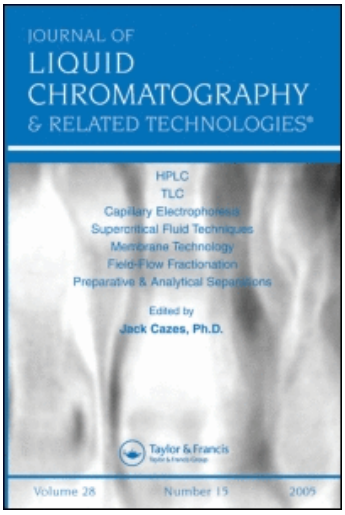

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Joseph Sherma[a]; Barry P. Sleckman[a]; Daniel W. Armstrong[b]
[a] Department of Chemistry, Lafayette College, Easton, PA [b] Department of Chemistry, Georgetown University, Washington, DC

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CHROMATOGRAPHY OF AMINO ACIDS ON REVERSED PHASE THIN LAYER PLATES

Joseph Sherma and Barry P. Sleckman
Department of Chemistry
Lafayette College
Easton, PA 18042
and
Daniel W. Armstrong
Department of Chemistry
Georgetown University
Washington, DC 20057

ABSTRACT

The separation of 19 amino acids was studied on reversed phase thin layers, including C_{18} chemically bonded silica gel, impregnated silica gel, and acetylated cellulose. Normal aqueous-organic solvents, aqueous micellar solutions, and reversed micellar solutions were tested as mobile phases. The only practical system that provided a reversal in migration sequence compared to silica gel and cellulose included a C_{18} layer impregnated with HDBS, and this reversal was apparently due to an ion exchange mechanism.

INTRODUCTION

In an earlier paper (1), separations of 18 amino acids were compared on silica gel, cellulose, and ion exchange thin layers. This paper extends the study of amino acid separations to reversed phase (RP) TLC, including chemically bonded C_{18} silica gel, impregnated silica gel, and acetyl cellulose layers, and conventional aqueous-organic and micellar mobile phases. Comparisons among these systems and with the adsorption, normal-phase partition, and ion exchange systems studied earlier (1) are reported.

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EXPERIMENTAL

Standard solutions of individual amino acids and mixtures were prepared at a concentration of 500 ng/µl of each compound in water. Initial zones were applied to the precoated thin layer plates (20 x 20 cm) specified below using 1 µl Drummond Microcap micropipets.

Plates were developed in standard, rectangular glass chambers that were lined with paper and pre-equilibrated with mobile phase for at least 10 minutes before inserting the spotted layer. Plates were used as received from the manufacturer, with no pretreatment. In general, development was carried out to a point 15 cm above the origin line. The chromatograms were oven dried at 100°C, sprayed with 0.1% ninhydrin in acetone, and heated again for 5 minutes or longer to detect the amino acids as colored spots on a white background.

Layers were impregnated with the surfactants HDBS and CTAB by attaching a clip to the top of the plate and dipping into ethanolic solutions contained in a metal Thomas-Mitchell dip tank (Arthur H. Thomas Co.). After 5 minutes of soaking, the plate was removed and placed in a hood until dry.

RESULTS AND DISCUSSION

RP Systems with Conventional Mobile Phases

Table 1 lists amino acids studied and their R_F values in 12 different chromatographic systems. System A consisted of a Whatman KC_{18} chemically bonded reversed phase plate developed with n-propanol-water (7:3 v/v). This mobile phase was chosen as optimum for amino acid TLC on KC_{18} plates after evaluation of 45 different 2-, 3-, and 4-component solvent mixtures containing water, acetonitrile, methanol, ethanol, n-propanol, formamide, THF, DMSO, pyridine, methyl cellosolve, acetic acid, hydrochloric acid, heptane, hexane, isopropanol, t-butanol, acetone, or methylene chloride. Solvent proportions were chosen to provide

TABLE 1

R_F x 100 Data in RP Systems

	Systems											
	A	B	C	D	E	F	G	H	I	J	K	L
Cystine	NV	14	NV	NV	NV	NV	NV	NV	NV	NV	ND	10
Cysteine	NV	ND	18	5.5	12	13	NV	78	NV	62	ND	ND
Arginine	0.60	13	3.6	17	19	16	67	54	79	21	ND	17
Histidine	6.3	12	21	5.5	15	11	43	33	36	32	25	11
Serine	30	26	37	52	35	36	83	78	71	80	59	44
Asparagine	31	ND	31	47	24	21	NV	78	NV	80	ND	ND
Glutamine	44	ND	33	50	33	35	83	78	71	80	59	ND
Threonine	47	30	37	49	47	45	85	78	64	80	56	47
Alanine	50	32	36	51	42	41	85	78	64	80	48	47
Aspartic acid	53	25	41	44	29	29	85	78	79	80	ND	23
Proline	59	24	27	42	55	51	83	78	NV	66	NV	ND
Glutamic acid	61	30	41	50	42	40	83	78	79	80	ND	44
Valine	66	44	42	51	75	71	79	78	50	62	44	54
Methionine	69	47	46	50	75	71	79	78	64	52	44	58
Isoleucine	71	49	47	50	63	60	79	77	50	45	40	54
Tyrosine	72	49	50	59	71	66	79	78	64	61	50	58
Leucine	72	52	49	52	77	72	79	71	64	40	40	58
Phenylalanine	72	52	50	49	77	71	62	60	71	35	40	54
Tryptophan	74	54	54	51	69	70	47	46	71	29	40	50

ND = No data
NV = Not visualized

The systems are described in the text.

similar mobile phase strengths (p') as defined by Snyder (2). Both nonaqueous and aqueous mixtures were tested, but water was found to be necessary to move many of the compounds from the origin and to prevent streaking. Other mobile phases that provided good resolution with compact spots included pyridine-water (8:2 v/v), pyridine-H_2O-THF or DMSO (16:4:1 v/v), pyridine-water-acetic acid (16:4:1 v/v), n-propanol-water-THF (16:4:1 v/v), t-butanol-H_2O (7:3 v/v), isopropanol-water (7:1 v/v), and methylene chloride-isopropanol-water (3:6:1 and 4:5:1 v/v). All of these mobile phases and almost every other one containing water gave the same sequence of migration as shown for System A in Table 1, although individual R_F values differed somewhat. Figure 1 illustrates a typical separation carried out on a KC_{18} layer.

The sequence of R_F values on KC_{18} silica gel (System A) was identical to that found when Whatman K6 silica gel was developed with the same solvent, n-propanol-water (7:3 v/v) (System B, Table 1). The absence of order reversal on the reversed phase layer indicated that perhaps the same separation mechanism was operating both on conventional silica gel and on chemically bonded silica gel [and, apparently, on fibrous and micro-crystalline cellulose, which also gave the same migration sequence (1)].

To determine if the chemically bonded RP layer was unique, the amino acids were developed with n-propanol-water (7:3 v/v) on an Analtech reversed phase plate containing a long-chain hydrocarbon impregnated support layer. As seen in Table 1 (System C), the migration sequence was again generally unchanged, indicating a similar separation mechanism. Cystine and cysteine were difficult to detect at the 500 ng level on both reversed phase layers.

An RPTLC plate designated OPTI-UP C12 (3), containing a layer of silica gel chemically bonded with C_{12} rather than C_{18} groups, was developed with n-propanol-water (7:3 v/v), and the same general migration sequence was obtained (System D, Table 1).

Two bonded reversed phase acetyl cellulose layers (4) were also developed with the same solvent. These were Baker Flex 10%

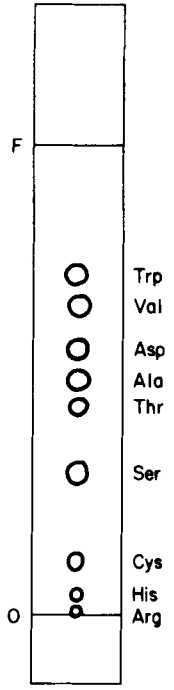

Figure 1. Separation of a 9-component amino acid mixture (500 ng each) on a Whatman KC_{18} thin layer developed with n-propanol-water (7:3 v/v) in a lined, pre-equilibrated chamber. F = solvent front, O = origin.

acetylated cellulose (plastic-backed) (System E) and Analtech 20% acetylated cellulose (glass-backed) (System F). Again, the same general sequence of migration was observed for these two systems. Spotting of samples was difficult on the acetylated cellulose layers. On the 10% acetylated layer, water solutions remained beaded on the surface of the origin for some time before finally being absorbed into the layer. Water solutions could not be spotted on the 20% acetylated layer, so standards were prepared in 96% ethanol containing 0.1 N HCl.

Development times for a 15 cm run with n-propanol-water (7:3 v/v) in Systems A-F were as follows:

(A)	KC_{18}	3.5 hours
(B)	Silica gel	3.5 hours
(C)	Hydrocarbon impregnated silica gel	5.0 hours
(D)	C_{12}	7.5 hours
(E)	10% Acetylated cellulose	3.5 hours
(F)	20% Acetylated cellulose	4.0 hours

The ninhydrin reagent produced purple, red, and tan spots on silica gel and KC_{18} chemically bonded silica gel plates, while all spots appeared some shade of purple on cellulose (1). The intensity of colors produced by the ninhydrin reagent for 500 ng of the amino acids varied in the order, silica gel > KC_{18} > cellulose. The general diffuseness of developed zones varied in the opposite order, cellulose > KC_{18} > silica gel. On the 10% acetylated cellulose layer, all compounds that were detected appeared as faint tan zones. For detection on 20% acetylated cellulose, ninhydrin was dissolved in ethanol rather than in acetone because spraying with the latter solvent caused the layer to peel. Again, all detected spots appeared as a faint tan color.

Mechanism of Separation of RP Layers

The similar order of migration of amino acids on silica gel, cellulose, 10% and 20% acetylated cellulose, C_{18} and C_{12} chemically bonded silica gel, and hydrocarbon-impregnated silica gel indicated that a similar mechanism might be operative in all of these systems. The possibility of a mechanism based on solubility in the alcohol-water mobile phase was considered, but attempts to correlate R_F values with amino acid solubility in alcohol-water mixtures were not successful. For example, Nozaki and Tanford (5) reported that solubilities of tyrosine, leucine, phenylalanine, and tryptophan in 60% ethanol-water were 0.02, 0.62, 1.23, and 1.40 g/100 g, respectively, but R_F values for all of these compounds on the KC_{18} reversed phase layer were 0.72–0.74. In addition, histidine has a higher solubility than asparagine

(0.5 compared to 0.2 g/100 g), but the R_F value of histidine was 0.063 and of asparagine 0.31.

Further studies were done to check the possibility of separations due to a precipitation mechanism, which sometimes occurs when binary or higher order mobile phases are used and concurrently fractionated during development. Single amino acids were spotted across different thin layer plates at an angle to the bottom edge. If the distance of migration of the amino acids was totally dependent on precipitation from the mobile phase, the final position of all spots, regardless of the location of spotting, would be a horizontal line parallel to the solvent front (0° angle) (6, 7). If no fractionation of the mobile phase occurs and a classic adsorption or partition mechanism was operative then all spots would move an exact distance dictated by their R_F. Hence, one would expect the developed spots to lie along a slanted line with a theoretical angle that could be predicted from the angle of the origin line and the R_F value of the spotted compound. The results of these experiments are shown in Table 2.

The developed spots did not lie on a line parallel to the solvent front (0°), but were usually somewhere between this and the theoretical line. The results on silica gel were similar to those on the reversed phase media, indicating a similar mechanism that was possibly some combination of adsorption, partition, and/or solubility. However, an adsorption mechanism is unexpected on the chemically bonded C_{18} layers because of the 10%-12% carbon loading and the fully covered (capped) silanized silica gel particles (8). The migration sequences in Table 1 and the results in Table 2 indicate that the mechanism, whatever it is, is similar on silica gel and chemically bonded and impregnated RP layers, and that it is not conventional reversed phase partition and solubility-based.

Indeed, one should realize that the definitions of "reversed" phase" and "normal phase" are based on ideal or nearly ideal systems. For example, reversal is easily obtained when separating a nonpolar, hydrophobic substance such as anthracene from a relatively

TABLE 2

Results of Study of TLC Mechanism on Silica Gel and RP Layers

Layer*	Amino Acid (R_F)	Angle of Spotting (°)	Predicted Angle (°)	Experimental Angle (°)
Silica gel	Serine (0.26)	20	17	13
	Alanine (0.32)	14	11	9
	Tryptophan (0.54)	14	8	5
C_{18}	Serine (0.30)	20	17	12
	Alanine (0.50)	15	10	8
	Leucine (0.72)	21	9	3
	Tryptophan (0.74)	15	6	4
C_{12}	Serine (0.52)	21	8	8
	Leucine (0.52)	21	8	8
Analtech RP	Serine (0.37)	19	14	10
	Leucine (0.49)	19	10	9.5

* All developments with n-propanol-water (7:3 v/v)

polar, hydrophilic substance such as sodium picrate (9). Unfortunately, in real life many separations cannot be easily predicted solely on the basis of polarity. The amino acids are classic examples of amphiphilic compounds having hydrophobic and hydrophilic parts, the ability to hydrogen-bond, to act as acids or bases, etc. Further complicating the picture is the fact that the structure or nature of the stationary phase (particularly the reversed phase) is not fully understood. Thus, a reversed stationary phase may appear hydrophobic and essentially deactivated to a nonpolar solute such as anthracene, but may not to an amino acid. The results of this work indicate that the reversed phase separation of amino acids (and probably a host of other compounds) is a complicated process that can not be explained by traditional idealized notions of "reversed" or "normal" phase TLC.

RP Systems with Micellar Mobile Phases

Pseudophase chromatography, in which micellar or cyclodextrin solutions are used as mobile phases in TLC or HPLC, has been described by Armstrong and coworkers (9-15). Amino acids were studied on polyamide thin layers using a reversed micellar mobile phase containing sodium dioctylsulfosuccinate, and R_F values for 20 compounds were reported (11). In general, the more polar amino acids had the highest R_F values, while the less polar ones had lower R_F values. Since this behavior is characteristic of reversed phase TLC, it was decided to evaluate pseudophase TLC on C_{18} layers (15) for amino acid separations.

Development on a KC_{18} plate with a mobile phase containing 0.015 M SDS (sodium dodecylsulfate) micelle-forming surfactant produced the R_F values shown in Table I (System G). Sodium chloride (0.5 M) was also included in the mobile phase to retain the binding of the layer in the totally aqueous solution. Little resolution of the compounds was obtained, and severe streaking occurred for the zones with R_F values greater than

0.78, which accounted for the majority of compounds. Many compounds were not detected below a level of 2 μg, which was the amount of each acid spotted to obtain the data in Table 1. The plate required 20 minutes of heating rather than 5 minutes to produce spots of reasonable intensity with ninhydrin, and the spot colors were orange and green in some cases in addition to the usual tan and purple produced with conventional mobile phases on the C_{18} layer. Apparent sequence reversals were noted for some compounds compared to non-micelle developments on KC_{18} layers, but results were difficult to assess because of the trailing mentioned above. For example, alanine had a lower R_F than phenylalanine when developed with propanol-water, but a relatively higher R_F with the SDS mobile phase. A 12 cm development with SDS required 2 hours. Virtually identical results as those just described for SDS were obtained when development was carried out with a mobile phase consisting of saturated aqueous CTAB-H_2O (1:19 v/v) also containing 0.5 M NaCl (System H, Table 1). CTAB (cetyltrimethylammonium bromide) is also a commonly used micelle-forming surfactant.

The reversed micellar surfactant sodium dioctylsulfosuccinate, which was used by Armstrong to develop amino acids on polyamide (11), was tested as a mobile phase [1.3 M DOSS in cyclohexane-water (50:4 v/v)] on C_{18} layers. The data in Table 1 (System I) show that, with a few exceptions, R_F values were all in the range between 0.64 and 0.71. The mobile phase was very viscous, and a 7 cm development required 26 hours. Attempts to lower the viscosity of the mobile phase by dilution resulted in streaked zones. The plate required 20 minutes of heating before 500 ng zones were reasonably intense; a standard array of purple and tan spots was produced, except for a few compounds that were orange. Zones were generally round and compact rather than streaked as with the micelle mobile phases. The resolution obtained was not as good as that reported (11) using this mobile phase with a polyamide layer.

RP Systems with Micellar Impregnation

Lepri et al. (16) have reported the TLC of amino acids on Merck C_2, C_8, and C_{18} plates impregnated with dodecylbenzenesulfonic acid (HDBS) and developed with 1 M acetic acid + 0.2 M HCl in methanol-water (1:1 v/v). Our results with this system using Whatman C_{18} layers are shown in Table 1 (System J). Plates were impregnated by dipping into a 4% solution of HDBS in 96% ethanol; development for 15 cm required 2.5 hours. Again, many compounds were not detected at levels below 2 μg, and plates required 20 minutes of heating to produce reasonably intense spots even for this amount. Although the acids with higher R_F values were badly streaked, this system gave the most evidence of a reversal of R_F values compared to those on C_{18} silica gel, cellulose, and silica gel with conventional aqueous-organic solvents. Although R_F values were different than those reported by Lepri et al. (16), the general sequence of migration for the common acids studied was similar. However, spots were not nearly as tight as illustrated in the figures of Lepri et al. (16). Development was also carried out with HDBS incorporated into the mobile phase instead of being impregnated into the plate. In this case, the mobile phase appeared to "demix", and all of the spots appeared in a narrow band at or very near the solvent front with virtually no resolution.

When the mobile phase was changed to contain 1 M acetic acid + 0.2 M HCl in methanol-water (7:3 v/v) (Table 1, System K), R_F values on the HDBS impregnated layer were generally lower, and streaking was much less of a problem. A development of 7 cm required 55 minutes. R_F values were in the same order as reported by Lepri et al. (16), but the spots were still not as compact as those pictured by these workers. The migration sequence was again reversed compared to C_{18} silica gel, cellulose, and silica gel.

The migration sequence on the HDBS-impregnated layer closely paralleled that which was found earlier (1) for Fixion

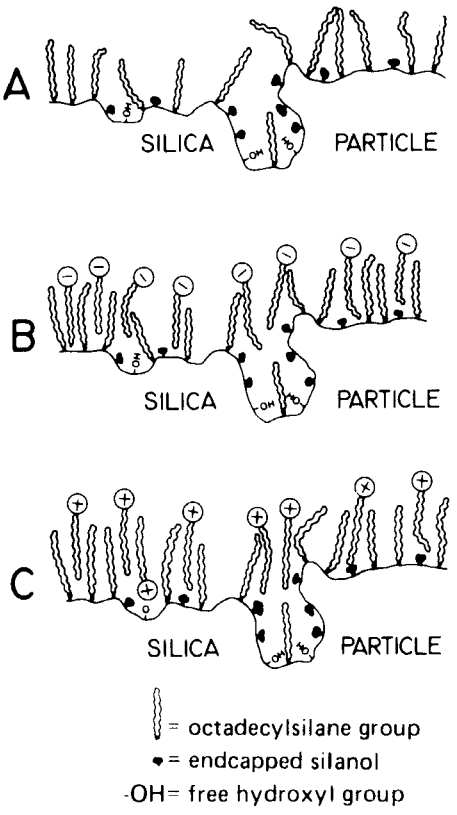

Figure 2. Schematic diagrams of (A) the surface of a C_{18} reversed phase particle, (B) a reversed phase particle impregnated with an anionic surfactant, and (C) a reversed phase particle impregnated with a cationic surfactant. Stationary phase B can behave as a cation exchanger and phase C as an anion exchanger. Surfactant counterions and solvent molecules residing in the bonded layers are not shown.

strong acid cation exchange layers. This suggests that the mechanism involved on these layers may be cation exchange with the impregnated sulfonic acid. This was confirmed by chromatographing the amino acids on a KC_{18} layer impregnated with CTAB from a 10% solution of this compound in 96% ethanol. Development was with methanol-water (9:1 v/v) + 1 M acetic acid + 0.2 M HCl (System L, Table 1). The results with CTAB were very different from those with HDBS, with the migration order for the amino acids being generally, but not uniformly, reversed. The impregnated surfactant probably caused an anion exchange mechanism on the CTAB-impregnated layer. Figure 2 illustrates the formation of cation-exchange and anion-exchange layers by impregnation of KC_{18} with HDBS and CTAB, respectively.

Conclusions

The only reversed phase system that was found to provide a reversal of migration sequence compared to silica gel, cellulose, and C_{18} silica gel as well as relatively compact spots and reasonable development time included a C_{18} layer impregnated with dodecylbenzenesulfonic acid. The charge of the impregnated surfactant head groups undoubtedly plays an important role in this system, and the reversal of migration is very likely due to an ion exchange mechanism. This system would, therefore, be useful in helping confirm the presence of an unknown amino acid in a sample. However, TLC on reversed phase layers has no advantages compared to adsorption, normal phase partition, or Fixion ion-exchange TLC (1) for analyses of mixtures of amino acids. The migration sequences on reversed phase layers are generally the same as on cellulose and silica gel, and an adsorption and/or partition mechanism may be operative on these RP layers. These results indicate that it is sometimes impossible to predict the relative separation behavior of many compounds (e.g., amino acids on the basis of chemical notions of "normal" or "reversed" phase chromatoqarphy.

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