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# Use of Carbohydrates as Eluent in Thin Layer Chromatographic Separation of Amino acids on Conventional Stationary Phases

**Ali Mohammad* and Nazrul Haq**

*Analytical Research Laboratory, Department of Applied Chemistry, Aligarh Muslim University, Aligarh, U.P, India*

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## Abstract

Thin layer chromatographic studies of amino acids were performed on three differentially charged surfaces of silica gel, alumina and cellulose with 40% aqueous solution of five carbohydrates namely dextrose, fructose, maltose, lactose and sucrose. 40% dextrose-alumina and 40% dextrose-cellulose TLC systems were identified as most favorable for selective separation of glutamic acid and tryptophan from the mixture of other amino acids. In addition to this, several combinations of amino acids have been resolved on silica gel and alumina layers with 40% dextrose as eluent. The lowest detectable limit of glutamic acid and tryptophan, stability of mixtures of amino acids and reproducibility of $R_F$ values were determined. The proposed method is environmentally acceptable because of the use of non-toxic nature of eluents used.

**Key words**: TLC, amino acids, carbohydrates, eluent, separation

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## Introduction

Because of biological and physiological importance of amino acids, several analytical technique such as high performance liquid chromatography [1], gas chromatography [2], thin layer chromatography [3], cyclic voltametry [4], electrophoresis [5], viscometric [6] and micellar electrokinetic chromatography [7], have been used for their analysis. Among chromatographic techniques, thin layer chromatography (TLC) has been the most popular for routine analysis of amino acids because of several attractive features such as wider choice of mobile and stationary phases, flexibility in sample detection, the open and disposal nature of thin layer

chromatographic plates, low solvent consumption, minimal sample clean up, reasonable resolution power and the ability to handle large number of samples simultaneously.

The extensive survey of literature of last twenty years on TLC of amino acids reveals that most of the studies performed so far include the use of eluents belonging to the following main groups [8-28]

1. Organic solvents (urea , carboxylic acids, alcohols, ketones, dimethyl sulphoxide, ethyl acetate, heptanes, chloroform, pyridine, benzene, toluene , xylene, carbon tetrachloride, methylene dichloride and their mixture).
2. Inorganic solvents (aqueous solvents of Li, Na, K, Rb and Cs salts).
3. Mixed aqueous-organic solvents (alcohols, ketones, acetonitrile, chloroform, and/or pyridine mixed with water, acetate buffer and carboxylic and mineral acids).
4. Chiral solvents (α or β- cyclodextrin and ethyl (s) - (+) - lactate).
5. Surfactant mediated solvents (aqueous solutions of (2- ethyl hexyl) sodium, sulfosuccinate, sodium dodecyl sulphonate and N- cetyl N,N, N-tri methyl ammonium bromide and their microemulsions).

Of the above eluents, organic and mixed aqueous-organic solvent systems have been found most useful for the separation of amino acids. However, the toxic nature of most of the solvents imposes a restriction on their frequent use. Though inorganic solvent systems are not as toxic as organic solvent systems but they are not capable to resolve the multicomponent mixtures of amino acids. Surfactant mediated solvent systems are quite complex and hence the interpretation of the chromatographic behavior of analyte becomes difficult. To the best of our knowledge, so far there have been only two studies [29-30] with carbohydrates other than cyclodexdextrin as a component of mobile phase.

It was, therefore, worthwhile felt to study the chromatographic behavior of amino acids through differentially charged surfaces of alumina, silica gel and cellulose using aqueous solutions of carbohydrates as eluents. The major advantage of these eluents is that they are non-toxic and ecofriendly and also worth mentioning is the simplicity of these systems (monocomponent nature). Another reason for selecting these carbohydrates as mobile phase was the physiologically important interaction of amino acids and sugars (in the formation of glycoproteins) and formation of amino acid-sugar adducts [31].

Based on the differential migration, we have obtained some important separations of amino acids on silica gel and alumina with 40% dextrose as eluent in addition to the selective separation of glutamic acid and tryptophan from other amino acids on alumina and cellulose stationary phase respectively with fructose as mobile phase.

## Results and Discussion

In this study, 5-50% aqueous solutions of five carbohydrates namely dextrose, fructose, sucrose, maltose and lactose were used as mobile phases and three differentially charged surfaces viz; alumina G (neutral and inorganic surface),silica gel G (acidic and inorganic surface) and cellulose(neutral and organic surface) were used to observe the mobility trend of amino acids. Mobility ($R_F$) of all amino acids studied with 5-35% solution of carbohydrates was in the range

of 0.94-1.00. So, these eluents were not useful for chromatographic studies. Similarly, eluents with carbohydrate concentration greater than 40% were found not useful for separation because there was very slight change in the $R_F$ of amino acids. So, 40% aqueous solution of these carbohydrates was selected for chromatography. The results obtained for the mobility of amino acids on S1-S3 stationary phases are presented in Tables 1-3.

**Table 1: Mobility of amino acids on cellulose (S1) TLC plates with aqueous solution of carbohydrates as mobile phase.**

| **Mobile phase →<br>Amino acids ↓** | **40% dextrose** | **40%fructose** | **40% sucrose** | **40% lactose** | **40% maltose** |
|---|---|---|---|---|---|
| Leucine | 0.95 | 0.93 | 0.87 | 0.95 | 0.93 |
| Norleucine | 0.93 | 0.92 | 0.95 | 0.95 | 0.95 |
| Isoleucine | 0.95 | 0.93 | 0.94 | 0.93 | 0.95 |
| Valine | 0.96 | 0.97 | 0.96 | 0.95 | 0.96 |
| Proline | 0.93 | 0.94 | 0.87 | 0.90 | 0.92 |
| Alanine | 0.92 | 0.94 | 0.89 | 0.91 | 0.93 |
| Tryptophan | 0.68 | 0.52 | 0.72 | 0.75 | 0.76 |
| Methionine | 0.92 | 0.95 | 0.91 | 0.94 | 0.94 |
| Glutamic acid | 0.92 | 0.88 | 0.93 | 0.93 | 0.89 |
| Serine | 0.92 | 0.91 | 0.83 | 0.92 | 0.92 |
| Tyrosine | 0.88 | 0.76 | 0.83 | 0.84 | 0.80 |

**Table 2: Mobility of amino acids (a) on alumina (S2) TLC plates with aqueous solution carbohydrates as mobile phase.**

| Mobile phase →<br>Amino acids ↓ | 40% dextrose | 40%fructose | 40% sucrose | 40% lactose | 40% maltose |
|---|---|---|---|---|---|
| Leucine | 0.56 | 0.50 | 0.61 | 0.50 | 0.58 |
| Norleucine | 0.58 | 0.55 | 0.53 | 0.50 | 0.63 |
| Isoleucine | 0.59 | 0.58 | 0.53 | 0.49 | 0.64 |
| Valine | 0.60 | 0.64 | 0.52 | 0.65 | 0.64 |
| Alanine | 0.51 | 0.55 | 0.46 | 0.48 | 0.52 |
| Tryptophan | 0.24 | 0.54 | 0.44 | 0.29 | 0.29 |
| Methionine | 0.39 | 0.49 | 0.40 | 0.44 | 0.49 |
| Glutamic acid | 0.29 | 0.22 | 0.28 | 0.22 | 0.20 |
| Serine | 0.30 | 0.35 | 0.25 | 0.24 | 0.32 |
| Tyrosine | 0.36 | 0.55 | 0.41 | 0.37 | 0.30 |

(a) Proline could not be detected on alumina layer

On neutral and organic cellulose stationary phase (S1), all amino acids, irrespective of polarity/charge, due to lack of ionic interaction with the stationary phase, show $R_F$ in the range of 0.85-1.00 except tryptophan and tyrosine (Table 1). Tyrosine has slightly lower $R_F$ than other amino acids. Amongst all amino acids, tryptophan shows lower $R_F$ (0.50-0.75) with all eluents. This may be due to the formation of sugar-tryptophan adduct [31].

From Table 2, it is clear that all non-polar amino acids except methionine and tryptophan show $R_F$ in the range of 0.50- 0.65 on alumina (S2).All polar amino acids are strongly adsorbed and show lower $R_F$ as compared to non-polar amino acids. This may be due to the interaction of polar group of these amino acids with alumina which has got high adsorption efficiency. Tryptophan, due to the reason stated above is showing still lesser mobility. Methionine with thioether linkage in its molecule interacts with alumina showing less mobility. Table 3 shows the mobility of amino acids on silica gel (S3) stationary phase.

**Table 3: Mobility of amino acids on silica gel (S3) TLC plates with aqueous solution of carbohydrates as mobile phase.**

| Mobile phase → Amino acids ↓ | 40% dextrose | 40%fructose | 40% sucrose | 40% lactose | 40% maltose |
|---|---|---|---|---|---|
| Leucine | 0.64 | 0.67 | 0.69 | 0.69 | 0.81 |
| Norleucine | 0.65 | 0.67 | 0.62 | 0.70 | 0.78 |
| Isoleucine | 0.70 | 0.67 | 0.65 | 0.70 | 0.71 |
| Valine | 0.85 | 0.78 | 0.83 | 0.75 | 0.85 |
| Proline | 0.65 | 0.69 | 0.67 | 0.73 | 0.73 |
| Alanine | 0.98 | 0.87 | 0.81 | 0.82 | 0.81 |
| Tryptophan | 0.84 | 0.84 | 0.79 | 0.86 | 0.84 |
| Methionine | 0.76 | 0.80 | 0.76 | 0.79 | 0.80 |
| Glutamic acid | 0.98 | 0.97 | 0.87 | 0.95 | 0.85 |
| Serine | 0.96 | 0.86 | 0.78 | 0.97 | 0.73 |
| Tyrosine | 0.93 | 0.96 | 0.71 | 0.92 | 0.85 |

From the results presented in Table 3, it is clear that all non –polar amino acids except alanine show $R_F$ in the range of 0.60-0.85 on silica gel 'G' (S3) stationary phase. Alanine shows slightly higher $R_F$ (0.81-0.98). This behavior of alanine may be attributed to the absence of any extra ionizable group in its structure. All polar amino acids have higher mobility as compared to non-polar amino acids.

The comparative mobility pattern of all amino acids examined with M1 and M2 mobile phases on all stationary phases S1-S3 has been presented in Figures 1 and 2.

From these figures, it is clear that amino acids are more strongly adsorbed on alumina compared to other stationary phases. This is due to the fact that the most important interaction affecting sample retention on the surface of the stationary phase in planar chromatography is the hydrogen

bonding [32] and mean hydroxyl group density of alumina is about 13µmol/$m^2$ [33] whereas mean hydroxyl group density of silica gel is about 08µmol/$m^2$ [34].

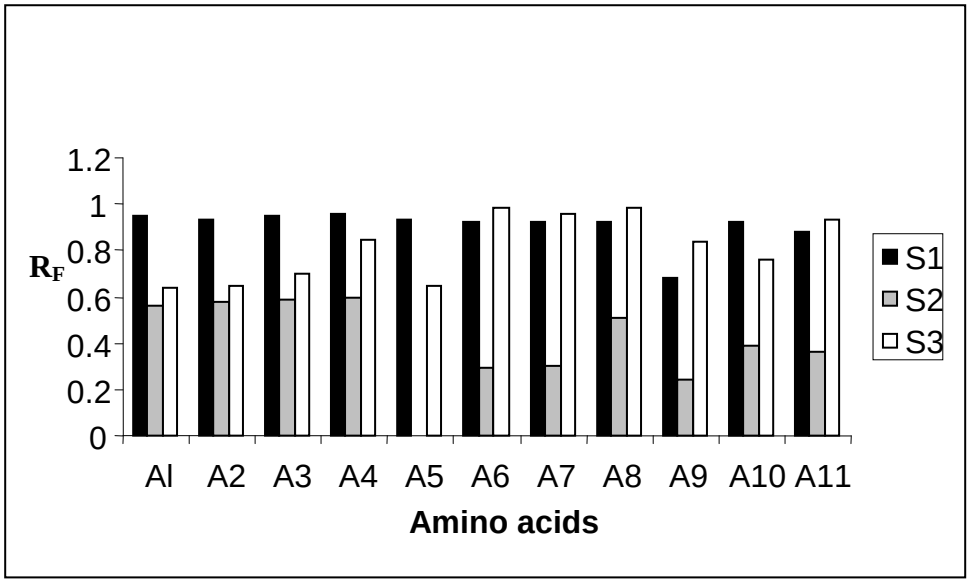


**Figure 1: Comparative mobility of amino acids on S1-S3 stationary phases with M1 mobile phase.**

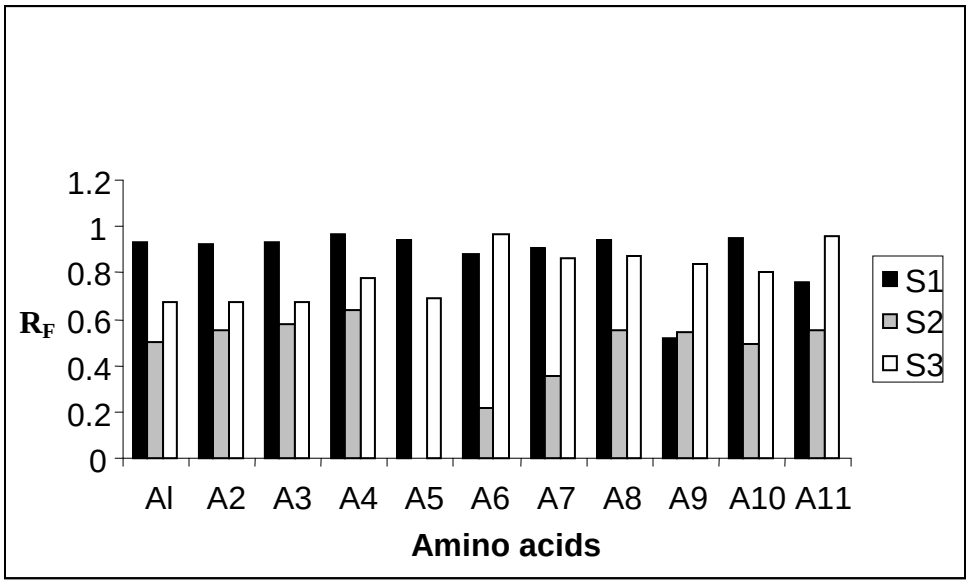


**Figure 2: Comparative mobility of amino acids on S1-S3 stationary phases with M2 mobile phase.**

Because the mobility of glutamic acid on S2 and tryptophan on S1 stationary phase is quite different from all other amino acids under study when M2 was used as eluent, S2-M2 and S1-M2 TLC systems were identified as the most favorable TLC system for selective separation of glutamic acid and tryptophan from the complex mixture containing amino acids of different

nature. When the mixture of glutamic acid and other amino acids under study were chromatographed with S2-M2 TLC system, other amino acids moved from the origin (point of application) to the middle of the plate($R_F$= 0.56) leaving glutamic acid near the origin($R_F$=0.26).Thus, glutamic acid was selectively separated from other amino acids. It seems that amino acids exists as ion- pairs, i.e., in a molecular form during the separation process. Similarly, tryptophan was selectively separated from other amino acids on S1-M2 TLC system. But here tryptophan moved up to the middle of the plate($R_F$=0.54) while mixture of all amino acids moved up to the top of the plate($R_F$=0.82) with the eluant.

As a result of the differential migration of amino acids on S1-S3 layers with M1 andM2 eluents, various combinations of amino acids were resolved from their complex mixtures. The experimentally achieved separations of amino acids belonging to the same group or different groups have been summarized in Table 4.

**Table 4: Separations of amino acids experimentally achieved on different stationary phases (S.P) with various mobile phases (M.P).**

| S.P | M.P | Amino acids separated | Remarks |
|---|---|---|---|
| Silica gel | 40% dextrose | Valine(0.83)- Leucine (0.63),Valine(0.84)- Norleucine(0.67), Valine(0.84)- Isoleucine(0.71) , Alanine(0.95)- Proline(0.65) , Alanine(0.95) - Leucine(0.67), Alanine(0.95) - Norleucine(0.66) , Alanine(0.93) - Isoleucine(0.65), Alanine(0.96) - Valine(0.84), Alanine(0.95) - Methionine(0.77) | Intra group separation of non polar amino acids |
| Silica gel | 40% dextrose | Glutamic acid(0.98)- Leucine (0.65), Glutamic acid(0. 96) - Norleucine(0.66), Glutamic acid(0. 97) - Isoleucine(0.72), Glutamic acid(0. 97) - Valine(0.85), Glutamic acid(0. 95) - Methionine(0.76), Glutamic acid(0. 97) – Proline(0.66) | Inter group separation of acidic amino acids from non polar amino acids |
| Silica gel | 40% dextrose | Serine(0.94) -Proline(0.68), Tyrosine(0.93) - Proline(0.65), Serine(0.93) - Leucine (0.67), Serine (0.95) -Norleucine(0.65), Serine(0.93) - Isoleucine(0.72), Serine(0.94) - Valine(0.80) Serine(0.93)- Methionine(0.76) | Inter group separation of non polar amino acids from polar amino acids |
| Alumina | 40% fructose | Glutamic acid(0.98)- Other amino acids(0.56) | Selective separation of glutamic acid from other amino acids |
| Alumina | 40% dextrose | Leucine (0.54)- Tryptophan(0.25), Leucine (0.55)- Tyrosine(0.33), Leucine (0.58)- Serine(0.27), Norleucine(0.55)- Serine(0.28), Isoleucine(0.50)- Serine(0.28), Valine(0.60)- Serine(0.30), Valine(0.55)- Tyrosine(0.35) , Isoleucine(0.57)- | Inter group separation of non polar amino acids from polar |

| | | | |
|---|---|---|---|
| | | Tyrosine(0.32) , Norleucine(0.55)- Tyrosine(0.36), Valine(0.58) – Tryptophan(0.26),Isoleucine(0.61)- Tryptophan(0.23),Norleucine(0.56)- Tryptophan(0.25) | amino acids |
| Cellulose | 40% fructose | Tryptophan(0.54)- Other amino acids(0.82) | Selective separation of tryptophan from other amino acids |
| Alumina | 40% dextrose | Leucine (0.58)- methionine(0.34) , Norleucine(0.55)- methionine(0.35), Isoleucine(0.59)- methionine(0.37), Valine(0.59)- Methionine(0.39) | Inter group separation of acidic amino acids from non polar hydrophobic amino acids |

The lowest possible detectable amounts of tryptophan and glutamic acid with S1-M2 and S2-M2 TLC systems were 0.15µg and 0.20 µg/spot, respectively.

Stability is an important and essential aspect of validation in thin layer chromatographic analysis. Highly sensitive samples should not decompose during development of the chromatogram and should be stable in solution and on the adsorbent. The intensity of spots on the chromatogram should be constant at least for 1h. No significant change in the intensities of the spots was observed after storage of developed plates, protected from light for periods up to 72h, indicating good stability of the mixture on the adsorbent.

Because no change in the $R_F$ values of the spots was observed when $R_F$ values of spots obtained from the freshly prepared solutions were compared with those obtained on 30 consecutive days, it was concluded that the solutions were sufficiently stable.

Another important property of the method is its reproducibility, defined as the precision under different conditions, for example when the method was performed by different analysts and by the same analyst on different days. The variation in $R_F$ values of individual amino acids measured by three different analysts and by the same analyst on three different days did not differ by more than 0.15(±15%) from the average $R_F$ value indicating a good reproducibility.

*Effect of surfactant in the mobile phase*
Surfactant was found to have a positive effect on the mobility of amino acids when present in mobile phase. CTAB enhanced the mobility of all amino acids by 1.5-11% [35].

*Effect of metals*
No significant effect on the mobility of amino acids was observed with any of the metal solution in the mobile phase.

*Effect of organic additives*
Organic additives increased the mobility of all amino acids up to 10%.This may be due to the increased solubility of amino acids in organic solutions.

## Material and Methods

***Experimental:*** All experiments were performed at 30 ± 1 °C.
*2.1 . Apparatus*
A thin layer chromatographic applicator (Toshniwal, India), 20cm x 3cm glass plates and 24cm x 6cm glass jars were used for the development of chromatographic plates. A glass sprayer was used to spray reagent on the plate to detect the spot. Tripette (Werthlim, Germany) was used for spotting of analyte.

*2.2. Chemicals and reagents*
Silica gel 'G', alumina (neutral) 'G', cellulose 'G', N-cetyl N,N,N-trimethyl ammoniumbromide (CTAB), potassium nitrate, cadmium nitrate, aluminium nitrate and amino acids (CDH, India), maltose, fructose, sucrose, dextrose, lactose propanol, acetone, and acetonitrile (Merck, India) were used.

*2.3. Amino acids studied*

| **Amino acids** | **Mol. wt.** | **Extra ionizable group present** | **$pK_1$ of α-COO- group** | **$pK_2$ of α-$NH_3$- group** | **$pK_3$ of extra ionizable group** | **pI** |
|---|---|---|---|---|---|---|
| **Non-polar amino acids** | | | | | | |
| Leucine(A1) | 131 | | 2.4 | 9.6 | | 6.0 |
| Norleucine (A2) | 131 | | 2.4 | 9.6 | | 6.0 |
| Isoleucine (A3) | 131 | | 2.4 | 9.7 | | 6.1 |
| Valine(A4) | 117 | | 2.3 | 9.6 | | 6.0 |
| Proline(A5) | 115 | Pyrollidine | 2.0 | 10.6 | | 6.3 |
| Alanine(A6) | 85 | | 2.3 | 9.7 | | 6.0 |
| Tryptophan (A7) | 204 | Indole group | 2.4 | 9.4 | | 5.9 |
| Methionine (A8) | 149 | Thioether group | 2.3 | 9.2 | | 5.8 |
| **Polar uncharged** | | | | | | |
| Serine(A9) | | Hydroxyl group | 2.2 | 9.2 | | 5.7 |
| Tyrosine (A10) | | Phenol | 2.2 | 9.1 | 10.1 | 5.7 |
| **Acidic** | | | | | | |
| Glutamic acid(A11) | | γ-carboxyl group | 2.2 | 9.6 | 4.3 | 5.9 |

*2.4. Test solutions*

The test solutions (1% w/v) of all the amino acids under study were prepared by dissolving appropriate weight (0.1 g) in double distilled water (10 ml).

*2.5. Detector*

Amino acids were detected using 0.3 %( w/v) ninhydrin solution prepared in acetone.

*2.6. Stationary phases*

The stationary phases investigated were S1 (Cellulose 'G'), S2 (Alumina 'G') and S3 (Silica gel 'G').

*2.7. Mobile phases*

40% aqueous solutions of carbohydrates viz; dextrose, fructose, maltose, sucrose and lactose and 1:1 v/v mixtures with aqueous solution of surfactant, aqueous solutions of metal salts and organic solvents were used to investigate the mobility trend of amino acids.

| S.No | Mobile phase | Composition |
|---|---|---|
| 1 | Aqueous carbohydrates solution | 40% aqueous solutions of dextrose(M1), fructose (M2), sucrose (M3), maltose(M4) and lactose(M5) |
| 2 | Mixed aqueous carbohydrates-metal salt solution | M1 + 5% $KNO_3$(M6) , M1 + 5% $CdNO_3$(M7), M1 + 5% $Al_2NO_3$(M8) |
| 3 | Mixed aqueous carbohydrates- organic solution(1:1) | M1 + propanol, M1 + acetone, M1 +acetonitrile |
| 4 | Mixed aqueous carbohydrates-surfactant solution | M1 + CTAB |

*2.8. Preparation of TLC plates*

A TLC applicator (Toshniwal, India) was used to coat 20cm x 3cm glass plates with the stationary phases. The desired stationary phase (20g) was homogenized with 60 ml double distilled water by constant shaking for 5 min and the resulting slurry was coated immediately onto 20cm x 3cm glass plates as a 0.25mm layer by means of a TLC applicator. The plates were dried at room temperature. After drying, the plates were activated by heating at 100 ± 1 $^0$C for 1h in an electrically controlled oven. After activation, the plates were cooled to room temperature and then stored in a closed chamber (30 $^o$C) before use.

*2.9. Procedure*

Thin layer chromatography was performed on silica gel, alumina and cellulose layers in glass jars. Test solutions (0.050 µl) were applied by means of a tripette (Werthlim, Germany) 2cm above the lower edge of the plates. The spots were dried at room temperature (30$^o$C). Chromatography was performed in 24cm x 6cm glass jars with lids. Chambers were presaturated

with mobile phase vapour for 10min. before introducing the plates for development. The plates were developed in the chosen solvent system by the ascending technique. The solvent ascent was fixed 10cm from the point of application in all cases. After development, the plate was withdrawn from glass jars and dried at room temperature, and a glass sprayer was used to apply the detector (ninhydrin) to the plates to locate the positions of analyte spots. All amino acids except proline appeared as violet spots on heating TLC plates for 15-20min. at 60 $^{o}$C, proline appears as yellowish spot. The retention sequence of amino acids by different stationary phases under different experimental conditions was measured in terms of retardation factor (i.e. $R_F$ value). For determining $R_F$ value, the $R_L$ ($R_F$ of leading front) and $R_T$ ($R_F$ of trailing front) values for each spot were determined and overall the $R_F$ value was calculated as

$$R_F = (R_L + R_T) \times 0.5$$

For the mutual separation of closely related amino acids, equal volumes of amino acids to be separated were mixed and 0.050 µl of the resultant mixture was loaded on TLC plates. The plates were developed to 10cm height with 40% aqueous dextrose solution (M1), the spots were detected and the $R_F$ values of the separated amino acids were determined.

*2.10. Limit of detection*

The limit of detection of glutamic acid and tryptophan was determined by spotting different amounts of these amino acids on the TLC plates coated with alumina (S2) and and cellulose 'G' (S1), developing the plates with M2 mobile phase and detecting the corresponding spots. The method was repeated with successively decreasing amounts of tryptophan and glutamic acid until no spot was detected. The minimum detectable amounts of amino acids were taken as limit of detection.

*2.11. Chromatographic parameters*

The stability of the colour intensity of amino acid spots on the chromatogram was observed visually. The developed chromatoplates were protected from light and the spot intensities were compared after every 12h for a period of 100h.

To investigate the stability (ageing effect) of mixture of amino acids under study, the mixture was chromatographed using M1 – S3 system after every 24h for the duration of 10 days. The $R_F$ values of amino acids so obtained after every 24h interval were compared with the values obtained from the freshly prepared mixture of amino acids.

The reproducibility of $R_F$ values was checked by determining the $R_F$ of the same sample by three independent analysts and by the same analyst on different days under identical experimental conditions, in the same laboratory, using the same apparatus.

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