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Use of sodium bis (2−ethyl hexyl) sulfosuccinate (AOT) anionic surfactant mobile phase systems in thin−layer chromatography of amino acids : simultaneous separation of thioamino acids

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SUMMARY

Silica gel has been used as stationary phase in combination with surfactant−mediated eluents for thin layer chromatography of 12 amino acids. Several combinations of mobile phase systems comprising of different constituent and ratio have been tested for rapid and reliable separation of amino acids. The results obtained with mobile phase M_7 were compared with those obtained with mobile phases M_2+DMSO+methanol (3:2:7; v/v), M_2+DMSO+ethanol (3:2:7; v/v), M_2+DMSO+1−propanol (3:2:7; v/v) Thin layer chromatography system constituting silica gel as stationary phase and 0.001 M aqueous AOT (Sodium bis(2−ethylhexyl) sulfosuccinate) and 1−butanol (3:2:7; v/v) as mobile phase was identified as most favourable system for the separation of coexisting L−methionine (L−Met), L−cystein (L−Cys) and L−cystine (L−Cys−Cys). The proposed method is rapid and suitable for identification and separation of L−Met, L−Cys and L−Cys−Cys in the presence of many common heavy metals. The separation of microgram quantities of L−Met from milligram quantities of L−Cys or vice−versa was reported. Parameter such as limits of detection was also studied. Quantitative determination of L−Cys were done.

Keywords: TLC, Separation, Amino Acids, TLC−Spectrophotometry, AOT.

INTRODUCTION

Thin layer chromatography (TLC) being simple and cost effective has been used by several workers (1−4) as an analytical tool for rapid analysis of amino acids and heavy metal cations. Most of the workers have used silica gel (5−7), alumina (8, 9), cellulose and cellulose derivatives (10,11), chitin and chitosan (12,13) polyamide (14), as layer materials in combination with aqueous, mixed−organic and mixed aqueous−organic solvents as mobile phase. Salting out reversed−phase TLC has been successfully employed for rapid analysis of dansylated amino acids by T. Cserhati et al. (15). Interesting separations of racemic aromatic amino acids have been reported on cellulose layers using concentrated aqueous solutions of α or β cyclodextrins (16,17). Ravi Bhushan and co−workers have achieved improved separations of closely related amino acids on surface−modified silica gel layers (18,19).

Micellar liquid chromatography (MLC) involving the use of surfactant ions above their critical micelle concentration (CMC) in mobile phase has been the focus of numerous separation studied (20−23) since its inception in 1977 by Armstrong and co−workers (24). With the aim of utilizing advantageous features such as inexpensiveness, non−toxicity and non−inflammability of surfactant−mediated mobile phase, a novel microemulsion system consisting of sodium dodecyl sulfate (SDS), as one of the components, was proposed by A. Mohammed et al. (25) to achieve certain important separations of amino acids on silica gel layer.

Traditionally, the enhanced separation efficiency of micellar

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systems has been achieved by adding small quantities of organic additives e. g. 1−propanol or 1−pentanol (26,27). However, in the present investigation we have realized improved separation efficiency for coexisting sulfur bearing amino acids with a hybrid mobile phase system consisting of micelles of AOT [Sodium bis (2−ethyl hexyl) sulfosuccinate, anionic surfactant], DMSO (dimethyl sulfoxide) and 1−butanol. Interestingly best separation was possible only when the concentration of 1−butanol was kept above 50% in the mobile phase. Thus deviating from earlier findings (26,27), our results establish higher concentration of alcohol in combination with micellar systems for achieving analytically different separations.

To the best of our knowledge, no work has been reported on the use of AOT micellar solution containing 1−butanol and DMSO as mobile phase in the analysis of amino acids by silica TLC.

DMSO was selected because of our past experience (28) that as being an aprotic dipolar solvent with hard oxygen and soft sulfur, which provide numerous separation of inorganic substances by solvating cationic species in preference to anionic species. Recently, DMSO has been utilized by S.D. Sharma et al. (29) as the mobile phase for TLC of amino acids on titanium tungstate.

The aim of this study is to separate L−Cys−Cys, L−Cys and L−Met from their coexisting mixture with TLC technique. The separation of sulfur bearing amino acids is very important because of the following reasons.

(a) L−Cys−Cys is dimeric form of L−Cys and it is easy for these amino acids to interchange under favourable conditions.

(b) As far as we are aware, the mutual separation of L−Cys−Cys, L−Cys and L−Met by TLC has not been reported.

(c) L−Met and L−Cys both are necessary for human being, but only L−Met is considered as essential amino acid and could be taken from food and drugs, whereas body forms L−Cys under metabolic process of L−Met present in adequate amount. Food and drugs are considered as contaminated, if L−Cys is present even in small quantity and it can be identified by separating from L−Met by the proposed TLC technique.

Though HPLC, GC and electrophoresis techniques are available for the analysis of amino acids, TLC being inexpensive is more suitable for routine analysis. Furthermore, TLC would be amendable as a pilot technique for column chromatography.

EXPERIMENTAL

All experiments were performed at 30+5 ℃ apparatus.

Apparatus : A TLC applicator (Toshniwal, India) was used for coating silica gel on 20 × 3.5 cm glass plates. The chromatography was performed in 24 × 6 cm glass jars. A glass sprayer was used to spray reagent on the plates to locate the position of the spot of analyst.

Chemicals and Reagents : Amino acids and DMSO (CDH, India), methanol, ethanol, 1−propanol and 1−butanol (Qualigens, India), Silica gel 'G' (E. Merck, India) and Aerosol−OT (BDH, England). All reagents were of Analytical Reagent grade.

Amino acids studied : L−tryptophan (L−Trp), L−iso−leucine (L−Ile), L−hydroxy proline (L−Hyp), L−proline (L−Pro), L−arginine (L−Arg), L−lysine (L−Lys), L−cysteine (L−Cys), L−cystine (L−Cys−Cys), L−methionine (L−Met), L−valine (L−Val), glycine (Gly) and L−serine (L−Ser).

Test solutions :

All the test solutions (1%) except L−Cys solution were prepared in demineralized double distilled water with a specific conductivity ($k=2 \times 10^{-6}$ ohm^{-1} at 25 ℃). The 1% solution of L−Cys was prepared in 0.1% aqueous HCl solution.

Detector :

0.3% ninhydrin solution in acetone was used to detect all the amino acids.

Stationary Phase: Silica gel 'G'.

Mobile Phase: The following solvent systems were used as mobile phase.

Symbol	Composition
M_1	0.0001M aqueous AOT
M_2	0.001M aqueous AOT
M_3	0.01M aqueous AOT
M_4	M_2 + 1−butanol (9.5:0.5 v/v)
M_5	M_2 + DMSO + 1−butanol (1:2:9; v/v)
M_6	M_2 + DMSO + 1−butanol (2:2:8; v/v)
M_7	M_2 + DMSO + 1−butanol (3:2:7; v/v)
M_8	M_2 + DMSO + 1−butanol (4:2:6; v/v)
M_9	M_2 + DMSO + 1−butanol (3:2:9; v/v)
M_{10}	M_2 + DMSO + 1−butanol (3:2:8; v/v)
M_{11}	M_2 + DMSO + 1−butanol (3:2:6; v/v)
M_{12}	H_2O + DMSO + 1−butanol (3:2:7; v/v)
M_{13}	M_2 + DMSO + methanol (3:2:7; v/v)
M_{14}	M_2 + DMSO + ethanol (3:2:7; v/v)
M_{15}	M_2 + DMSO +1−propanol (3:2:7; v/v)

Preparation of TLC Plates :

The plates were prepared by mixing silica gel with water in 1:3 ratio with constant shaking until homogeneous slurry was obtained. The resultant slurry was applied on the glass plates with the help of a Toshniwal applicator to give a 0.25 mm thick layer. The plates were dried in air at room temperature and then activated by heating for 1 h at 100±5 ℃ in an electrically controlled oven. The

activated plates were stored in a close chamber at room temperature until used.

Procedure :

Test solutions (approx. 10 μl) were applied by means of micropipets approximately about 2.0 cm above the lower edge of the plates. The plates were developed in the chosen solvent system by the ascending technique. The solvent ascent was fixed to 10 cm in all cases. After development was complete the plates were withdrawn from glass jars and dried at room temperature followed by spraying with freshly prepared ninhydrin solution. All amino acids except L−Pro and L−Cys−Cys appeared as violet spots on heating TLC plates for 15−20 minutes at 100±5 ℃. L−Pro and L−Cys−Cys produce yellow spots. The R_L (R_F of leading front) and R_T (R_F of trailing front) values for each spot were determined and the R_F value was calculated as :

$$R_F = \frac{R_L + R_T}{2}$$

Separation :

For the mutual separation, equal amounts of L−Cys, L−Cys−Cys and L−Met were mixed and 20 μl of the resultant mixture was loaded on the TLC plates. The plates were developed with mobile phase M_7 the spots were detected and the R_F values of the separated amino acids were determined.

Interference :

Investigating the effect of metal cations on the mobility of amino acids are an important aspect because in metalloprotein, amino acids and metal cations are the building blocks. For investigating the interference of heavy metal cations on the separation of co−existing L−Cys, L−Cys−Cys and L−Met. An aliquot (10 μl) of foreign substance was spotted along with the mixture (10 μl) of L−Cys, L−Cys−Cys and L−Met and chromatography was performed as described above with M_7. The spots were detected and the R_F values of amino acids were determined.

Microgram Separation of L−Cys from L−Met and Vice Versa :

For this study TLC plate was first spotted with 0.01 ml of the L−Cys (10 μg) solution and then with 0.01 ml from a series of standard solutions of L−Met containing 0.1 mg−1.2 mg per 0.01 ml onto the same TLC plate. Simultaneously another chromatoplate was first spotted with 0.01 ml of L−Met (10 μg) solution and then with 0.01 ml of the standard solutions containing 0.1 mg− 1.8 mg L−Cys per 0.01 ml onto the same TLC plate. The spots were dried and the plates were developed with M_7 and the separated spots were visualized. The R_L and R_T values were calculated for both the sulfur containing amino acids.

Limit of detection:

The identification limits of various amino acids including L−Cys, L−Cys−Cys and L−Met were determined by spotting different amounts of amino acids on the TLC plates. The plates were detected as described above. The method was repeated with successive lowering of the amount of amino acid until spots could no longer be detected. The minimum amount of amino acid that could be detected was taken as the limit of detection.

Quantitative determination of the L−Cys by TLC−spectrophotometric methods

For spectrophotometric determination, 0.01 ml of L−Cys of different strength (0.5−3.5%) containing 0.05−0.35 mg L−Cys were treated with 5 ml of methanol and 2 ml of 0.3% ninhydrin (in acetone) and heated in a oven at 1000 C for 30 mins. After cooling, the solution was diluted up to 10 mlwith methanol. The absorbance of the developed color was measured spectrophotometically against at 530 nm (λ_{max}) using 1 cm cells and a standard curve was constructed.

The devised TLC method was applied to the determination of L−Cys after their chromatographic separation from L−Met and L−Cys−Cys. For this purpose, 0.01 ml of L−Cys of different strength (0.75−3.25%) containing 0.10−0.30 mg L−Cys were spotted onto TLC plates, followed by spotting 0.1 mg each of the L−Met and L−Cys−Cys solution onto the same spots with a micropipette and the development was made as usual with M_7. Pilot chromatograms were run under similar conditions to ascertain the actual position of the spots on pilot plates, which were detected using the ninhydrin reagent. The same portion of the experimental plates was scratched out and L−Cys present in these portions were extracted with small volume of methanol, 5 ml being used for complete elution. A chromogenic reagent (2 ml of 0.3% ninhydrin in acetone) was added to the filtrate and heated in oven at 100 ℃ for 30 mins. After cooling, the solution was diluted to 10 ml with methanol. The absorbance of the purple−pinks (pinkish purple) color so developed was measured spectrophotometrically against a blank at 530 nm (λ_{max}) using 1 cm cells. The L−Cys content after its separation from L−Met and L−Cys−Cys was determined from the standard curve.

RESULTS AND DISCUSSION

Optimization of mobile phase system :

In order to select a most appropriate concentration of an anionic surfactant, we used 0.0001M−0.01M aqueous solutions of AOT. This concentration range was selected to keep the concentration of surfactant below, near and above critical micelle concentration (CMC) value. The reported CMC value of AOT is 0.00064M.

Although in pure water, most of amino acids produce diffused spots. L−Cys−Cys remains at the point of application in the form of highly compact spot.

The result of the mobility pattern of amino acids on silica layer developed with M_1−M_3 mobile phase e.g. 0.0001− 0.01 M AOT are summarized in Table 1. It is evident from this table that all the studied amino acids except L−Cys−Cys show higher mobil-

Table 1. R_F value of Amino Acids on Slica Gel Layers Developed with Different Mobile Phases.

Amino acid	M_1	M_2	M_3	M_{12}
L−Trp	0.94	0.91	0.90	0.85
L−Ile	0.88	0.54	0.80	0.78 T
L−Hyp	0.82	0.78	0.75	0.70
L−Pro	0.85	0.80	0.78	0.65
L−Arg	0.79	0.77	0.74	0.72 T
L−Lys	0.83	0.80	0.75	0.69 T
L−Cys	0.97	0.93	0.88	0.85
L−Cys−Cys	0.00	0.0	0.0	0.0
L−Met	0.90	0.88	0.83	0.82 T
L−Val	0.86	0.83	0.78	0.66
Gly	0.91	0.89	0.84	0.78 T
L−Ser	0.97	0.94	0.90	0.85

T = Tailing Spot (R_L−R_T>0.30)

ity (or high R_F value) regardless the concentration level of AOT. L−Cys−Cys remain at the point of application (R_F=0.0) and hence it can be selectively separated from all other amino acids mentioned in Table 1. The AOT in water acts as tailing reducer producing more compact spots. It appears that amino acids are preconcentrated in hydrophobic pool of AOT micelles via non−covalent interactions similar to SDS (30). Thus amino acids are available at enhanced concentration level to react easily with ninhydrin producing more compact and brighter spots.

Taking into consideration the compactness and the clearer detection of spots the mobile phase M_2 (0.001M AOT) is selected for further studies.

Synergistic Effect of 1−butanol on Mobility of Amino Acid :

The improved chromatorgarphic performance of surfactant mediated mobile phase in the presence of alcohols have been reported (3). Therefore, we added 1−butanol in M_2 (0.001M aqueous AOT) and the resultant mobile phases were used to understand the mobility pattern of amino acids. The results obtained with M_2 and M_4 have been plotted in Figure 1 as ΔR_F values (R_F in M_2−R_F in M_4). From Figure 1, it is clear that in general the mobility of almost all the amino acids is decreased in the presence of 1−butanol as evident by positive values of ΔR_F. Contrary to our hope this lowering in mobility of amino acids does not produce improved separations.

As the mutual solubility of 1−butanol and 0.001 M AOT (M_2) is very low (maximum solubility of 1−butanol in AOT and vice versa was 9.5:0.5; v/v) at normal room temperature, we concluded that it would not be possible to achieve important separations using mobile phase M_4 until mutual solubility of these two partially miscible liquids is enhanced. For this purpose, unique solubilizing property of a polar aprotic solvent (e. g. DMSO) was utilized by adding it as a third component into M_4. The resulting solvent systems (M_5−M_8) containing a fixed volume of DMSO and variable volumes of AOT (0.001M) and 1−butanol were tested. The R_F values of amino acids obtained on silica gel 'G' layers developed with M_5−M_8 are summarized in Table 2.

It is clear from Table 2 that mobility of most of the amino acids increases with the decrease in volume ratio of 1−butanol. Amino acid such as L−Pro (R_F range 0.33−0.37) and L−Cys−Cys (RF=0.0) can be selectively separated from most of amino acids us-

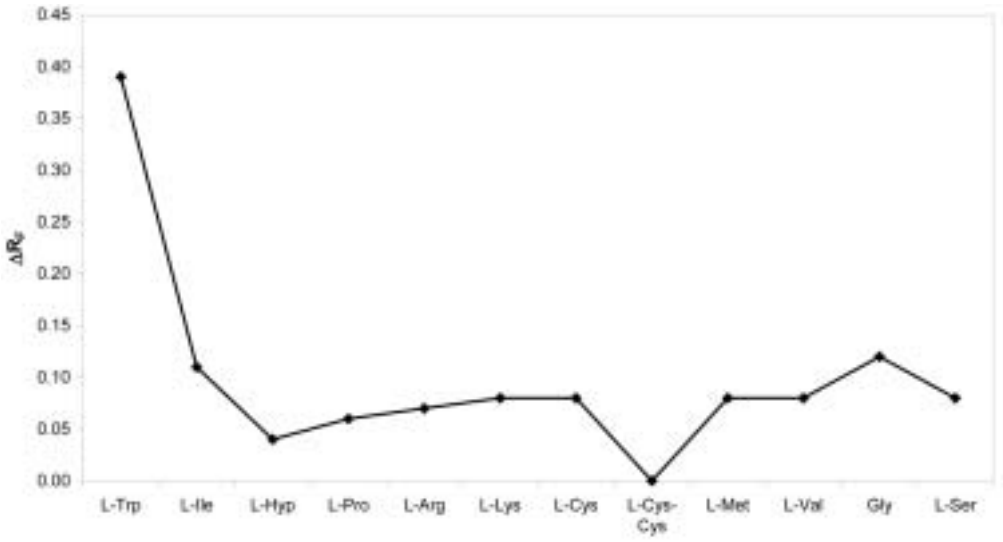

Figure 1. ΔR_F between R_F values obtained for the amino acids by use of mobile phases M_2 and M_4. [ΔR_F=R_F(M_2)−R_F(M_4)]

Table 2. R_F value of Amino Acids on Silica Layers Developed with Mixed Mobile Phases Consisting of Fixed Volume of DMSO and Variable volumes of AOT (0.001M) and 1−butanol.

Amino acid	M_5	M_6	M_7	M_8
L−Trp	0.45	0.50	0.66	0.75
L−Ile	0.55	0.58	0.63	0.72
L−Hyp	0.21	0.23	0.32	0.41
L−Pro	0.33	0.33	0.34	0.37
L−Arg	0.06	0.09	0.19	0.21
L−Lys	0.04	0.06	0.12	0.20
L−cys	0.09	0.17	0.23	0.45
L−Cys−Cys	0.0	0.0	0.0	0.0
L−met	0.42	0.45	0.53	0.58
L−val	0.36	0.40	0.49	0.56
Gly	0.14	0.19	0.30	0.38
L−Ser	0.15	0.18	0.31	0.38

ing M_5 to M_8. Whereas M_7 was found useful for specific separation of L−Cys−Cys (RF=0.0) from L−Met (R_F=0.53) and L−Cys (R_F= 0.23).

The higher mobility of L−Met compared to L−Cys in 1−butanol containing mobile phase (M_7) may be explained on the basis of size of hydrophobic tail. The structures of amphipathic molecules (e.g. amino acids and AOT) are stabilized by hydrophobic interaction among non−polar regions (31). Thus L−Met being non−polar than L−Cys (Polar) migrates faster on TLC plates developed with M_7 containing 1−butanol (moderately polar solvent) as one of the components of mobile phase. In case of L−Cys−Cys, there is no mobility and it remained at the point of application (R_F=0). L−Cys−Cys is a covalently linked dimeric amino acid, in which two L−Cys molecules are joined by a disulfide bond. The ends of the L−Cys−Cys molecule interact with free hydroxyl group of silica gel analogous to phenol (32).

Thus M_7 was considered the most suitable mobile phase as it facilitates the mutual separation of sulfur containing amino acids L−Cys, L−Cys−Cys and L−Met Figure 2.

Importance of AOT and 1−butanol in Mobile Phase:

To understand the importance of AOT in M_7 for the specific separation of L−Cys−Cys from L−Met and L−Cys, the composition of M_7 was changed and the resultant solvent system (M_{12}) was used as eluent. The separation of L−Cys and L(Met is hampered in the absence of AOT (M_{12}) due to overlapping of L−Met and L−Cys spots Table 1.

In order to show more clearly the effect of 1−butanol, the

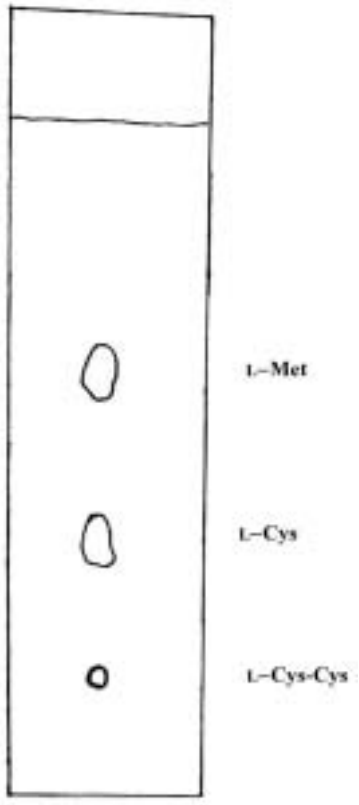

Figure 2. Separation pattern of coexisting L−Met, L−Cys and L−Cys−Cys with M_7 Mobile Phase on Silica Layers.

chromatography of amino acids was performed with mobile phase systems (M_7, M_9−M_{11}) prepared by varying the concentration of 1−butanol and keeping the concentration of AOT and DMSO constant. The result obtained with M_7, M_9 to M_{11} are presented in Figure 3. It is clear from Figure 4 that mobility of most of the amino acids increases with the decrease in volume ratio (or concentration) of 1−butanol, except L−Cys−Cys (R_F=0), whereas mobility of L−Pro fluctuates within the range of 0.33 to 0.36.

Effect of Added Alcohols in the Mobile Phase:

In order to examine the influence of different alcohols on the separation of L−Cys, L−Cys−Cys and L−Met, 1−butanol in M_7 was replaced by methanol, ethanol and 1−propanol. The resultant mobile phases (M_{13}−M_{15}) were used to examined mobility of sulfur containing amino acids from their mixture on silica layer. The results obtained with M_{13}−M_{15} were compared with M_7 and are presented in Figure 4 From this figure it is evident that the L−Cys co−migrates with L−Met imposing a restriction on mutual separation of L−Cys from L−Met where TLC plates were developed with M_{13} −M_{15}. Though, M_{13}−M_{15} systems are capable to provide binary separation of L−Cys−Cys from L−Cys or L−Met but their separation from three component mixture is not possible. However, a very reliable and clear separation of coexisting L−Cys, L−Cys−Cys and L−Met is always possible with M_7, therefore it may be con-

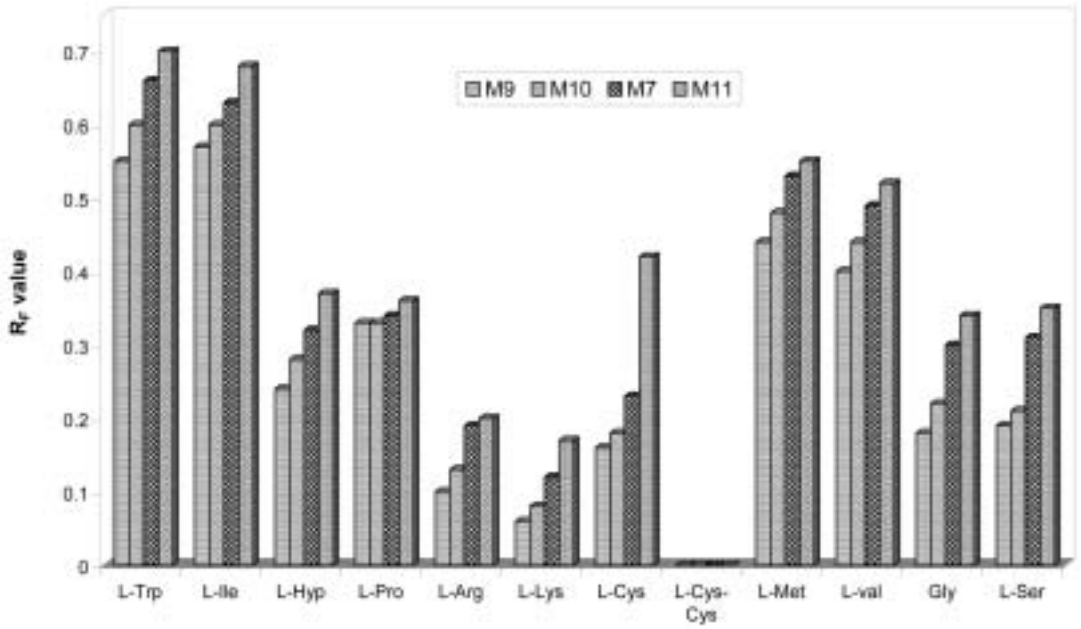

Figure 3. Effect of 1−butanol with Mobile phase system M_7, M_9−M_{11}.

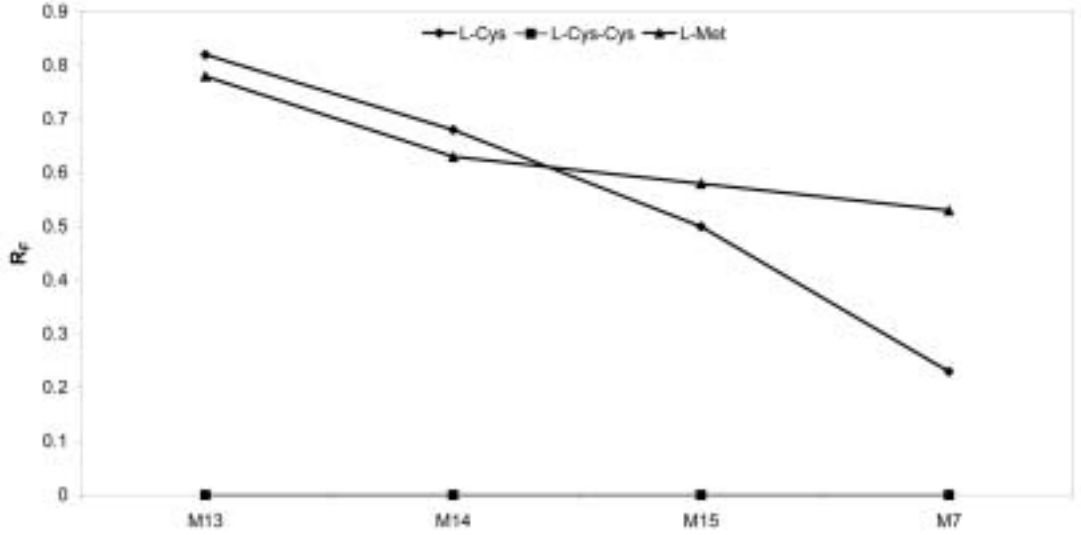

Figure 4. Effect of Added Alcohol with 0.001M AOT and DMSO

cluded that alcohols (methanol, ethanol and 1−propanol) are not favourable for the separation of sulfur containing amino acids.

Effect of Impurities:

From the data listed in Table 3, it is evident that the RF value of L−Met is decreased considerably in the presence of Pb^{2+} whereas the R_F value of L−Cys and L−Cys−Cys remain almost unchanged. Though the separation of L−Cys, L−Cys−Cys and L−Met from their mixture is possible in the presence of Pb^{2+}, but the separation is hampered in the presence of Hg^{2+} in the sample. The separation is not altered by the presence of other metal cations studied (Table 3).

Effect of Loading Amount of Analyte:

It was observed that 10 μg of L−Cys can easily be separated from 1.2 mg of L−Met, Similary 10 μg of L−Met can be separated from 1.8 mg of L−Cys. Thus milligram quantities of one amino acid can be successfully separated from microgram amounts of the other amino acid using the proposed TLC system.

Limit of Detection:

The lowest possible detectable microgram amounts along with dilution limits amino acids (give in parenthesis) on silica layer were L−Met (0.125, 1.8 × 104), L−Cys (0.20, 1:5 × 104) and L−Cys−Cys (1.0, 1:1 × 104).

Quantitative determination of L−Cys by TLC−spectrophotometric methods :

Table 3. Separation of L−Cys−Cys, L−Cys and L−Met from their Mixtures, in the Presence of Metals Cation as Impurities on Silica Layers Developed :

Metals cation	Separation (R_F)		
	L−Cys	L−Cys−Cys	L−Met
Fe^{3+}	0.21	0.0	0.53
Cu^{2+}	0.23	0.0	0.52
Ni^{2+}	0.22	0.0	0.55
Co^{2+}	0.23	0.00	0.54
UO_2^{2+}	0.23	0.0	0.55
Al^{3+}	0.21	0.0	0.55
Cd^{2+}	0.23	0.0	0.51
Zn^{2+}	0.23	0.0	0.52
Pb^{2+}	0.21	0.0	0.44
Hg^{2+}	0.25	0.0	0.31 T
VO^{2+}	0.21	0.0	0.52

T = Tailed spot ($R_L - R_T > 0.30$)

Table 4. Spectrophotometric Determination: Amounts of L−Cys after TLC separation for L−Met and L−Cys amino acids.

Sample	Amount loaded (mg)	Amount recovered(mg)	% Error
1	0.75	0.70	6.6
2	1.25	1.21	3.2
3	1.75	1.70	2.85
4	2.25	2.17	3.55
5	2.75	2.68	2.54
6	3.25	3.15	3.07

Table 5. Experimentally Achieved Separations of Amino Acids on Plain Silica Layers Developed with Different Mobile Phases:

M_5	L−Cys−Cys (0.0)−L−Hyp (0.20)−L−Ile(0.55)
M_6	L−Cys−Cys (0.0) −L−Hyp (0.23) −L−Ile (0.56).
M_7	L−Cys−Cys (0.0)−L−Trp (0.64) −L−Ile (0.62).
M_8	L−Cys−Cys (0.0)−L−Arg (0.20) / L−Lys (0.19).

Upto 3.25 mg, L−Cys could be determined spectrophot−ometrically using 0.3% ninhydrin in acetone as the chromogenic reagent. The percentage error was not greater than 6.60. The result obtained are tabulated in Table 4.

Applications:

Several important separations of amino acids were experimentally achieved on silica gel layers developed with a variety of mobile phase systems. These separations have been listed in Table 5.

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