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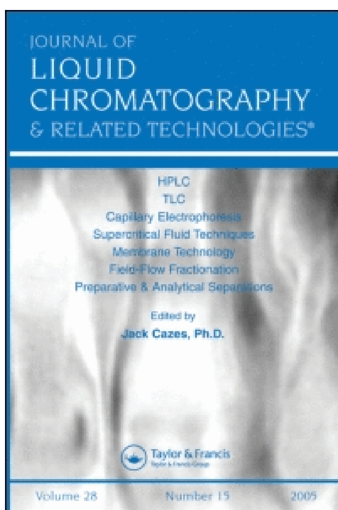

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A Comparison of Amino Acid Separations on Silica Gel, Cellulose, and Ion Exchange Thin Layers

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A COMPARISON OF AMINO ACID SEPARATIONS ON SILICA GEL, CELLULOSE, AND ION EXCHANGE THIN LAYERS

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ABSTRACT

Separations of amino acids on silica gel, high performance silica gel, preadsorbent silica gel, microcrystalline and fibrous cellulose, and Fixion strong acid cation exchange layers were compared under standardized conditions using a mixture of the nine essential acids and a more complex 18-component mixture. Fixion layers were evaluated using three different development conditions, and loading studies of raw urine were carried out on all of the layers. Tables and figures are presented illustrating which amino acids can be resolved in each of the chromatographic systems.

INTRODUCTION

The analysis of biochemical and biomedical samples for their amino acid content inevitably depends upon use of some form of chromatography. Thin layer chromatography (TLC) has become a widely accepted method for this purpose, and the literature contains many TLC systems for the separation and detection of amino acids (1). This paper describes results of the first direct, comparative study, carried out under standard conditions, of the TLC systems from the literature (1-4) that were experimentally determined to give the best results for

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the amino acid standards being used. Adsorption TLC on silica gel, normal-phase partition TLC on cellulose, and ion exchange TLC on Fixion strong acid cation exchange layers were used to separate two model standard mixtures, one containing the nine "essential" amino acids needed in the diet of most animals, including man (5), and the other containing 18 amino acids. The advantages and disadvantages of each system are reported below, along with R_F and resolution data. A comparison of the ability of the systems to separate amino acids in raw urine samples is also reported.

A recent paper (6) has been published that reports the TLC of amino acids on chemically bonded reversed phase plates impregnated with dodecylbenzenesulfonic acid. The reversed phase TLC of amino acids was not included in our present comparative study but will be the subject of a separate paper to be published in the near future.

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EXPERIMENTAL

The following amino acid standards were purchased from Analabs:

Essential amino acids

d,l-leucine (Leu)
d,l-isoleucine (Ile)
l-triptophane (Trp)
d,l-methionine (Met)
d,l-valine (Val)
d,l-lysine·HCl (Lys)
l-histidine (free base) (His)
d,l-phenylalanine (Phe)
d,l-threonine (Thr)

Nonessential amino acids

d,l-alanine (Ala)
d,l-serine (Ser)
l-tyrosine (Tyr)
d,l-glutamic acid monohydrate (Glu)
d,l-aspartic acid (Asp)
l-arginine (Arg)
glycine anhydride (Gly)
l-proline (Pro)
l-cystine (Cys)

Individual standard solutions were prepared by weighing and quantitative dilution at concentrations of 500 ng of amino acid/1.0 μl of water or 0.1 N HCl in 96% ethanol. Gly was insoluble in the acidic ethanol and was prepared only in water. Mixture solutions were prepared at the same concentration level by appropriate dilution of individual concentrated stock standard solutions.

The following layers and mobile phases were studied:

(A) Baker-Flex 20 x 20 cm cellulose sheets; 2-butanol-acetic acid-water (3:1:1)
(B) Baker-Flex 20 x 20 cm microcrystalline cellulose sheets; 2-butanol-acetic acid-water (3:1:1)
(C) Whatman 20 x 20 cm K6 silica gel plates; n-butanol-acetic acid-water (3:1:1)
(D) Whatman 10 x 10 cm high performance silica gel plates; n-butanol-acetic acid-water (3:1:1)
(E) Fixion 20 x 20 cm strong acid ion exchange sheets (Na^+ form); run buffer: 84 g citric acid monohydrate + 16.0 g NaOH + 5.8 g NaCl + 54.0 g ethylene glycol + ca. 4 ml of conc. HCl (added dropwise to give pH 3.3); equilibration buffer: run buffer diluted 30 times (pH 3.8)

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Some experiments were also performed using Whatman K5DF and LK5DF (preadsorbent) silica gel plates.

Samples of individual solutes and mixtures were applied at 500 ng levels (1 µl) using a 10 µl Drummond Dialamatic micro-dispenser calibrated from 1-10 µl. Urine was applied in quantities of 1-20 µl. Initial zones were applied as tight spots one cm from the bottom of each layer, except for the preadsorbent plate to which samples were applied by streaking across the inert spotting zone. Sample solutions prepared in water were used for all layers except Fixion, for which acidic ethanol solutions were applied according to the manufacturer's instructions (7).

Silica gel and cellulose layers were developed in paper-lined rectangular glass N-tanks that had been pre-equilibrated with solvent for 20 minutes prior to inserting the layer. The Fixion layers were developed by three different procedures. In each case, 1 cm wide lanes on each side of the sheet were scraped free of resin, and the layers were developed in unlined, unequilibrated tanks. In the system designated FxA, the sheet was developed without prior treatment. In system FxB, the layer was pre-equilibrated for 16 hours with the equilibration buffer by attaching filter paper to the top of the layer to allow a continuous flow of the solution. After this period, a 1 cm strip was cut from the bottom edge of the layer in direct contact with the equilibration buffer, the layer was air dried, spotted, and developed in the run buffer. In system FxC, the plate was pre-equilibrated as above, dried, spotted, and developed in the run buffer at 45°C rather than at ambient temperature. A Fotodyne Repro-Tank with preconditioning lid was used for development at the elevated temperature. The sheet was supported with a clamp above the mobile phase, the tank was placed inside an oven at 45°C, and after 15 minutes the layer was lowered into the mobile phase for development. All layers were developed for a distance of 17 cm beyond the origin.

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Chromatograms were completely dried in a chromatography oven at 100°C, and the cooled plates were sprayed with Whatman ninhydrin reagent supplied in an aerosol bottle. Heating at 90-100°C for 5 minutes produced blue to purple zones of amino acids. Although 500 ng of the amino acids were routinely spotted, the detection limits with ninhydrin reagent generally ranged from 50-100 ng.

Results and Discussion

Table 1 gives the R_F values of the amino acids in the seven TLC systems studied, and Tables 2-8 give resolution possibilities of each pair of acids in these systems. The resolution (R) values were calculated in the usual manner, by dividing the distance between spot centers by one-half of the sum of the diameters of the two spots. The higher the R value, the greater was the separation obtained between the two compounds. When this calculated value was greater than 1.50, the compounds were considered to be completely resolved and a "c" was placed in the table. Numerical values below 1.5 are specified in the table; compound pairs with an R value >1.0 were almost completely resolved, while R values below 1.0 indicate incomplete resolution. Values are missing for proline in Tables 6-8 because this compound is not detected on Fixion layers with ninhydrin reagent.

The listings at the bottom of each of the Tables 2-8 are separations of the 18-component amino acid mixture (Separation A) and the essential amino acid mixture (Separation B) carried out in each system. In each case, the specific acids selected for separation could have been different, using the R_F and resolution data as a guide. However, the number of amino acids in the respective mixtures represents the maximum that each system can separate. Figure 1 shows the actual separations of the essential amino acids in six of the systems.

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TABLE 1

hR_F (R_F x 100) Values for Amino Acids

	TLC System						
	A	B	C	D	FxA	FxB	FxC
Ala	41.9	29.0	32.4	28.8	50.9	51.2	53.6
Ser	26.9	16.1	26.4	24.1	67.1	64.7	67.1
Tyr	50.0	36.1	49.4	45.9	11.9	13.9	15.5
Glu	34.4	22.6	30.0	28.2	34.5	29.4	30.6
Asp	26.3	14.8	25.3	21.8	71.5	68.2	68.6
Arg	25.6	11.0	12.9	10.0	1.8	2.2	2.2
Gly	29.4	14.8	25.9	23.5	55.6	52.4	53.6
Leu	75.0	63.9	51.8	48.8	21.8	17.8	19.4
Ile	73.1	60.0	49.4	47.1	27.8	22.2	23.3
Trp	55.6	36.1	54.1	51.8	1.8	2.2	2.2
Met	41.0	22.5	47.3	43.5	28.0	27.2	25.0
Val	63.1	48.4	43.5	41.2	42.5	35.0	34.4
Lys	18.1	7.1	10.0	7.1	7.5	5.0	5.6
His	20.0	7.1	11.7	7.1	10.6	8.9	10.0
Phe	67.5	54.8	52.4	50.0	14.4	11.1	11.7
Thr	32.5	21.3	30.0	27.6	67.1	60.6	57.2
Cys	6.9	3.2	14.1	7.1	55.9	50.0	57.9
Pro	43.8	33.5	24.1	21.2	-	-	-

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The mobile phases used were selected after an exhaustive literature search (1-4, 7, 8) and subsequent experimental evaluation of the most promising solvents reported. Because of their aqueous nature, development times tended to be relatively long in all cases. The following times were required for 17 cm developments: System A - 7 hours, B - 11 hours, C - 4.5 hours, D - 7.5 hours, FxA - 6.5 hours, FxB - 6.0 hours, FxC - 2 hours. Whatman K5DF and LK5DF silica gel required 6.5 hours for a 17 cm development.

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TABLE 2

Resolution Data for System A

	Pro	Cys	Thr	Phe	His	Lys	Val	Met	Trp	Ile	Leu	Gly	Arg	Asp	Glu	Tyr	Ser
Ala	0.43	c	c	c	c	c	c	0.0	c	c	c	c	c	c	1.47	c	c
Ser	c	c	1.43	c	1.11	1.44	c	1.26	c	c	c	0.77	0.0	0.0	c	c	
Tyr	1.00	c	c	c	c	c	c	0.73	1.05	c	c	c	c	c	c		
Glu	1.88	c	0.38	c	c	c	c	1.00	c	c	c	1.07	1.18	c			
Asp	c	c	1.42	c	1.11	1.33	c	1.30	c	c	c	0.76	0.0				
Arg	c	c	0.91	c	0.69	1.00	c	1.22	c	c	c	0.47					
Gly	c	c	0.67	c	c	c	c	1.15	c	c	c						
Leu	c	c	c	c	c	c	c	c	c	1.35							
Ile	c	c	c	1.11	c	c	c	c	c								
Trp	c	c	c	c	c	c	1.16	0.85									
Met	0.0	c	0.90	c	1.30	c	1.42										
Val	c	c	c	0.67	c	c											
Lys	c	c	c	c	0.25												
His	c	c	c	c													
Phe	c	c	c														
Thr	c	c															
Cys	c																

Separation A: Leu - Phe - Trp - Ala - Glu - Ser - Lys - Cys -Tyr

Separation B: Leu - Phe - Trp - Thr - Lys

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TABLE 3

Resolution Data for System B

	Pro	Cys	Thr	Phe	His	Lys	Val	Met	Trp	Ile	Leu	Gly	Arg	Asp	Glu	Tyr	Ser
Ala	0.88	c	1.38	c	c	c	c	0.74	0.96	c	c	c	c	c	1.33	1.05	c
Ser	c	c	0.95	c	1.5	1.5	c	0.76	c	c	c	0.0	0.69	0.0	1.29	c	
Tyr	0.16	c	c	c	c	c	c	0.63	0.15	c	c	c	c	c	c		
Glu	c	c	0.0	c	c	c	c	0.0	c	c	c	1.33	c	1.33			
Asp	c	c	1.33	c	c	c	c	0.85	c	c	c	c	0.70				
Arg	c	1.00	c	c	0.43	0.43	c	1.25	c	c	c	0.70					
Gly	c	c	c	c	c	c	c	0.85	c	c	c						
Leu	c	c	c	1.47	c	c	c	c	c	0.50							
Ile	c	c	c	0.95	c	c	c	c	c								
Trp	0.43	c	c	c	c	c	c	1.18									
Met	0.43	c	c	c	c	c	c										
Val	c	c	c	1.05	c	c											
Lys	c	0.63	c	c	0.0												
His	c	0.63	c	c													
Phe	c	c	c														
Thr	c	c															
Cys	c																

Separation A: Leu - Phe - Tyr - Val - Glu - Asp - Lys

Separation B: Leu - Phe - Val - Trp - Thr - Lys

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TABLE 4

Resolution Data for System C

	Pro	Cys	Thr	Phe	His	Lys	Val	Met	Trp	Ile	Leu	Gly	Arg	Asp	Glu	Tyr	Ser
Ala	c	c	0.33	c	c	c	c	c	c	c	c	1.33	c	c	0.40	c	1.45
Ser	0.60	c	1.09	c	c	c	c	c	c	c	c	0.0	c	0.0	c	c	
Tyr	c	c	c	1.11	c	c	c	0.98	c	0.0	0.80	c	c	c	c		
Glu	c	c	0.20	c	c	c	c	c	c	c	c	1.40	c	c			
Asp	0.60	c	1.09	c	c	c	c	c	c	c	c	0.0	c				
Arg	c	0.44	c	c	0.40	1.00	c	c	c	c	c	c					
Gly	c	c	1.09	c	c	c	c	c	c	c	c						
Leu	c	c	c	0.18	c	c	c	c	0.91	0.67							
Ile	c	c	c	1.11	c	c	c	0.98	c								
Trp	c	c	c	0.60	c	c	c	c									
Met	c	c	c	c	c	c	0.67										
Val	c	c	c	1.25	c	c											
Lys	c	c	c	c	0.80												
His	c	1.00	c	c													
Phe	c	c	c														
Thr	c	c															
Cys	c																

Separation A: Trp - Ile - Val - Ala - Ser - Cys - Lys

Separation B: Trp - Ile - Val - Thr - Lys

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TABLE 5

Resolution Data for System D

	Pro	Cys	Thr	Phe	His	Lys	Val	Met	Trp	Ile	Leu	Gly	Arg	Asp	Glu	Tyr	Ser
Ala	c	c	0.33	c	c	c	c	c	c	c	c	1.23	c	c	0.36	c	1.27
Ser	0.66	c	1.17	c	c	c	c	c	c	c	c	0.0	c	0.85	c	c	
Tyr	c	c	c	1.09	c	c	1.43	0.77	1.50	0.0	0.83	c	c	c	c		
Glu	c	c	0.0	c	c	c	c	c	c	c	c	1.40	c	c			
Asp	0.0	c	c	c	c	c	c	c	c	c	c	0.88	c				
Arg	c	c	c	c	c	c	c	c	c	c	c	c					
Gly	0.36	c	1.27	c	c	c	c	c	c	c	c						
Leu	c	c	c	0.36	c	c	c	1.36	0.66	0.31							
Ile	c	c	c	0.83	c	c	1.33	0.71	1.23								
Trp	c	c	c	0.36	c	c	c	c									
Met	c	c	c	c	c	c	0.67										
Val	c	c	c	c	c	c											
Lys	c	0.0	c	c	0.0												
His	c	0.0	c	c													
Phe	c	c	c														
Thr	c	c															
Cys	c																

Separation A: Trp - Ile - Val - Ser - Glu - Arg - Lys

Separation B: Trp - Ile - Val - Thr - Lys

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TABLE 6

Resolution of Data for System FxA

	Pro	Cys	Thr	Phe	His	Lys	Val	Met	Trp	Ile	Leu	Gly	Arg	Asp	Glu	Tyr	Ser
Ala	–	0.94	c	c	c	c	c	c	c	c	c	0.76	c	c	c	c	c
Ser	–	c	0.0	c	c	c	c	c	c	c	c	c	c	0.60	c	c	
Tyr	–	c	c	0.0	1.00	c	c	c	c	c	c	c	c	c	c		
Glu	–	c	c	c	c	c	c	1.25	c	1.33	c	c	c	c			
Asp	–	c	0.78	c	c	c	c	c	c	c	c	c	c				
Arg	–	c	c	c	c	1.50	c	c	0.0	c	c	c					
Gly	–	0.10	c	c	c	c	c	c	c	c	c						
Leu	–	c	c	c	c	c	c	1.38	c	1.00							
Ile	–	c	c	c	c	c	c	0.11	c								
Trp	–	c	c	c	c	1.50	c	c									
Met	–	c	c	c	c	c	c										
Val	–	c	c	c	c	c											
Lys	–	c	c	c	c												
His	–	c	c	c													
Phe	–	c	c														
Thr	–	c															
Cys	–																

Separation A: Thr – Gly – Val – Glu – Met – Leu – Phe – His – Lys – Arg

Separation B: Thr – Val – Met – Leu – Phe – His – Lys – Trp

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TABLE 7

Resolution Data for System FxB

	Pro	Cys	Thr	Phe	His	Lys	Val	Met	Trp	Ile	Leu	Gly	Arg	Asp	Glu	Tyr	Ser
Ala	-	0.0	c	c	c	c	c	c	c	c	c	0.38	c	c	c	c	c
Ser	-	c	0.27	c	c	c	c	c	c	c	c	c	c	0.71	c	c	
Tyr	-	c	c	0.99	c	c	c	c	c	c	1.14	c	c	c	c		
Glu	-	c	c	c	c	c	1.29	c	c	c	c	c	c	c			
Asp	-	c	1.00	c	c	c	c	c	c	c	c	c	c				
Arg	-	c	c	c	c	c	c	c	0.0	c	c	c					
Gly	-	0.29	c	c	c	c	c	c	c	c	c						
Leu	-	c	c	c	c	c	c	1.02	c	0.89							
Ile	-	c	c	c	c	c	c	0.12	c								
Trp	-	c	c	c	c	c	c	c									
Met	-	c	c	c	c	c	c										
Val	-	c	c	c	c	c											
Lys	-	c	c	c	c												
His	-	c	c	1.00													
Phe	-	c	c														
Thr	-	c															
Cys	-																

Separation A: Asp - Thr - Gly - Val - Met - Leu - Tyr - His - Lys - Trp

Separation B: Thr - Val - Met - Leu - Phe - His - Lys - Trp

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TABLE 8

Resolution Data for System FxC

	Pro	Cys	Thr	Phe	His	Lys	Val	Met	Trp	Ile	Leu	Gly	Arg	Asp	Glu	Tyr	Ser
Ala	-	1.07	c	c	c	c	c	c	c	c	c	0.13	c	c	c	c	c
Ser	-	c	0.27	c	c	c	c	c	c	c	c	c	c	0.46	c	c	
Tyr	-	c	c	c	c	c	c	c	c	c	1.00	c	c	c	c		
Glu	-	c	c	c	c	c	0.63	1.29	c	c	c	c	c	c			
Asp	-	c	0.83	c	c	c	c	c	c	c	c	c	c				
Arg	-	c	c	c	c	c	c	c	0.0	c	c	c					
Gly	-	c	c	c	c	c	c	c	c	c	c						
Leu	-	c	c	c	c	c	c	1.33	c	0.88							
Ile	-	c	c	c	c	c	c	0.40	c								
Trp	-	c	c	c	c	c	c	c									
Met	-	c	c	c	c	c	c										
Val	-	c	c	c	c	c											
Lys	-	c	c	c	c												
His	-	c	c	1.00													
Phe	-	c	c														
Thr	-	c															
Cys	-																

Separation A: Asp - Thr - Gly - Val - Met - Leu - Tyr - His - Lys - Trp

Separation B: Thr - Val - Met - Leu - Phe - His - Lys - Trp

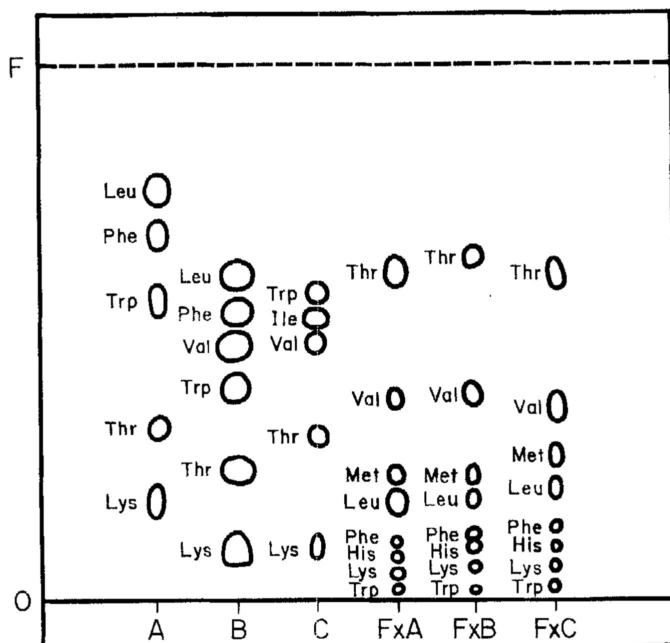

FIGURE 1. Separations of selected essential amino acids by cellulose, silica gel, and ion exchange TLC. See the Experimental section for descriptions of the TLC systems. F = solvent front, O = origin.

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Although acidic ethanol was specified (7) as the solvent for sample solutions on ion exchange layers, no differences were found when aqueous solutions were tested. However, the former solutions were routinely used for the Fixion layers, except for Gly as explained above.

A review of the data tables and the figure clearly indicates the superiority of the Fixion systems for resolution of complex amino acid mixtures. In each of the Fixion systems, ten acids from the 18-component mixture and 8 of the 9 essential acids could be separated. This is in comparison to the best non ion-exchange systems which were capable of separating nine of the 18-component mixture (System A) and 6 of the 9 essential acids (System B).

The tables and Figure 1 also show that the resolution ability for the three Fixion systems increased in the sequence FxC (pre-equilibration + elevated temperature) > FxB (pre-equilibration) > FxA. This is demonstrated by the R values for the pair Phe and Tyr, which are >1.50, 0.99, and 0.0, respectively. The increase in resolution, however, does not hold for all amino acid pairs. For example, the resolution between Ala and Gly was 0.76 in FxA, 0.38 in FxB, and 0.13 in FxC. The overall resolution advantages achieved as one progresses from system FxA to FxC are accompanied by additional work, causing the chromatography to become more tedious and time consuming. In addition, all Fixion layers require the preparation of a run buffer with carefully controlled pH (3.3 ± 0.1), and the chromatograms are characterized by uneven solvent fronts and high R_F zones that drift laterally, making interpretation of final results somewhat difficult.

Final spot sizes on developed chromatograms were in the following ranges: System A, 6-10 mm; B, 7-15 mm; C, 3-7 mm; D, 2-6 mm; FxA, FxB, FxC, 4-8 mm. The microcrystalline cellulose system (B) gave the largest and most irregularly shaped spots. The silica gel HPTLC system (D) gave somewhat smaller spots than for silica gel TLC (System C), but resolution values were essentially the same for the two model mixtures on the two silica gel layers.

Biological samples such as blood and urine to be analyzed for amino acids are often applied to TLC plates with a minimum of sample preparation (9-11). In order to evaluate the ability of cellulose, silica gel, Fixion, and preadsorbent silica gel layers to resolve the amino acids in such samples, 1-20 µl of freshly collected, untreated urine was directly spotted on each layer, which was developed and sprayed as described above. Good resolution of amino acids was obtained with the FxA ion exchange system for 15 µl of urine, which was the highest loading capacity found (Systems FxB and FxC were not tested). The loading capacities for fibrous and microcrystalline cellulose layers (Systems A and B) were 5 µl and 1 µl, respectively, and

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for Whatman K5 silica gel and LK5D preadsorbent silica gel were 5 μl and 10 μl, respectively. Outside of the preadsorbent silica gel plate, the Fixion layer was the easiest to spot with the larger volumes of raw urine. The thickness of the layers tested was 0.12 mm for Fixion, 0.25 mm for silica gel, and 0.10 mm for cellulose.

Although our comparative tests consistently indicated that the Fixion layers were superior to cellulose and silica gel for amino acid separations, their use required more experimental manipulation and preparation than the other layers. The data in this paper should be of great value to scientists separating and detecting amino acids by unidimensional TLC since it enables one to choose the most convenient applicable system for the compounds of interest. If only a limited number of amino acids is to be separated and they are adequately resolved on silica gel or cellulose, it would be advantageous to use these less tedious and time consuming systems rather than Fixion. For more complex separations, the Fixion layers will usually be required. For example, examination of the data for the individual pairs of compounds indicates that the compounds Ala, Phe, and Thr can be completely resolved in all three Fixion systems, as well as in (cellulose) System A. On the basis of convenience, the latter would probably be the best choice for separation of a mixture of these three amino acids. An approach such as this, involving comparison of data for all possible compound pairs, can be expanded to include as many amino acids as are of interest in any situation.

One dimensional TLC is advantageous when densitometric scanning is to be used for quantitation. Samples and standards can be run in parallel on the same plate under identical conditions for more accurate and precise comparison, which cannot be done in 2D-TLC. The data in this paper can indicate if one-dimensional TLC will provide adequate resolution to allow *in situ* quantitation of acids in any particular mixture.

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The data can also be used as an aid in identification of amino acids in a sample. A match in R_F values between samples and standards in systems with diverse separation mechanisms, such as is the case with silica gel, cellulose, and ion exchange layers, is much more valuable for confirming identity than would be non-independent data from the use of several mobile phases on the same layer (12). Any differences in colors produced by reaction with ninhydrin would also be significant evidence.

After this research was completed, a paper was published reporting "improved separation" of amino acids by microcrystalline cellulose TLC (13). Like the mobile phase used in our Systems A and B, the optimum solvent systems in this study were composed of butanol, acetic acid, and water. However, we did not test the exact mobile phases recommended by Dale and Court (13).

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