMIDE-Cu

The mobile phase contained 0.3 m*M* $CuCl_2$; $T = 35°C$.

Acid	*Experiment a*			*Experiment b*		
	k'_L	k'_D	α	k'_L	k'_D	α
Ala	0.90	1.25	1.39	0.81	1.09	1.35
Val	6.19	13.9	2.25	6.11	13.3	2.17
Pro	2.96	20.8	7.0	2.63	17.4	6.6

of k' on T for the two tyrosine isomers. The two lines diverge slightly. The value of $\Delta\Delta H$ is +84 cal/mole and that of $\Delta\Delta S$ is −0.7 e.u./mole. The selectivity in this case increases slightly with increasing temperature. Davankov *et al.* found similar behavior for tyrosine in one publication[9] and the opposite in another[8]. In general, however, these authors have found that α values of the enantiomers do not change with temperature. In the present work the selectivity was found almost always to decrease with increasing temperature.

Davankov *et al.*[8,9] have maintained that the alkyl side-chain of the chiral reagent protrudes into the reversed-phase layer and that the third contact point is



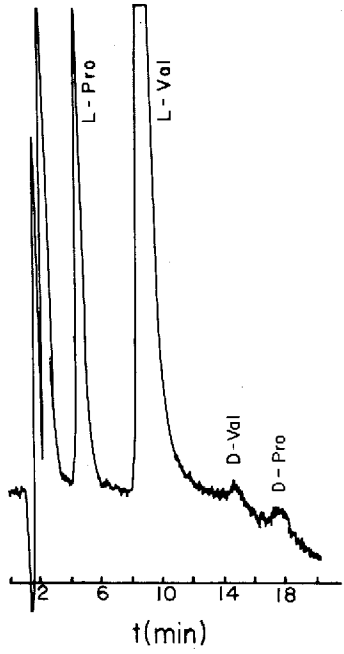


Fig. 6. Separation of some enantiomers of amino acids using adsorbed aspartyloctylamide. The mobile phase contained 0.3 m*M* $CuCl_2$.

Fig. 7. Detection of D-isomers in the presence of L-isomers. Conditions as in Fig. 6. 0.005 a.u.f.s.

between the side-chain of the amino acid enantiomer and the bonded alkyl groups. With water-rich mobile phases, however, the bonded phase is most likely collapsed on to the silica gel surfaces. In such a case the chiral reagent is most likely adsorbed on the bonded phase. The third contact point, then, could be between the alkyl part of the resolving agent and the amino-acid enantiomer. Space-filling models show that this is possible[3]. However, if the chiral reagent wets the octadecyl chains, then protrusion into the reversed phase is possible. This point has to be investigated further.

In all the cases studied the elution order of the enantiomers was the same whether the chiral reagent was in the mobile phase or adsorbed on the stationary phase. This fact tends to corraborate the hypothesis that the diastereomers, and hence the enantioselectivity, are made in the stationary phase.

The utilization of adsorbed chiral reagent

To be a viable method the system should be reproducible. Table V shows chromatographic data repeated twice as follows. Aspartyl-*n*-octylamide ($6 \cdot 10^{-4}$ *M*) and $CuCl_2$ ($3 \cdot 10^{-4}$ *M*) were dissolved in methanol–water (40:60) solution which was passed through the column (*ca.* 500 ml). After the solutes were injected, the column was washed with pure methanol containing Cu^{2+} until no chiral recognition was observed. Then, the same solution containing the reagents was again passed through the column and the solutes were re-chromatographed. Table V shows that the reproducibility of α is quite good. Greater variation occurred in the retention of the D-isomers, since they are much more sensitive to small changes in the amount of the chiral reagent[7]. Fig. 6 shows typical separation with the aspartyl-*n*-octylamide adsorbed phase.

A possible application of the method is to detect one isomer in the presence of

Fig. 8. Separation of the products of reflux in HCl. Conditions as in Fig. 7.

the other. Fig. 7 shows a chromatogram of 5 % D-Val and D-Pro in the presence of the L-isomers; 5 ng/10 μl of each L-isomer were injected. If greater amounts are introduced to the column, lower relative concentrations of the D-isomers can easily be detected. The method can easily be extended to 0.5–1 % impurity of one isomer in the presence of the other.

Fig. 8 shows a chromatogram of a mixture of three amino-acids after refluxing in hydrochloric acid for 20 h. The reflux started with pure D-isomer of each amino acid. The chromatogram shows that some of the D-Pro is converted into the L-isomer. This is not surprising since proline can racemize quite easily. Thus the present technique could be applied to study the kinetics of the racemization.

In summary, the data indicate that chiral recognition and enantioselectivity take place in the stationary phase. When the chiral reagent is added to the mobile phase, it is most likely extracted into the alkyl stationary phase.

This is supported by the facts that the retention order of the enantiomers is independent of whether the resolving agent is in the mobile phase or loaded on the support and also that resolution can take place even under extreme conditions.

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