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REVERSED-PHASE AND SOAP THIN-LAYER CHROMATOGRAPHY OF AMINO ACIDS

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SUMMARY

The chromatographic characteristics of 33 amino acids have been studied using soap thin-layer chromatography (TLC). The influence of the type of detergent, the organic solvent and the acid concentration in the eluent on the chromatographic behaviour of the amino acids was investigated. Many interesting separations that cannot be effected by ion-exchange TLC have been performed.

INTRODUCTION

The use of layers of silanized silica gel impregnated with anionic and cationic detergents has given very interesting thin-layer chromatographic (TLC) results for many classes of organic compounds[1]. We have extended this technique to the behaviour of amino acids. These compounds have already been studied on layers of algin icacid[2], of ion exchangers with cellulose, paraffin and polystyrene matrices[3–5] and, more recently, on columns of hydrophobic supports as the stationary phase and aqueous and aqueous–organic solvents containing "ion-pairing" reagents[6] or anionic detergents in the sodium form[7,8] as eluents.

In this paper we report studies on 33 amino acids using triethanolamine dodecylbenzenesulphonate (which has already be employed in previous work[1]), detergents in the sodium form and dodecylbenzenesulphonic acid in order to show the influence of the detergent form on the retention of the amino acids.

EXPERIMENTAL

The test compounds were dissolved in a 1:1 mixture of methanol and 0.1 *M* hydrochloric acid. The amount of substance deposited on the layer was between 0.5 and 1 μg.

The amino acids were detected by spraying the wet layers with a solution of 1% ninhydrin in a 5:1 mixture of pyridine and glacial acetic acid 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,

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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 the silanized silica gel was suspended. The detergents used were triethanolamine dodecylbenzenesulphonate (DBS), sodium lauryl-etherosulphate (Na-LES), sodium dioctylsulphosuccinate (Na-DSS) (Serva, Heidelberg, G.F.R.) and dodecylbenzenesulphonic acid (H-DBS) (ICN Pharmaceutical, Plainview, NY, U.S.A.). Sodium dodecylbenzenesulphonate could not be used owing to its low solubility in ethanol.

The amino acids used were glycine (Gly), alanine (Ala), β-alanine (β-Ala), serine (Ser), 2-amino-*n*-butyric acid (n-But), threonine (Thr), norvaline (n-Val), valine (Val), norleucine (n-Leu), leucine (Leu), isoleucine (Ile), proline (Pro), methionine (Met), dihydroxyphenylalanine (DOPA), 4-iodophenylalanine (MIP), ethionine (Eth), tryptophan (Trp), phenylalanine (Phe), tyrosine (Tyr), 3,4-3-iodotyrosine (MIT), 3,5-diiodotyrosine (DIT), 3,5-dibromotyrosine (DBrT), 3,5-diiodothyronine (T_2), 3,3',5-triiodothyronine (T_3), 3,3',5,5'-tetraiodothyronine (T_4), aspartic acid (Asp), asparagine (Asp-NH_2), glutamic acid (Glu), taurine (Tau), citrulline (Cit), lysine (Lys), arginine (Arg) and histidine (His).

All measurements were carried out at 25°C. The migration distance was 11 cm unless otherwise stated.

RESULTS AND DISCUSSION

Influence of type and concentration of detergent

In Table I are reported the chromatographic characteristics of the amino acids on layers of silanized silica gel alone and impregnated with 4% Na-LES, Na-DSS and H-DBS solutions, 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 most amino acids run with the solvent front, with the exception (although with high R_F values) of the halogenated derivatives of phenylalanine and tyrosine, the leucine isomers, phenylalanine, ethionine and tryptophan.

Such behaviour is similar to that observed on columns of C_8-bonded silica eluting with a solution at pH 2.50 containing 10% *n*-propanol[7]. The iodo derivatives of thyronine are strongly retained, and can be separated from each other and from all others, which is different to the results on impregnated layers, where such compounds remain at the starting point.

In the presence of Na-LES a general increase in the retention of basic amino acids and those with an aromatic ring in the side-chain is observed. For the other compounds the retention is insufficient for separations to be effected.

On layers impregnated with Na-DSS the amino acids are more strongly retained than with Na-LES. However, acidic amino acids and those with an aliphatic side-chain shorter than C_5 exhibit high R_F values (≥ 0.9) and therefore the layer is not suitable for their separation. Several amino acids give rise to elongated spots on both layers. Also, DBS did not give the results expected on the basis of its previous use with other classes of organic compounds[1]. The retention power of the layer impregnated with this detergent, is in fact, intermediate between that of layers impregnated with Na-LES and Na-DSS.

The selectivity towards amino acids is similar with the three detergents. Further, on layers impregnated with DBS, the detection of the less retained amino acids ($R_F \geq 0.8$) is adversely affected by a violet colour in the vicinity of the solvent

TABLE I

R_F VALUES OF AMINO ACIDS ON LAYERS OF SILANIZED SILICA GEL ALONE (1) AND IMPREGNATED WITH 4% Na-LES (2), 4% Na-DSS (3) and 4% H-DBS (4)

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

Amino acid	*Layer*			
	1	*2*	*3*	*4*
Gly	0.96	0.96	0.94	0.54
Ala	0.96	0.96	0.94	0.52
β-Ala	0.96	0.73	0.47	0.35
Ser	0.96	0.96	0.94	0.64
n-But	0.95	0.94	0.89	0.39
Thr	0.96	0.96	0.94	0.59
n-Val	0.92	0.84	0.65*	0.21
Val	0.93	0.88*	0.71*	0.30
n-Leu	0.85	0.65*	0.35	0.12
Leu	0.87	0.68*	0.41	0.13
Ile	0.91	0.75*	0.51*	0.15
Pro	0.96	0.96	0.92*	0.46
Met	0.95	0.85	0.63	0.21
Eth	0.86	0.67	0.46	0.12
Trp	0.71	0.36	0.15	0.07
Phe	0.79	0.56	0.37	0.12
Tyr	0.89	0.87	0.70	0.27
Dopa	0.92	0.90	0.78*	0.41
MIP	0.53	0.19	0.08	0.03
MIT	0.78	0.48	0.30	0.11
DIT	0.63	0.29	0.13	0.05
DBrT	0.70	0.40	0.24	0.08
T_2	0.23	0.00	0.00	0.00
T_3	0.12	0.00	0.00	0.00
T_4	0.05	0.00	0.00	0.00
Asp-NH_2	0.96	0.95	0.92*	0.59
Asp	0.96	0.96	0.95	0.69
Glu	0.96	0.96	0.95	0.65
Tau	0.96	0.96	0.96	0.96
Cit	0.96	0.95	0.90*	0.49
Lys	0.96	0.56	0.14	0.06
Arg	0.96	0.44	0.08	0.04
His	0.96	0.53	0.13	0.06

* Elongated spot.

front, which can be ascribed to the reaction between ninhydrin and triethylanolamine displaced during the elution process.

In the presence of H-DBS most amino acids are strongly retained, with the exception of taurine, which contains a sulphonic group in the molecule and runs almost with the solvent front, in a similar manner to the results observed on layers of alginic acid[2] and polystyrene-based ion-exchangers[3]. The spots are very compact for all amino acids and therefore the data given here refer to layers impregnated with H-DBS.

The use of lower percentages of detergent does not give any improvement from an analytical standpoint, as a marked decrease in the retention of the amino acids with a decrease in the concentration of detergent on the layer is observed.

Influence of the acidity of the eluent

The chromatographic behaviour of the amino acids on layers impregnated with 4% H-DBS solution is considerably affected both by the pH of the eluent and by its ionic strength. On the basis of the acid–base characteristics of these compounds, with a change in the apparent pH of the eluent the degree of protonation of the amino and of the carboxylic group can be varied. As elongated spots are obtained with alkaline eluents and the amino acids are only slightly retained, the change in the apparent pH of the eluent is interesting from an analytical point of view only in acidic media; the results are reported in Table II.

TABLE II

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

Eluents: (1) 0.5 *M* HCl + 1 *M* CH_3COOH in 30% CH_3OH (pH 0.7); (2) 0.1 *M* HCl + 1 *M* CH_3COOH in 30% CH_3OH (pH 1.25); (3) 0.1 *M* NaCl + 1 *M* CH_3COOH in 30% CH_3OH (pH 2.75); (4) 0.1 *M* CH_3COONa + 1 *M* CH_3COOH in 30% CH_3OH (pH 4.10); (5) 0.1 *M* CH_3COONa + 0.1 *M* CH_3COOH in 30% CH_3OH (pH 5.10); (6) 1 *M* CH_3COONa + 0.1 *M* CH_3COOH in 30% CH_3OH (pH 6.10).

Amino acid	*Eluent*						$pK_{(-COOH)}$[a]
	1	*2*	*3*	*4*	*5*	*6*	
Gly	0.83	0.70	0.72	0.76	0.76	0.83	2.34
Ala	0.74	0.64	0.68	0.74	0.77	0.82	2.34
β-Ala	0.8(	0.64	0.65	0.64	0.67	0.81	3.60
Ser	0.85	0.76	0.76	0.79	0.77	0.86	2.21
n-But	0.65	0.48	0.55	0.67	0.73	0.82	—
Thr	0.83	0.72	0.77	0.77	0.77	0.85	2.15
n-Val	0.45	0.26	0.37	0.52	0.60	0.75	2.36
Val	0.54	0.33	0.44	0.60	0.67	0.74	2.32
n-Leu	0.24	0.15	0.17	0.36	0.40	0.70	2.39
Leu	0.26	0.17	0.19	0.39	0.42	0.65	2.36
Ile	0.31	0.20	0.24	0.45	0.48	0.70	2.26
Pro	0.63	0.48	0.64	0.65	0.66	0.77	1.99
Met	0.42	0.28	0.34	0.50	0.51	0.74	2.28
Eth	0.26	0.16	0.20	0.31	0.36	0.57	—
Trp	0.13	0.08	0.10	0.22	0.22	0.40	2.38
Phe	0.21	0.15	0.17	0.32	0.28	0.49	1.83
Tyr	0.45	0.34	0.44	0.60	0.57	0.76	2.20
Dopa	0.59	0.47	0.57	0.72	0.66	e.s.*	—
MIP	0.05	0.03	0.03	0.07	0.05	0.12	—
MIT	0.20	0.12	0.13	0.31	0.24	0.49	—
DIT	0.08	0.04	0.05	0.11	0.11	0.30	2.12
DBrT	0.15	0.08	0.08	0.24	0.19	0.43	2.17
Asp-NH_2	0.85	0.73	0.73	0.73	0.75	0.82	2.02
Asp	0.86	0.79	0.79	0.80	0.80	0.86	1.88, 3.85
Glu	0.83	0.76	0.76	0.80	0.85	0.90	2.19, 4.25
Tau	0.96	0.96	0.96	0.96	0.96	0.96	1.5
Cit	e.s.	0.60	0.68	0.69	0.71	e.s.	2.43
Lys	0.47	0.14	0.24	0.26	0.27	0.76	2.18
Arg	0.28	0.08	0.13	0.13	0.14	0.62	2.01
His	0.40	0.11	0.22	0.22	0.22	e.s.	1.78

* e.s. = elongated spot.

Table II also gives the pK_a values of the carboxylic groups of amino acids. It can be seen that in the pH range 0.7–1.25 the prevailing species of the amino acids is the cationic form, whereas in the pH range 5.1–6.1 the amino acids are completely in the zwitterionic form.

When the pH is increased, keeping the concentrations of counter ion, glacial acetic acid and methanol constant, a general increase in the R_F values is observed (Table II), and it is therefore evident that the zwitterionic form of the amino acids is less retained than the cationic form. At the lowest pH (0.7), however, an increase in the R_F values is observed compared with those at pH 1.25, as hydrogen ion act as a counter ion.

On plotting the R_M values of the amino acids as a function of the apparent pH of the eluent in the pH range 0.7–1.55 (that is, in the pH range in which the cationic form prevails), straight lines are obtained for most compounds; the slopes are between 0.5 and 0.7 for neutral and acidic amino acids and between 1 and 1.1 for basic amino acids (lysine, arginine and histidine). Although these values are about half the theoretical values[1], the straight lines show that the retention of the amino acids in strongly acidic solutions is greatly affected by an ion-exchange process. Such a process seems to act even in the presence of the zwitterionic form. In fact, the differences between the R_F values in columns 5 and 6 in Table II, obtained with eluents of pH 5.1 and 6.1 and differing only in the counter ion (Na^+) concentration, can be explained only with the presence of an ion-exchange process on the layer.

It should be noted that the influence of the apparent pH of the eluent on the TLC characteristics of the amino acids is similar to that observed in column chromatography in the same pH range even if under different conditions of both the eluent composition and the stationary phase[7].

Influence of the nature and concentration of the organic solvent

The study of the influence of the nature and concentration of the organic solvent on the retention of amino acids was carried out keeping the acetic acid and the sodium chloride concentrations constant at 1 and 0.1 *M*, respectively. The replacement of methanol with ethanol, *n*-propanol and acetonitrile did not give significant differences. An increase in the methanol concentration, in contrast, resulted in an increase in the R_F values towards a constant value, as observed in previous work[1]. The strongest resolving power of the layer was achieved at a low methanol content (20–30%).

Use of water–organic solvent mixtures as eluents

In order to study the behavior of amino acids in the absence of counter ions derived from both salts and strong acids, we used water–methanol, water–acetic acid and water–formic acid mixtures as eluents. Independent of the kind of organic solvent, mixtures with a water–organic solvent ratio of less than 1 cannot be used. In fact, with high percentages of organic solvent (70%), the eluent does not run uniformly and elongated or irregular spots are achieved for those compounds which are less retained. Therefore, the data in Table III refer to eluents with percentages of organic solvent between 10 and 50%.

From the data in Table III it is apparent that, together with the increase in the percentage of organic solvent, a general increase in the R_F values and, for some

TABLE III

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

Amino acid	*Eluent*					
	H_2O–CH_3OH (7:3)	*H_2O–CH_3OH (1:1)*	*H_2O–CH_3COOH (9:1)*	*H_2O–CH_3COOH (7:3)*	*H_2O–CH_3COOH (1:1)*	*H_2O–HCOOH (7:3)*
Gly	0.60	0.61	0.58	0.60	0.70	0.70
Ala	0.56	0.60	0.43	0.49	0.65	0.54
β-Ala	0.31*	0.45	0.32	0.38	0.62	0.59
Ser	0.66	0.65	0.66	0.66	0.69	0.77
n-But	0.50	0.53	0.25	0.31	0.51	0.32
Thr	0.64	0.62	0.56	0.59	0.63	0.63
n-Val	0.35	0.43	0.09	0.14	0.38	0.15
Val	0.44	0.48	0.13	0.20	0.43	0.18
n-Leu	0.20	0.32	0.03	0.07	0.30	0.07
Leu	0.21	0.34	0.03	0.07	0.30	0.07
Ile	0.23	0.37	0.04	0.09	0.33	0.09
Pro	0.55	0.56	0.32	0.37	0.56	0.32
Met	0.29	0.38	0.11	0.20	0.44	0.20
Eth	0.14	0.32	0.04	0.11	0.34	0.11
Trp	0.05	0.24	0.02	0.08	0.29	0.05
Phe	0.12	0.28	0.05	0.07	0.32	0.07
Tyr	0.37	0.44	0.13	0.25	0.61	0.25
Dopa	0.46	0.54	0.24	0.42	0.68	0.38
MIP	0.03	0.13	0.02	0.02	0.14	0.02
MIT	0.08	0.29	0.03	0.10	0.41	0.07
DIT	0.04	0.21	0.02	0.03	0.26	0.03
DBrT	0.07	0.27	0.03	0.08	0.35	0.06
T_2	0.00	0.00	0.00	0.00	0.00	0.00
T_3	0.00	0.00	0.00	0.00	0.00	0.00
T_4	0.00	0.00	0.00	0.00	0.00	0.00
Asp-NH_2	0.62	0.55	0.59	0.59	0.65	0.70
Asp	0.71	0.63	0.73	0.73	0.76	n.d.**
Glu	0.69	0.61	0.63	0.68	0.74	0.75
Tau	0.94	0.91	0.96	0.96	0.91	0.96
Cit	0.56	0.55	0.39	0.46	0.63	0.40
Lys	0.07	0.18	0.02	0.09	0.16	0.07
Arg	0.03	0.12	0.02	0.07	0.12	0.05
His	0.06	0.15	0.02	0.08	0.16	0.07

* Slightly elongated spot.
** n.d. = not determined.

amino acids, even a levelling of such values are observed. With a 7:3 water–methanol the trend of the R_F values is similar to that obtained eluting with 1 *M* acetic acid in 30% methanol (see Table I), apart from a general slight increase in the R_F values. On replacing methanol with the same percentage of acetic acid, the layer exhibits a higher selectivity towards the amino acids, especially towards the aliphatic acids. Further, more compact spots are obtained and therefore water–acetic acid mixtures are suitable for analytical purposes.

On changing from acetic to formic acid, the greatest differences occur with

the lower aliphatic amino acids, which exhibit higher R_F values. The use of formic acid, therefore, does not give any particular advantage over acetic acid.

Analytical applications

The sequence of R_F values for amino acids using soap TLC is different from that observed on thin layers of ion exchangers[2–4].

Separations of amino acids such as Phe–Tyr–Dopa, Gly–Ala, Leu–Ile and Ser–Thr, which can be carried out only with difficulty on ion exchangers, can be easily achieved on layers impregnated with H-DBS.

Among the numerous separations that may be effected on the basis of the R_F values reported in Tables II and III, we carried out those more interesting from an analytical standpoint that cannot be effected on layers of ion exchangers with cellulose[2] or polystyrene[5] matrices. Fig. 1 shows the separation of C_2–C_6 *n*-alkyl-α-amino acids with water–acetic acid (9:1) as eluent. Under the same experimental conditions, the pairs aspartic acid–glutamic acid and serine–threonine have also been separated. The last pair was also separated on the same layers with water–formic acid (7:3) as eluent. The separation of the two acidic amino acids, however, is difficult with soap TLC.

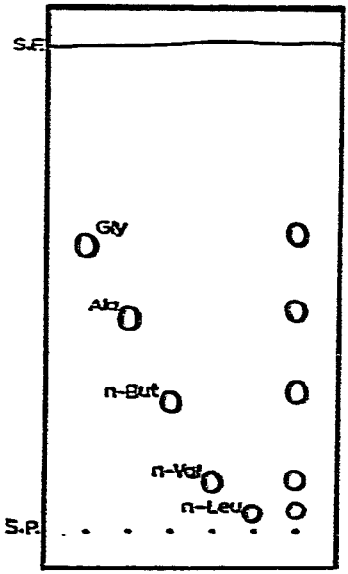

Fig. 1. Thin-layer chromatogram of *n*-alkyl-α-amino acids on silanized silica gel impregnated with 4% H-DBS solution. Migration distance: 13 cm. Eluent: water–acetic acid (9:1). S.P. = start ponit; S.F. = solvent front.

Fig. 2 shows the two separations of different amino acids (the more interesting of which concerns the aromatic amino acids and their iodo derivatives) obtained by eluting with water–acetic acid (1:1).

Fig. 3 shows the separation of some pairs of isomeric amino acids with 0.1 *M* sodium acetate + 1 *M* acetic acid in 30% methanol as eluent.

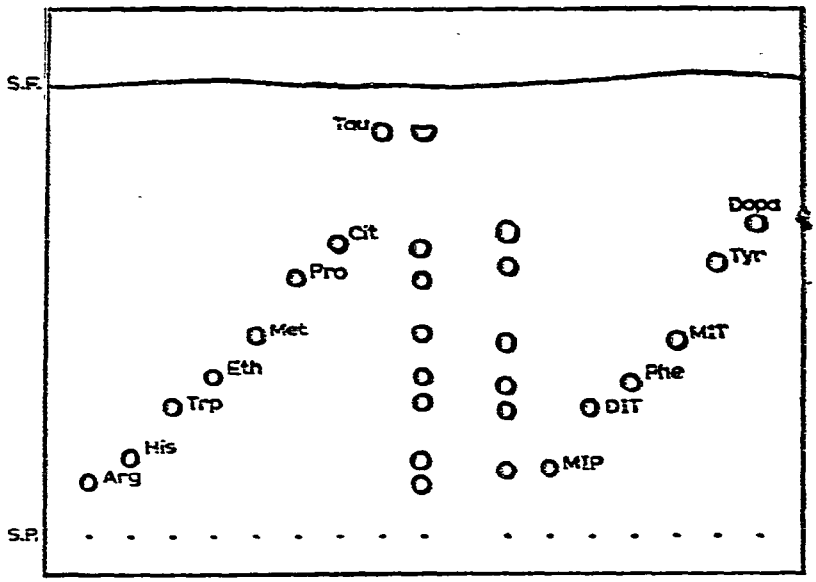

Fig. 2. Thin-layer chromatogram of α-amino acids on silanized silica gel impregnated with 4% H-DBS solution. Migration distance: 12 cm. Eluent: water–acetic acid (1:1). S.P. = start point; S.F. = solvent front.

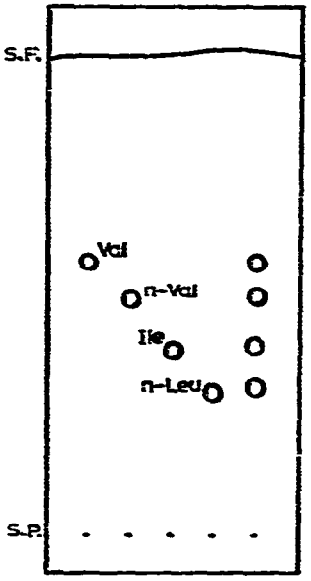

Fig. 3. Thin-layer chromatogram of pairs of isomeric α-amino acids on silanized silica gel impregnated with 4% H-DBS solution. Migration distance: 12.5 cm. Eluent: 0.1 *M* sodium acetate solution in water–methanol–acetic acid (64.3:30:5.7). S.P. = start point; S.F. = solvent front.

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