Journal of Chromatography, 268 (1983) 493–501
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,022

CHROMATOGRAPHIC BEHAVIOUR OF AMINO ACIDS, THEIR DERIVATIVES AND PEPTIDES ON LAYERS OF AMMONIUM TUNGSTOPHOSPHATE AND SILANIZED SILICA GEL

L. LEPRI*, P. G. DESIDERI and D. HEIMLER
Institute of Analytical Chemistry, University of Florence, Via G. Capponi 9, 50121 Florence (Italy)
(Received May 31st, 1983)

SUMMARY

Amino acids and derivatives, peptides, tryptamine and its derivatives were studied on layers of ammonium tungstophosphate (AWP) and on plates of silanized silica gel untreated or impregnated with detergents, and eluted with aqueous solutions of ammonium nitrate or nitric acid and with acetic acid–methanol–water mixtures respectively. The changes in the carboxylic group have a significant effect on the retention on AWP layers; on plates of silanized silica gel, only the methyl or ethyl derivatives have different affinities towards the stationary phase. The distance between the $-NH_3^+$ group and the carboxylic group or groups in oligomers of the different amino acids determines the retention on AWP layers in the case of neutral (Met, Val, Tyr, Phe) and acidic (Glu) amino acids; on Sil C_{18}-50 the differences between the oligomers of neutral amino acids, which are correlated to their hydrophobicities, can be pointed out. The trends in R_M *versus* the number of amino acid residues are reported and discussed both on AWP and silanized silica gel layers. Interesting separations of closely related compounds were carried out. The AWP layers are particularly suited to the separation of the methyltryptophan isomers.

INTRODUCTION

Ammonium tungstophosphate (AWP) has proved to be very useful for the rapid separation of nitrogen-containing organic compounds, such as amino acids[1], primary aromatic amines[2], dinitrophenyl-amino acids[3], peptides[4] and their diastereoisomers[5] and aliphatic mono- and polyamines[6]. It has been pointed out that, in the case of aliphatic diamines and of glycine and alanine oligomers, the affinity sequence is closely correlated to the distance between the two $-NH_3^+$ groups involved in the exchange reaction, or between the $-NH_3^+$ group and the carboxylic group. The steric hindrance of a methyl group on the same carbon atom bonded to the exchanging $-NH_3^+$ group plays an important rôle in the retention (*cf.*, alanine and glycine, and 1,2-diaminopropane and 1,2-diaminoethane[3,4]).

It was therefore of interest further to elucidate the retention mechanism, and to examine the behaviour of amino acids, their esters and other derivatives, of hydrophobic (Met, Val, Tyr and Phe) or strongly polar (Glu) amino acid oligomers and of tryptamine and tryptophan derivatives containing a methyl group directly

0021-9673/83/$03.00

bonded to the nitrogen atom or at different distances from the amino group. The behaviour of these compounds has also been studied on ready-for-use plates of silanized silica gel (Sil C_{18}-50 and OPTI UP-C_{12}) untreated or impregnated with anionic and cationic detergents in order to test the usefulness of reversed-phase chromatography.

EXPERIMENTAL

The standard solutions of amino acids, their derivatives and peptides (1–2 mg/ml) were prepared by dissolving the compounds in water–methanol (1:1). The amount deposited on the layer was between 0.2 and 0.5 μg in the case of Sil C_{18}-50 (Macherey, Nagel & Co., Düren, F.R.G.) and between 0.5 and 1 μg for ammonium tungstophosphate (2 μg for Phe_4). The compounds were visualized by spraying the layers with a 1% ninhydrin solution in pyridine–acetic acid (5:1) and heating the plates for 5 min at 100°C. N-Methyltryptamine, gramine and N-methyltryptophan were detected by spraying a 1% *p*-dimethylaminobenzaldehyde solution in concentrated hydrochloric acid–methanol (1:1) and heating the layers at 50°C for 20 min. The AWP and its layers were prepared as described previously[1,2]. Only a AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$ mixture in the 4:2 ratio was used.

In order to obtain reproducible R_F values, the plates must be used within 24 h of their preparation. The Sil C_{18}-50 plates were impregnated with N-dodecylpyridinium chloride (N-DPC) or with dodecylbenzenesulphonic acid (HDBS) as previously described[7]. The migration distance was 6 cm for the ready-for-use plates and 10 cm in the case of the inorganic exchanger. The chromatographic measurements were carried out at 25°C.

The following abbreviations are used: –NHOH = hydroxamate; $-NHNH_2$ = hydrazide; –OMe = methyl ester; –OEt = ethyl ester; $-(OMe)_2$ = dimethylester; $-(OEt)_2$ = diethyl ester; Trm = tryptamine; Glu-NH_2 = glutamine.

RESULTS AND DISCUSSION

Ammonium tungstophosphate layers

Layers of AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$ in the ratio 4:2 were employed since these have been found to give the best results both in terms of the compactness of the spots and the resolution power[1,2]. The eluents used were aqueous solutions of ammonium nitrate at different concentrations and 0.5 *M* nitric acid (see Table I). With all the eluents the formation of a double front is observed. The first front merges with the second one at increasing concentrations of the salt or of the acid[4]. The data of Table I show that as the salt concentration in the eluent is increased the affinity sequence of the different compounds generally does not change.

As regards the amino acid derivatives, the behaviour of the γ-substituted compounds of glutamic acid is very interesting. On this exchanger, Glu-γ-NH_2, Glu-γ-$NHNH_2$ and Glu-γ-NHOH can easily be separated by eluting with 1 *M* ammonium nitrate (see Fig. 1), whereas Glu-γ-OMe and Glu-γ-OEt exhibit the same chromatographic behaviour. All these five γ-substituted derivatives have R_F values smaller than that of glutamic acid. Therefore the retention seems to be affected both by the kind of substituent group and by the negative charge due to the carboxylic group.

TABLE I

R_F VALUES OF AMINO ACIDS AND PEPTIDES ON THIN LAYERS OF AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$ AND Sil C_{18}-50 WITH DIFFERENT ELUENTS

n.d. = Not determined. Me = methyl; Et = ethyl.

Compound	*AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$*			*Sil C_{18}-50*
	1 M NH_4NO_3	*2 M NH_4NO_3*	*0.5 M HNO_3*	*Acetic acid–methanol–water (1:20:79)*
Glu	0.60	0.72	0.33	0.96
Glu-NH_2	0.49	0.58	0.16	0.95
Glu-γ-NHOH	0.40	0.57	0.04	0.95
Glu-γ-$NHNH_2$	0.57	0.64	0.21	0.95
Glu-γ-OMe	0.46	0.55	0.18	0.90
Glu-γ-OEt	0.46	0.55	0.18	0.79
Glu-$(OMe)_2$	0.27	0.35	0.08	0.43
Glu-$(OEt)_2$	0.27	0.35	0.08	0.34
Glu_2	0.35	0.47	0.13	0.96
γ-Glu-Glu	0.45	0.54	0.20	0.96
γ-Glu-Glu-NH_2	0.30	0.40	0.06	0.96
Glu_3	0.03	0.08	0.00	0.96
Asp	0.67	0.74	0.40	0.96
Asp-β-NHOH	0.61	0.68	0.31	0.95
Asp-$(OMe)_2$	0.37	0.45	0.10	0.52
Gly	0.66	0.74	0.40	0.96
Gly-NHOH	0.56	0.65	0.25	0.90
Gly-OMe	0.51	0.60	0.21	0.70
Ala	0.69	0.77	0.49	0.96
Ala-NHOH	0.63	0.72	0.35	n.d.
Ala-OMe	0.52	0.60	0.21	0.66
β-Ala	0.52	0.60	0.23	0.96
β-Ala-NHOH	0.47	0.56	0.16	0.91
β-Ala-OMe	0.41	0.50	0.12	0.79
Met	0.50	0.58	0.21	0.84
Met_2	0.14	0.22	0.05	0.60
Met_3	0.04	0.08	0.02	0.40
Val	0.67	0.78	0.46	0.90
Val_2	0.49	0.58	0.20	0.73
Val_4	n.d.	n.d.	n.d.	0.34
Tyr	0.57	0.64	0.27	0.76
Tyr_2	0.23	0.31	0.11	0.49
Tyr_3	0.06	0.10	0.06	0.19
Phe	0.53	0.58	0.19	0.65
Phe_2	0.14	0.23	0.05	0.19
Phe_3	0.03	0.04	0.00	0.02
Phe_4	0.00	0.00	0.00	0.00
Tyr-Gly	0.30	0.43	0.10	0.72
Tyr-Gly-Gly	0.15	0.27	0.03	0.72
Tyr-Pro-Phe-Pro	0.01	0.03	0.00	0.05
Tyr-Pro-Phe-Pro-Gly	0.01	0.03	0.00	0.07
Trp	0.26	0.36	0.07	0.43
N-Methyl-Trp	0.15	0.19	0.01	0.45
7-Methyl-Trp	0.23	0.34	0.07	0.28
6-Methyl-Trp	0.32	0.41	0.11	0.28

(Continued on p. 496)

TABLE I *(continued)*

Compound	*AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$*			*Sil C_{18}-50 Acetic acid–methanol–water (1:20:79)*
	1 M NH_4NO_3	*2 M NH_4NO_3*	*0.5 M HNO_3*	
5-Methyl-Trp	0.21	0.29	0.04	0.28
5-Methoxy-Trp	0.17	0.25	0.02	0.35
5-Hydroxy-Trp	0.32	0.43	0.11	0.61
Trm	0.06	0.11	0.02	0.30
N-Methyl-Trm	0.05	0.09	0.00	0.31
Gramine	0.00	0.00	0.00	0.34
5-Methyl-Trm	0.07	0.15	0.00	0.20
5-Methoxy-Trm	0.04	0.07	0.00	0.27

The behaviour of the dimethyl and diethyl esters of glutamic acid, which are more strongly retained than the corresponding monoderivatives, further supports this assumption.

The γ-substituted derivatives of glutamic acid containing nitrogen atoms are less strongly retained than the methyl and ethyl esters, eluting with 2 *M* ammonium nitrate. This trend is common to the hydroxamates of aspartic acid, glycine and alanine. The R_F sequence is always as follows: amino acid > hydroxamate > ester. This behaviour allows the separation of aspartic acid, glycine and alanine from their derivatives (see Fig. 1).

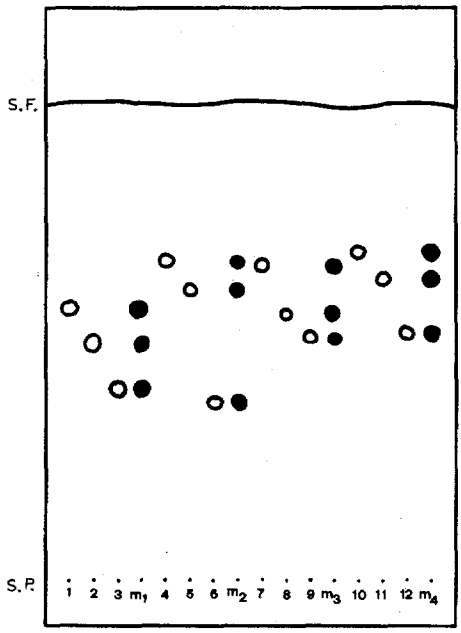


Fig. 1. Thin-layer chromatogram on AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$. Eluent: 1 *M* NH_4NO_3. Compounds: 1 = Glu-γ-$NHNH_2$; 2 = Glu-NH_2; 3 = Glu-γ-NHOH; 4 = Asp; 5 = Asp-β-NHOH; 6 = Asp-$(OMe)_2$; 7 = Gly; 8 = Gly-NHOH; 9 = Gly-OMe; 10 = Ala; 11 = Ala-NHOH; 12 = Ala-OMe. m_1 = Mixture of 1–3; m_2 = mixture of 4–6; m_3 = mixture of 7–9; m_4 = mixture of 10–12. S.P. = Starting point; S.F. = solvent front.

The hydroxamates and the dimethyl derivatives of aspartic and glutamic acids can easily be separated from each other, while the free amino acids can be separated only with difficulty. The same behaviour is observed in the case of glycine and alanine hydroxamates, and suggests the use of this exchanger in the separation of amino acid derivatives. It is interesting to note that the eluents are weakly acidic salt solutions and can be used also with unstable derivatives.

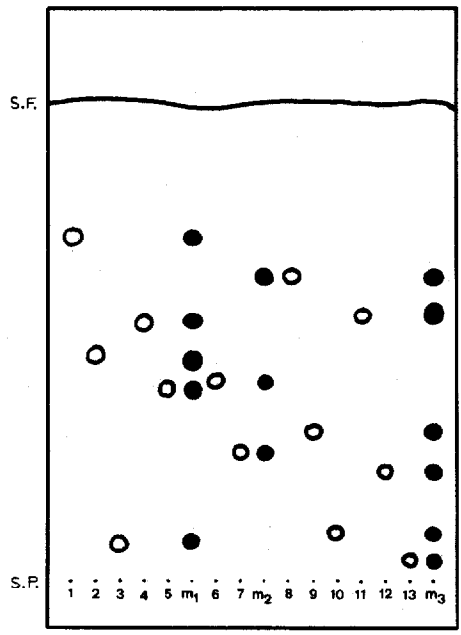
The distance between the amino group and the carboxylic group plays an important rôle in the retention of the oligomers of glutamic acid, methionine, valine, tyrosine and phenylalanine. The retention of these compounds increases with increasing number of amino acid sub-units. A typical example is that of the glutamic acid oligomers. Glu_3 is more strongly retained than Glu_2 which is more strongly retained than Glu, notwithstanding the increase in the number of carboxylic groups on changing from Glu to Glu_2 and Glu_3. On the basis of the number of carboxylic groups the affinity sequence of the three compounds should be the opposite of that observed. The structures of the compounds and, particularly, the distances between the $-NH_3^+$ group and the two nearest carboxylic groups, support the experimental sequence; in fact, the carboxylic group in the γ-position gives rise to a constant contribution while the other, whose distance from the $-NH_3^+$ group increases with increasing number of the amino acid residues, determines the affinity of the oligomers towards the stationary phase. The affinity sequence is not affected by the degree of dissociation of the carboxylic group which is the same when eluting with ammonium nitrate or with 0.5 *M* nitric acid (see Table I).

The smaller retention of γ-Glu-Glu with respect to Glu_2 indicates the higher influence of the carboxylic group in the α- than in the γ-position in decreasing the affinity towards the stationary phase. The sequence of the R_F values of the glutamic acid oligomers including γ-Glu-Glu-NH_2, is as follows: Glu > γ-Glu-Glu > Glu_2 > γ-Glu-Glu-NH_2 > Glu_3. The separation of these five compounds with 2 *M* ammonium nitrate as eluent is shown in Fig. 2. The separation of Tyr, Tyr-Gly and Tyr-Gly-Gly (see Fig. 2) is a further confirmation of the high selectivity of this exchanger towards compounds which differ in the distance between the $-NH_3^+$ group and the carboxylic group. Fig. 2 also shows the simultaneous separation of tyrosine and phenylalanine oligomers.

As regards the valine oligomers, it should be noted that Val_4 cannot be detected even at the 2-μg level; this demonstrates the limits of this exchanger in the case of compounds with numerous and/or long aliphatic side-chains[5,6].

The chromatographic behaviour of tryptophan, tryptamine (Trm) and their derivatives demonstrates the influence of the substituent group and of its position on the retention of these compounds. The introduction in the tryptophan molecule of a $-CH_3$, $-OCH_3$ or $-OH$ group (5-position) results in the following R_F sequence $-OH > -CH_3 > -OCH_3$ which has also been found in the case of methyl and methoxy derivatives of tryptamine and does not fit with that predicted on the basis of the substituent polarity.

The position of the methyl substituent results in a R_F sequence: 6-CH_3 > 7-CH_3 > 5-CH_3 $\gg$ N-CH_3. The strong retention of compounds with a methyl group bonded to the nitrogen atom, which has also been found in the case of the N-methyl derivatives of tryptamine, may be explained on the basis of the higher affinity of the N-alkylammonium ions with respect to ammonium ion towards the tungstophos-



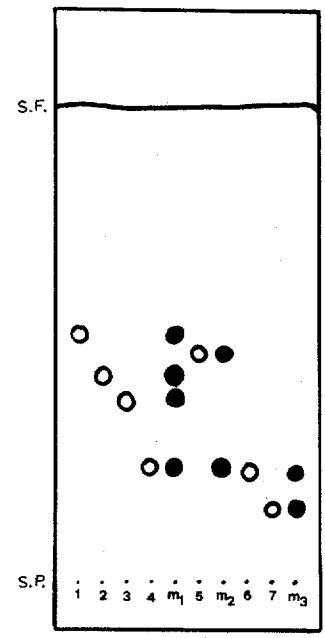


Fig. 2. Thin-layer chromatogram on AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$. Eluent: 2 *M* NH_4NO_3. Compounds: 1 = Glu; 2 = Glu_2; 3 = Glu_3; 4 = γ-Glu-Glu; 5 = γ-Glu-Glu-NH_2; 6 = Tyr-Gly; 7 = Tyr-Gly-Gly; 8 = Tyr; 9 = Tyr_2; 10 = Tyr_3; 11 = Phe; 12 = Phe_2; 13 = Phe_3. m_1 = Mixture of 1–5; m_2 = mixture of 6–8; m_3 = mixture of 8–13.

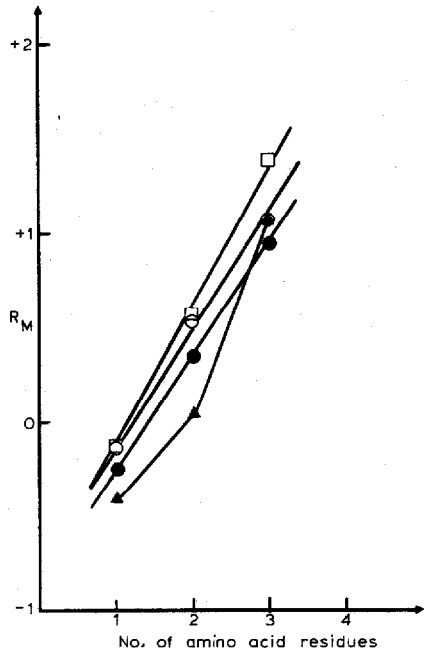
Fig. 3. Thin-layer chromatogram on AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$. Eluent: 4 *M* NH_4NO_3. Compounds: 1 = 6-methyl-Trp; 2 = 7-methyl-Trp; 3 = 5-methyl-Trp; 4 = N-methyl-Trp; 5 = Trp; 6 = Trm; 7 = N-methyl-Trm. m_1 = Mixture of 1–4; m_2 = mixture of 4 and 5; m_3 = mixture of 6 and 7.

phate anion[6]. The lower solubility of alkyl- and arylammonium molybdophosphate with respect to the ammonium salt is also known[8,9].

Gramine is more strongly retained than N-methyltryptamine; this is a further demonstration that an increase in the number of alkyl groups bonded to the nitrogen atom results in an increase in the affinity towards the inorganic exchanger. The affinity sequence is therefore as follows: $-\underset{\displaystyle CH_3}{\overset{\displaystyle |}{\underset{|}{N}}}-CH_3 > -\underset{\displaystyle H}{\overset{\displaystyle |}{N}}-CH_3 > -\underset{\displaystyle H}{\overset{\displaystyle |}{N}}-H$. The separations of the four isomers of tryptophan containing a methyl group and of tryptophan and tryptamine from their N-methyl derivatives are shown in Fig. 3.

Trends in R_M versus the number of amino acid residues. The influence on the chromatographic behaviour of the number of amino acid residues may be demonstrated by plotting the R_M values as a function of this number for the different oligomers. Fig. 4 shows such a plot for methionine, tyrosine, phenylalanine and glutamic acid oligomers. In the case of neutral amino acid oligomers, straight lines are obtained in agreement with a previous study concerning glycine and alanine oligomers[6].

The slopes of the straight lines (see Fig. 4), being remarkably steeper than those obtained for alanine (≈0.2) and glycine (≈0.3), show that the retention de-



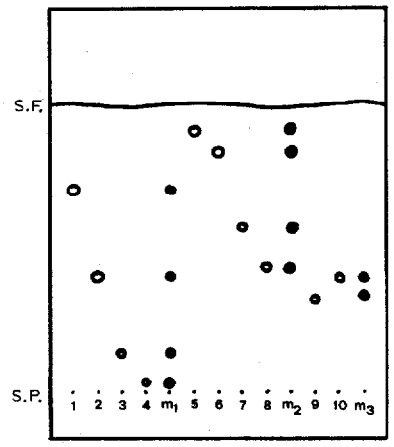


Fig. 4. Plot of R_M *versus* number of amino acid residues on layers of AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$. Eluent: 2 *M* ammonium nitrate. □, Phe (0.72); ○, Met (0.63); ●, Tyr (0.61); ▲, Glu. The numbers in parentheses refer to the slopes of the straight lines.

Fig. 5. Thin-layer chromatogram on Sil C_{18}-50. Eluent: acetic acid–methanol–water (1:40:59). Compounds: 1 = Phe; 2 = Phe_2; 3 = Phe_3; 4 = Phe_4; 5 = Glu-γ-OMe; 6 = Glu-γ-OEt; 7 = Glu-$(OMe)_2$; 8 = Glu-$(OEt)_2$; 9 = Tyr-Pro-Phe-Pro; 10 = Tyr-Pro-Phe-Pro-Gly. m_1 = Mixture of 1–4; m_2 = mixture of 5–8; m_3 = mixture of 9 and 10.

pends on the kind of amino acid residue. In particular, the retention of the different oligomers increases with increasing affinity of the constituent amino acids towards the exchanger. The trends for the glutamic acid oligomers never fit straight lines, regardless of the ammonium nitrate concentration in the eluent (from 1 to 4 *M*). This behaviour can be ascribed to the acidic characteristics of the amino acid which involve, on changing from Glu to Glu_2 and Glu_3, an increase in the number of carboxylic groups.

Plates of silanized silica gel

Table I (column 5) lists the R_F values of amino acids and peptides on layers of Sil C_{18}-50 eluted with acetic acid–methanol–water (1:20:79, v/v). Even when the eluent contains very little methanol, most polar amino acids and derivatives run with the solvent front. Furthermore, the hydroxamates can be detected only with difficulty. Therefore these plates are ill-suited for the separation of such compounds. Similar results are obtained on OPTI UP-C_{12} plates eluted with aqueous solutions.

The best results are achieved in the case of methyl and ethyl esters and of peptides formed by hydrophobic amino acid residues. The chromatogram in Fig. 5 shows the separation of the four phenylalanine oligomers, of the methyl and ethyl

esters of glutamic acid and of the two peptides Tyr-Pro-Phe-Pro and Tyr-Pro-Phe-Pro-Gly eluted with acetic acid–methanol–water (1:40:59). The high percentage of methanol (40%) accounts for the separation of the last two peptides and of Phe_3 from Phe_4 which could not be effected under the eluent conditions given in Table I.

The affinity sequence of the glutamic acid esters is as follows: Glu-$(OEt)_2$ > Glu-$(OMe)_2$ > Glu-γ-OEt > Glu-γ-OMe which agrees with the hydrophobicity sequence of the compounds. The influence of the hydrophobic characteristics of substituent groups on the retention is also seen from the comparison of the 5-substituted derivatives of tryptophan and tryptamine, whose R_F sequence is: –OH > $-OCH_3$ > $-CH_3$. In contrast to the behaviour on AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$ layers, the position of the methyl substituent in the molecule does not affect the retention (see 5-, 6- and 7-methyltryptophan). The presence of a methyl group directly bonded to the nitrogen atom (see tryptophan and N-methyltryptophan; tryptamine, N-methyltryptamine and gramine) does not involve large changes in the R_F values, apart from a slight decrease in retention. Such behaviour is not correlated to the degree of dissociation of the carboxylic group; in fact, on eluting with 0.1 *M* ammonia in 40% methanol, tryptophan, N-methyltryptophan and 5-methoxytryptophan have R_F =

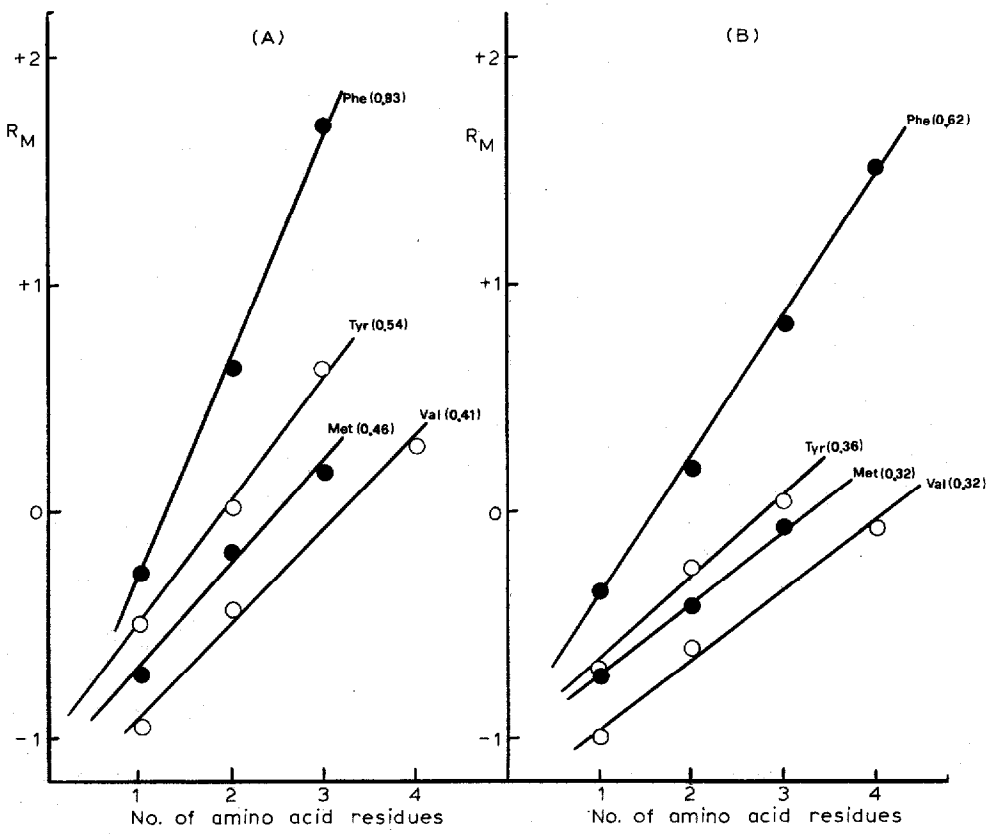


Fig. 6. Plots of R_M *versus* number of amino acid residues on sil C_{18}-50 plates. Eluents: A, acetic acid–methanol–water (1:20:79); B, acetic acid–methanol–water (1:40:59). The numbers in parentheses are the slopes of the lines.

TABLE II

SEPARATIONS OF AMINO ACIDS AND DERIVATIVES ON LAYERS OF Sil C_{18}-50 + 4% HDBS ELUTED WITH ACETIC ACID–METHANOL-WATER (1:20:79)

Me = methyl; Et = ethyl.

Mixture	R_F
Glu-γ-OMe/Glu-γ-OEt/Glu-$(OMe)_2$/Glu-$(OEt)_2$	0.89/0.65/0.15/0.04
Gly/Gly-NHOH/Gly-OMe	0.95/0.61/0.21
Ala/Ala-NHOH/Ala-OMe	0.95/0.60/0.30
β-Ala/β-Ala-NHOH/β-Ala-OMe	0.92/0.56/0.30

0.66, the three methyl derivatives R_F = 0.54 and 5-hydroxytryptophan R_F = 0.81. Tryptamine and its derivatives, on the contrary, remain at the starting point.

R_M versus number of amino acid residues. Fig. 6 reports the trends in R_M and the number of amino acid residues for the oligomers of valine, methionine, tyrosine and phenylalanine when eluted with acetic acid–methanol–water in the ratios 1:20:79 (A) and 1:40:59 (B). In both cases straight lines are obtained, similarly to the cases of column chromatography of alanine oligomers[10] and of the oligo-γ-glutamates of phthalic acid[11]. The slopes increase on changing from valine to phenylalanine (see the numbers in parentheses in Fig. 6) and are closely correlated to the different hydrophobic characteristics of the amino acid residues. An increase in the percentage of methanol in the eluent results in a smaller difference of the slope values, as expected.

Layers of Sil C_{18}-50 impregnated with anionic and cationic detergents

These layers have been employed in order to determine whether the impregnation would result in a stronger retention of polar compounds which on plates of silanized silica gel alone run with the solvent front. On plates impregnated with 4% N-DPC, eluted with 0.5 *M* sodium acetate in water–methanol (20%), slight differences are observed with respect to the untreated layers. More interesting results are achieved on Sil C_{18}-50 plates impregnated with 4% HDBS and eluted with acetic acid–methanol–water (1:20:79). Besides a more marked retention of the esters of acidic and neutral amino acids, a higher affinity of the hydroxamates towards the stationary phase is observed. Under these experimental conditions, glycine, alanine and β-alanine can be separated from their derivatives (see Table II).

REFERENCES

1 L. Lepri, P. G. Desideri and D. Heimler, *Ann. Chim. (Rome)*, 71 (1981) 89.
2 L. Lepri, P. G. Desideri and D. Heimler, *J. Chromatogr.*, 207 (1981) 29.
3 L. Lepri, P. G. Desideri and D. Heimler, *J. Chromatogr.*, 235 (1982) 411.
4 L. Lepri, P. G. Desideri and D. Heimler, *J. Chromatogr.*, 243 (1982) 339.
5 L. Lepri, P. G. Desideri, D. Heimler and S. Giannessi, *J. Chromatogr.*, 265 (1983) 328.
6 L. Lepri, P. G. Desideri, D. Heimler and S. Giannessi, *J. Chromatogr.*, 245 (1982) 297.
7 L. Lepri, P. G. Desideri and D. Heimler, *J. Chromatogr.*, 209 (1981) 312.
8 G. Schulze and S. Noack, *Z. Anorg. Allg. Chem.*, 426 (1976) 119.
9 G. Schulze and S. Noack, *Z. Anal. Chem.*, 294 (1979) 117.
10 I. Molnár and Cs. Horváth, *J. Chromatogr.*, 142 (1977) 623.
11 B. T. Bush, Jr., J. H. Frenz, Wr. Melander, Cs. Horváth, A. R. Cashmore, R. N. Dryer, J. K. Coward, J. O. Knipe and J. R. Bertino, *J. Chromatogr.*, 168 (1979) 343.