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REVERSED-PHASE AND SOAP THIN-LAYER CHROMATOGRAPHY OF PEPTIDES

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SUMMARY

The chromatographic behaviour of 38 peptides on layers of silanized silica gel alone and impregnated with anionic and cationic detergents has been investigated. On the basis of the comparison with the amino acid constituents, it has been possible to predict the sequence of the affinities of the peptides with the different solvents. Many separations have been carried out on layers impregnated with 4% dodecylbenzenesulphonic acid solution.

INTRODUCTION

With reversed-phase chromatography on thin layers of silanized silica gel impregnated with detergents (soap TLC), it has been possible to perform many separations of amino acids that can be effected only with difficulty or not at all on ion-exchange thin layers[1]. The type and concentration of the detergent on the silanized silica gel determines the separations that can be achieved. The retention of amino acids seems to be controlled by their interactions with both the hydrophobic side-chain and the functional group (ion-exchange) of the detergent.

In this work we investigated whether soap TLC can also be used for the separation of peptides. Interesting results have recently been achieved with reversed-phase chromatography on C_{18}-bonded silica columns[2] and on μBondapak alkylphenyl columns in the presence of ion-pairing reagents in the eluent[3–5].

EXPERIMENTAL

The compounds were dissolved in water–methanol (1:1). The amount of substance deposited on the layer was between 1 and 2 μg. The peptides were detected by spraying the wet layers with a solution of 1% ninhydrin in pyridine–glacial acetic acid (5:1) and then heating the layers at 100°C for 5 min.

The layers (thickness 300 μm) were prepared with a Chemetron automatic apparatus by mixing 20 g of silanized silica gel 60 HF (C_2) (Merck, Darmstadt, G.F.R.) in 50 ml of 95% ethanol with a known concentration of detergent. The detergent concentrations reported in the text refer to the alcoholic solution in which

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the silanized silica gel was suspended. The detergents used were triethanolamine dodecylbenzensulphonate (DBS), sodium dioctylsulphosuccinate (Na-DSS) (Serva, Heidelberg, G.F.R.), dodecylbenzensulphonic acid (H-DBS) (ICN Pharmaceutical, Plainview, NY, U.S.A.) and N-dodecylpyridinium chloride (N-DPC) (Merck). All measurements were carried out at 25°C. The migration distance was 11 cm unless otherwise stated.

The amino acids used were glycine (Gly), alanine (Ala), serine (Ser), isoleucine (Ile), leucine (Leu), proline (Pro), tyrosine (Tyr), phenylalanine (Phe), valine (Val), histidine (His), aspartic acid (Asp), arginine (Arg), tryptophan (Trp) and methionine (Met).

RESULTS AND DISCUSSION

Table I reports the chromatographic characteristics of 38 peptides on layers of silanized silica gel alone and impregnated with DBS, Na-DSS and H-DBS, eluting with an aqueous–organic mixture containing 5.7% acetic acid and 30% methanol (apparent pH = 2.75).

On layers of silanized silica gel alone the peptides with hydrophilic or basic amino acids (glycine, alanine, serine, arginine, histidine and aspartic acid) are not retained by the stationary phase and run with the solvent front. The peptides with one or more hydrophobic amino acid residues (valine, leucine, isoleucine, tyrosine and phenylalanine) are more retained. The strongest retention is observed with the pentapeptide, which is mainly formed by hydrophobic amino acids and therefore it can be easily separated from all others, which is different to the results observed on impregnated layers. The chromatographic behaviour of the peptides on this layer is similar to that observed on alkylphenyl and C_{18}-bonded silica columns eluting with acidic solutions[2,4].

On layers impregnated with 4% DBS a general increase in the retention of peptides, especially of those containing hydrophobic and basic amino acids, is observed. Some peptides (Gly–Pro and Ala–Pro), however, give rise to elongated spots and the detection of the peptides that are only slightly retained (Asp–Gly and Asp–Ala) is difficult owing to the violet colour which appears in the neighbourhood of the solvent front[1].

In the presence of 4% Na-DSS, although an increase in the retention with respect to DBS is observed, a change in the sequence of the R_F values for the different peptides is not achieved. On this layer several peptides give rise to non-compact spots (Gly–Gly, Gly–Ala, Ala–Gly) and others (Gly–Ser and Ala–Ser) give rise to a second spot with $R_F = 0.92$, which can probably be ascribed to the amino acid constituents that are formed on the layer by hydrolysis of the peptides. This last occurrence is probably related to the acid–base characteristics of this detergent, which is formed from the sodium salt of a weak acid.

On layers impregnated with 4% H-DBS, all peptides are strongly retained and the spots are very compact. For these reasons the study has been focused on layers impregnated with this detergent.

Table I also shows the influence of the H-DBS concentration on the chromatographic behaviour of the peptides. As the percentage of detergent is increased a sharp increase in the retention of all compounds, and in some instances even a change in the affinity sequence, is observed. The reversal of the sequence of the R_F values

TABLE I

R_F VALUES OF PEPTIDES ON THIN LAYERS OF SILANIZED SILICA GEL ALONE AND IMPREGNATED WITH DETERGENTS

Eluent: water–methanol–acetic acid (64.3:30:5.7).

Peptide	*Layer*					
	SiO_2	*SiO_2 + 4% DBS*	*SiO_2 + 4% Na-DSS*	*SiO_2 + 1% H-DBS*	*SiO_2 + 2% H-DBS*	*SiO_2 + 4% H-DBS*
Gly–Gly	0.96	0.76	0.60	0.88	0.68	0.44
Gly–Ala	0.96	0.68	0.55	0.85	0.62	0.39
Gly–Ser	0.96	0.81	0.71	0.93	0.75	0.53
Gly–Ile	0.83	0.22	0.14	0.38	0.22	0.09
Gly–Leu	0.81	0.20	0.12	0.32	0.20	0.08
Gly–Pro	0.96	e.s.*	0.51	0.75	0.56	0.28
Gly–Tyr	0.90	0.44	0.33	0.59	0.44	0.22
Gly–Phe	0.72	0.18	0.12	0.29	0.18	0.07
Ala–Ala	0.95	0.60	0.48	0.74	0.58	0.36
Ala–Gly	0.96	0.74	0.60	0.87	0.67	0.43
Ala–Ser	0.96	0.77	0.70	0.92	0.71	0.50
Ala–Val	0.90	0.37	0.25	0.52	0.37	0.20
Ala–Ile	0.77	0.21	0.14	0.33	0.21	0.09
Ala–Pro	0.92	0.48**	0.43	0.67	0.49	0.24
Ala–Tyr	0.90	0.40	0.30	0.54	0.41	0.20
Ala–Hys	0.96	0.16	0.06	0.38	0.12	0.02
Asp–Gly	0.96	n.d.***	0.92	0.95	0 78	0 58
Asp–Ala	0.96	n.d.	0.78	0.88	0.68	0.49
Phe–Gly	0.76	0.19	0.13	0.32	0.19	0.08
Phe–Ala	0.75	0.17	0.12	0.26	0.15	0.07
Arg–Gly	0.96	0.06	0.03	0.21	0.05	0.02
Arg–Asp	0.96	0.11	0.05	0.29	0.09	0.03
Ile–Gly	0.90	0.30	0.24	0.47	0.35	0.14
Leu–Leu	0.55	0.06	0.05	0.09	0.05	0.03
Leu–Val	0.71	0.13	0.11	0.20	0.12	0.07
Leu–Tyr	0.72	0.16	0.14	0.24	0.15	0.08
Gly–Gly–Gly	0.96	0.75	0.56	0.83	0.66	0.42
Gly–Gly–Ala	0.96	0.64	0.49	0.78	0.59	0.38
Gly–Ala–Gly	0.96	0.67	0.49	0.78	0.59	0.38
Gly–Ala–Ala	0.96	0.60	0.46	0.74	0.56	0.36
Ala–Ala–Ala	0.96	0.61	0.51	0.74	0.57	0.38
Gly–Gly–Phe	0.76	0.24	0.16	0.29	0.17	0.07
Gly–Leu–Tyr	0.63	0.13	0.12	0.17	0.12	0.06
Leu–Gly–Phe	0.47	0.05	0.04	0.09	0.05	0.02
Gly–Gly–Gly–Gly	0.96	0.75	0.56	0.83	0.64	0.39
Ala–Ala–Ala–Ala	0.96	0.63	0.55	0.75	0.59	0.41
Leu–Trp–Met–Arg	0.49	0.00	0.00	0.00	0.00	0.00
Leu–Trp–Met–Arg–Phe	0.11	0.00	0.00	0.00	0.00	0.00

* e.s. = elongated spot.
** Tailing.
*** n.d. = not determined.

for tetra-Gly and tetra-Ala on changing from 1% to 4% H-DBS on the layer should be noted.

Influence of eluent acidity

As with amino acids, the chromatographic behaviour of peptides is consider-

ably affected both by the apparent pH of the eluent and by the counter ion (Na^+) concentration.

On the basis of the acid–base characteristics of these compounds (Table II), species with different charges may exist in solution depending on the apparent pH of

TABLE II

R_F VALUES OF PEPTIDES ON THIN LAYERS OF SILANIZED SILICA GEL IMPREGNATED WITH 4% H-DBS

Eluents: (1) 0.1 *M* HCl + 1 *M* CH_3COOH in 30% CH_3OH (pH 1.25); (2) 0.05 *M* HCl + 1 *M* CH_3COOH in 30% CH_3OH (pH 1.55); (3) 0.1 *M* NaCl + 1 *M* CH_3COOH in 30% CH_3OH (pH 2.75); (4) 0.1 *M* NaCl + 0.1 *M* CH_3COOH in 30% CH_3OH (pH 3.30); (5) 0.1 *M* CH_3COONa + 0.1 *M* CH_3COOH in 30% CH_3OH (pH 5.10); (6) 1 *M* CH_3COONa in 30% CH_3OH (pH 8.15).

Peptide	*Eluent*						$pK_{(-COOH)}$[6]	$pK_{(-NH^+)}$[6]
	1	*2*	*3*	*4*	*5*	*6*		
Gly–Gly	0.68	0.60	0.70	0.70	0.69	0.83	3.22	8.17
Gly–Ala	0.60	0.55	0.64	0.64	0.71	0.85	3.17	8.23
Gly–Ser	0.73	0.70	0.75	0.76	0.75	0.85	2.92	8.10
Gly–Ile	0.17	0.15	0.19	0.13	0.32	0.67	—	—
Gly–Leu	0.14	0.12	0.16	0.11	0.28	0.62	3.18	8.29
Gly–Pro	0.42	0.36	0.49	0.49	0.59	0.79	2.81	8.65
Gly–Tyr	0.31	0.30	0.40	0.30	0.50	0 78	2.93	8.45
Gly–Phe	0.12	0.08	0.12	0.11	0.24	0.52	3.12	8.17
Ala–Ala	0.56	0.52	0.62	0.62	0.70	0.83	3.30	8.14
Ala–Gly	0.66	0.59	0.69	0.69	0.73	0.85	3.17	8.18
Ala–Ser	0.72	0.68	0.74	0.74	0.73	0.85	—	—
Ala–Val	e.s.*	0.27	0.36	0.34	0.52	0.78	—	—
Ala–Ile	0.15	0.13	0.19	0.16	0.30	0.71	—	—
Ala–Pro	0.37	0.32	0.43	0.43	0.55	0.78	3.04	8.38
Ala–Tyr	0.31	0.30	0.39	0.32	0.46	0.78	—	—
Ala–His	0.10	0.04	0.15	0.14	0.15	0.76	2.65	6.83, 9.51
Asp–Gly	0.74	0.71	0.75	0.77	0.77	0.87	2.10, 4.53	9.07
Asp–Ala	0.62	0.61	0.68	0.65	0.67	0.84	—	—
Phe–Gly	0.13	0.11	0.13	0.09	0.15	0.37	—	—
Phe–Ala	0.12	0.10	0.15	0.08	0.18	0.49	—	—
Arg–Gly	0.06	0.03	0.06	0.04	0.05	0.56	—	—
Arg–Asp	0.08	0.04	0.09	0.07	0.11	0.69	—	—
Ile–Gly	0.23	0.21	0.28	0.20	0.31	0.56	—	—
Leu–Leu	0.08	0.04	0.06	0.03	0.07	0.31	—	—
Leu–Val	0.11	0.09	0.13	0.08	0.21	0.56	—	—
Leu–Tyr	0.12	0.11	0.16	0.09	0.21	0.55	2.87	8.36
Gly–Gly–Gly	0.66	0.58	0.67	0.66	0.62	0.82	3.28	8.00
Gly–Gly–Ala	0.59	0.52	0.63	0.61	0.64	0.82	—	—
Gly–Ala–Gly	0.62	0.54	0.64	0.62	0.61	0.82	—	—
Gly–Ala–Ala	0.59	0.51	0.62	0.59	0.63	0.82	3.38	8.10
Ala–Ala–Ala	0.60	0.54	0.63	0.61	0.69	0.82	3.39	8.03
Gly–Gly–Phe	0.13	0.10	0.13	0.09	0.22	0.55	—	—
Gly–Leu–Tyr	0.09	0.08	0.10	0.06	0.14	0.40	—	—
Leu–Gly–Phe	0.04	0.03	0.03	0.02	0.04	0.20	—	—
Gly–Gly–Gly–Gly	0.62	0.56	0.67	0.65	0.59	0.78	—	—
Ala–Ala–Ala–Ala	0.62	0.57	0.69	0 66	0 63	0.80	3.42	7.94
Leu–Trp–Met–Arg	0.00	0.00	0.00	0.00	0.00	0.00	—	—
Leu–Trp–Met–Arg–Phe	0.00	0.00	0.00	0.00	0.00	0.00	—	—

* e.s. = elongated spot.

the eluent. On the other hand, as the peptides are generally hydrolysed with strongly alkaline or acidic eluents, giving rise to several spots on the layer or to elongated spots, the study was restricted to the pH range 1.25–8.15 (Table II). In this pH range the peptides change from the cationic to the zwitterionic form and, even if not completely, to the anionic form.

In the pH range in which the cationic form prevails (columns 1 and 2), an increase in the R_F values as the pH decreases is observed, as H^+ acts as a counter ion. On plotting the R_M values of the peptides as a function of the apparent pH of the eluent in the pH range 1.25–2.25, straight lines are obtained for most compounds. The $\Delta R_M/\Delta$pH slopes are between 0.3 and 0.5. These values are much lower than the theoretical values and are even lower than those obtained with amino acids[1]. For such peptides, however, the retention seems to be affected by an ion-exchange process.

The zwitterionic form of the peptides, which prevails at pH 5.1 (column 5), exhibits a smaller retention than the corresponding cationic form (see columns 3 and 4), as observed with amino acids[1]. As the apparent pH of the eluent is increased (column 6), a further decrease in retention by the layer is observed, in accordance with the prevalence of the anionic form of most peptides at such pH values.

As regards the behaviour of the single peptides, it is interesting that for those formed by hydrophilic amino acids an increase in pH results in a levelling of the R_F values, while for the others an increase in the resolving power is observed.

The affinity sequence of the different peptides can be predicted on the basis of the R_F values of the amino acid residues. In fact, the sequence of the oligomers Gly–Gly, Ala–Ala and Leu–Leu is identical with that of the corresponding amino acids under the same experimental conditions[1], apart from a sharper retention of the peptides. As the number of the amino acid residues is increased, such agreement no longer applies, as tetra-Ala, for instance, is less retained than tetra-Gly even in acidic solutions.

With dipeptides formed by two different amino acids, referring to the oligomer (*i.e.*, Gly–Gly or Ala–Ala) and replacing the final residue with other amino acids, the retention of the resulting peptides is correlated with that of the substituent amino acid. For instance, the sequence of R_F values Ala–Ser > Ala–Gly > Ala–Ala > Ala–Pro > Ala–Val > Ala–Ile > Ala–His, which is observed in acidic solutions, is identical with that found for the different amino acids (Ser > Gly > Ala, etc.) with the same eluents[1]. The replacement of the starting residue in the oligomer dipeptides generally does not affect the above sequence, but it results in a smaller change in the R_F values.

Influence of organic solvent concentration

The study of the influence of the organic solvent concentration on the retention of the peptides was performed keeping the acetic acid and sodium chloride concentrations constant at 1 and 0.1 *M*, respectively. As the percentage of methanol in the solution is increased, a decrease in retention is observed for most compounds, together with a reduction in the resolving power of the layer. Such a trend is similar to that observed for amino acids[1].

Use of water–organic solvent mixtures as eluents

We studied water–methanol, water–ethanol and water–acetic acid mixtures, which with amino acids yielded good results from an analytical standpoint. With peptides, the use of these eluents, especially water–acetic acid, gave useful results, particularly for dipeptides with low molecular weights, which are more clearly differentiated than with eluents used previously.

Table III gives the R_F values of peptides on silanized silica gel impregnated

TABLE III

R_F VALUES OF PEPTIDES ON THIN LAYERS OF SILANIZED SILICA GEL ALONE AND IMPREGNATED WITH ANIONIC OR CATIONIC DETERGENTS

Eluents: (1) water–acetic acid (7:3); (2) water–acetic acid (1:1); (3) 0.1 *M* CH_3COOH + 0.1 *M* CH_3COONa in 30% CH_3OH; (4) 1 *M* CH_3COOH in 30% CH_3OH.

Peptide	*SiO_2 + 4% H-DBS*		*SiO_2, eluent 3*	*SiO_2 + 4% N-DPC*	
	Eluent 1	*Eluent 2*		*Eluent 4*	*Eluent 3*
Gly–Gly	0.46	0.68	0.96	0.97	0.96
Gly–Ala	0.38	0.66	0.96	0.97	0.96
Gly–Ser	0.54	0.75	0.96	0.97	0.96
Gly–Ile	0.12	0.44	0.93	0.93	0.87
Gly–Leu	0.10	0.40	0.90	0.90	0.86
Gly–Pro	0.27	0.58	0.96	0.97	0.95
Gly–Tyr	0.27	0.69	0.94	0.84	0.77
Gly–Phe	0.10	0.41	0.78	0.81	0.69
Ala–Ala	0.35	0.64	0.96	0.97	0.96
Ala–Gly	0.43	0.70	0.96	0.97	0.96
Ala–Ser	0.51	0.72	0.96	0.97	0.96
Ala–Val	0.19	0.52	0.96	0.97	0.96
Ala–Ile	0.10	0.41	0.93	0.94	0.87
Ala–Pro	0.24	0.54	0.96	0.95	0.95
Ala–Tyr	0.26	0.62	0.96	0.88	0.81
Ala–His	0.03	0.18	0.96	0.97	0.96
Asp–Gly	0.58	0.79	0.96	0.94	0.88
Asp–Ala	0.46	0.71	0.96	0.94	0.89
Phe–Gly	0.10	0.42	0.79	0.86	0.74
Phe–Ala	0.08	0.41	0.81	0.89	0.81
Arg–Gly	0.02	0.13	0.96	0.97	0.96
Arg–Asp	0.02	0.18	0.96	0.97	0.96
Ile–Gly	0.15	0.48	0.94	0.96	0.95
Leu–Leu	0.03	0.26	0.57	0.80	0.71
Leu–Val	0.06	0.35	0.82	0.92	0.85
Leu–Tyr	0.10	0.49	0.74	0.79	0.69
Gly–Gly–Gly	0.39	0.67	0.96	0.97	0.96
Gly–Gly–Ala	0.34	0.67	0.96	0.97	0.96
Gly–Ala–Gly	0.35	0.67	0.96	0.97	0.96
Gly–Ala–Ala	0.34	0.67	0.96	0.97	0.96
Ala–Ala–Ala	0.36	0.68	0.96	0.97	0.96
Gly–Gly–Phe	0.07	0.43	0.80	0.78	0.66
Gly–Leu–Tyr	0.06	0.48	0.62	0.71	0.45
Leu–Gly–Phe	0.02	0.25	0.47	0.70	0.46
Gly–Gly–Gly–Gly	0.39	0.68	0.96	0.97	0.96
Ala–Ala–Ala–Ala	0.36	0.73	0.96	0.97	0.96
Leu–Trp–Met–Arg	0.02	0.05	0.32	0.79	0.58
Leu–Trp–Met–Arg–Phe	0.00	0.03	0.07	0.51	0.19

with 4% H-DBS eluting with mixtures containing 30% and 50% acetic acid; in both instances very compact spots are obtained. The former eluent is more suitable for the separation of hydrophilic dipeptides such as Gly–Gly, Gly–Ser and Ala–Ala, and the latter for the separation of hydrophobic dipeptides such as Leu–Leu, Leu–Val and Leu–Tyr.

Layers impregnated with cationic detergents

Table III gives the R_F values of peptides on layers of silanized silica gel impregnated with 4% N-DPC eluting with 1 *M* acetic acid in 30% methanol (apparent pH = 2.75) and with 0.1 *M* acetate buffer in 30% methanol (apparent pH = 5.10), and also results obtained on silanized silica gel with the eluent at pH 5.10.

On layers impregnated with N-DPC, the retention of the peptides eluting with the solution at pH = 2.75 is smaller than that observed in silanized silica gel alone, which is different to the results observed on layers impregnated with anionic detergents under the same elution conditions. Such behaviour can be ascribed to the repulsive forces between the positive charge of the peptide and that of the quaternary nitrogen atom of the cationic detergent. From an analytical standpoint, therefore, the use of these layers for the separation of peptides with hydrophilic amino acid residues and, generally, of those with low molecular weights does not offer any advantage over silanized silica gel alone, as such compounds are less well differentiated. For peptides with higher molecular weights and formed by hydrophobic amino acids, the smaller retention may offer the best separation conditions.

With the eluent at pH 5.10, an increase in retention is observed for those peptides formed by one or more hydrophobic amino acids and by aspartic acid. At this pH the peptides are prevalently in the zwitterionic form, which causes a decrease in the detergent–peptide repulsive forces and gives rise to an anion-exchange process with the counter ion of N-DPC.

With respect to the layers of silanized silica gel alone, where the above interactions are not possible, the peptides generally exhibit a stronger retention. However, the dipeptide Leu–Leu, the tetrapeptide Leu–Trp–Met–Arg and the pentapeptide are less retained under these elution conditions.

The use of layers impregnated with cationic detergents therefore seems very interesting for large peptides with one or more hydrophobic amino acid residues. A study of this subject is now being undertaken.

Analytical applications

Of the separations that are possible on the basis of the chromatographic data on layers impregnated with anionic detergents, we carried out some of those which are more interesting from an analytical standpoint.

Fig. 1 shows the separation of six dipeptides containing glycine as starting residue and that of Asp–Gly from Asp–Ala on layers of silanized silica gel impregnated with 4% H-DBS, eluting with 1 *M* acetic acid in 30% methanol. Under such elution conditions Gly–Gly has also been separated from Ala–Ala. The separation of these two peptides has also been performed by eluting with water–acetic acid (7:3).

Fig. 2 shows the separation of seven dipeptides containing alanine as starting

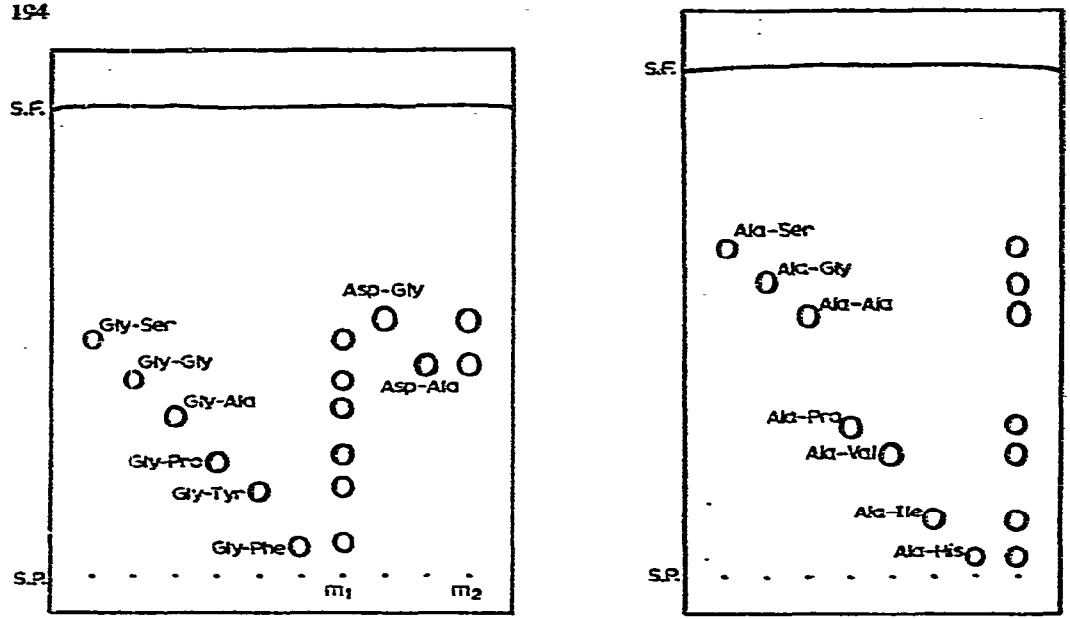

Fig. 1. Thin-layer chromatogram of peptides on silanized silica gel impregnated with 4% H-DBS solution. Migration distance = 12.5 cm. Eluent: water–methanol–acetic acid (64.3:30:5.7). m_1 = mixture of six peptides; m_2 = mixture of Asp–Gly and Asp–Ala. S.P. = starting point; S.F. = solvent front.

Fig. 2. Thin-layer chromatogram of peptides on silanized silica gel impregnated with 4% H-DBS solution. Migration distance = 13.5 cm. Eluent: 0.05 *M* HCl + 1 *M* CH_3COOH in 30% methanol (pH = 1.55). S.P. = starting point; S.F. = solvent front.

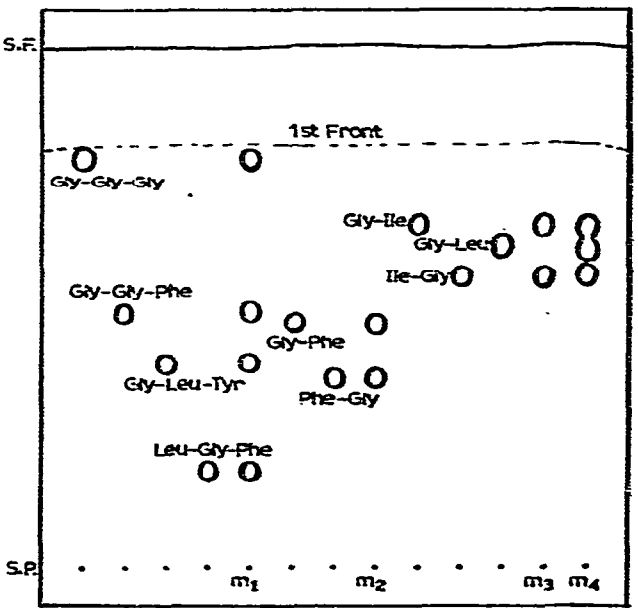

Fig. 3. Thin-layer chromatogram of tripeptides and pairs of isomeric dipeptides on silanized silica gel impregnated with 4% H-DBS solution. Migration distance = 14 cm. Eluent: 1 *M* sodium acetate solution in water–methanol (7:3). m_1 = mixture of tripeptides; m_2 = mixture of Gly–Phe and Phe–Gly; m_3 = mixture of Gly–Ile and Ile–Gly; m_4 = mixture of the three isomeric peptides. S.P. = starting point; S.F. = solvent front.

residue, eluting with 0.05 *M* hydrochloric acid + 1 *M* acetic acid in 30% methanol (apparent pH = 1.55). Such a separation cannot be effected on layers of silanized silica gel alone, which is more suitable for the separation of hydrophobic polypeptides. In this connection it should be noted that the separation of the peptides Gly–Gly–Gly, Gly–Gly–Phe, Gly–Leu–Trp, Leu–Gly–Phe, Leu–Trp–Met–Arg and Leu–Trp–Met–Arg–Phe) can be effected only on silanized silica gel alone, with 0.1 *M* acetate buffer in 30% methanol as eluent.

In Fig. 3 are shown the separations of the four above-mentioned tripeptides and of pairs of isomeric dipeptides on layers impregnated with 4% H-DBS, eluting with 1 *M* sodium acetate in 30% methanol. It should be noted that among the isomeric pairs of dipeptides the only separation not completely achieved is Gly–Ile/Gly–Leu. Similar behaviour is observed on the same layers in the separation of Gly–Ala from Ala–Gly, eluting with 1 *M* acetic acid in 30% methanol or with water–acetic acid (7:3).

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