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Thin Layer Chromatography of Amino Acids

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ABSTRACT

This review involves the thin layer chromatography of amino acids. Chromatography is by far the most useful general group of techniques available for the separation of closely related compounds in a mixture. Here the separation is effected by differences in the equilibrium distribution of the components between two immiscible phases, viz., the stationary and the mobile phases. Amino acids are generally considered the building blocks of proteins. Thin layer chromatography (TLC) is generally regarded as a common analytical method for screening, separation, and preliminary identification of compounds. TLC plays an important role in the quality control of food and drugs in order to investigate the ingredients and to detect the impurities and also for checking the purity and the stability of preparations. Amino acids also serve as an energy source, especially in times of starvation. Proline is one of the amino acid used as an energy reserve in blood-feeding insectsandincertainblood-feedingprotozoanparasites.Inthecaseofplants, these compounds are essential for the biosynthesis of numerous endogenous nitrogenous compounds. Thin-layer chromatography is a versatile separation technique and is compatible to almost all types of compounds available in nature. This review presents numerous chromatographic systems developed for the analysis of amino acids. The stationary phases, solvent systems, and detection reagents used by the various chromatographers are incorporated in this review. From the available literature, it is obvious that amino acids have been greatly analyzed by thin-layer chromatography.

Keywords: *Amino acids, Thin layer chromatography, Mobile Phase, Stationary phase, Analysis, Detection, Separation.*

INTRODUCTION

Amino acids are generally considered the building blocks of proteins. Amino acids are used in the manufacturing of many industrial products such as in food technology as a flavor enhancer, as well as in productionof biodegradable plastics, drugs, and chiral catalysts. The determination of amino acids from biological samples is very important because it highlights some anomalies correlated to various metabolic illnesses. Several chromatographic methods have been developed to meet the challenges of environmental mutations [1]. Because of the simplicity of the technique for the analysis of various useful and unwanted materials in the environmental samples, thin-layer chromatography (TLC) has a privileged position, especially in the field of biochemical analysis. These analyses provide solutions for some questions in the diagnostic therapy and, on the other hand, detect some biochemical modifications of diseases at the cellular level [2]. Thin-layer chromatography is a fast, inexpensive, and versatile separation technique with many practical considerations that contribute to its effectiveness. Thin layer chromatographic (TLC) technique readily provides qualitative information and with careful attention to details, it is possible to obtain quantitative data. Thin layer

chromatography is a technique used to separate and identify compounds of interest. A TLC plate is made up of a thin layer of silica adhered to glass or aluminum for support. The silica gel acts as the stationary phase and the solvent mixture acts as the mobile phase. In the ideal solvent system the compounds of interest are soluble to different degrees. Separation results from the partition equilibrium of the components in the mixture.In the simplest form of the technique, a narrow zone or spot of the sample mixture to be separated is applied near one end of the TLC plate and allowed to dry. The strip or plate is then placed with this end dipping in to the solvent mixture, taking care that the sample spot/zone is not immersed in the solvent. As the solvent moves towards the other end of the strip, the test mixture separates into various components. This is called as the development of TLC plates. The separation depends on several factors; (a) solubility: the more soluble a compound is in a solvent, the faster it will move up the plate. (b) attractions between the compound and the silica, the more the compound interacts with silica, the lesser it moves, (c) size of the compound, the larger the compound the slower it moves up the plate. The plate is removed after an optimal development time and dried and the spots/zones are detected using a suitable location reagent. An important characteristic used in thin layer chromatography is Rf value [3].

The plate is removed after an optimal development time and dried and the spots/zones are detected using a suitable location reagent. An important characteristic used in thin layer chromatography is Rf value [4].

$$Rf = \frac{\text{distance moved by the substance from origin}}{\text{distance moved by solvent from origin}}$$

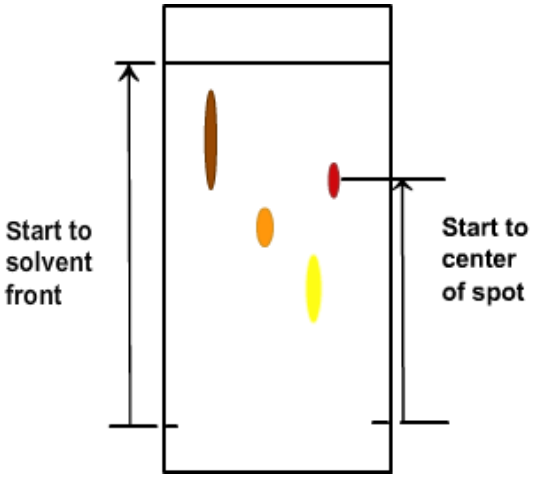

Fig. 1. Separation of Amino Acids

The isolation of amino acids can be carried out by different extraction methods from dry materials. The following solvents have been previously utilized for the extraction of amino acids: 5% NaCl solution, 75% ethyl alcohol, 0.25% NaOH, 0.25M HCl, meta siliconic acid or a CH3COOH HClH2O (18:1:1v:v:v) mixture. Hodisan *et al*. [6] extracted 0.5g dry plant in 10mL 1% HCl solution. The proteins were then removed from the extract by precipitation with a Na3P (W3O10)4 solution. After centrifugation, the solution was passed through an ion exchange Amberlite IR 120H column. The column was eluted with 40mL 10% ammonia solution [5-6]. The solution obtained was evaporated to dryness and the residue was redissolved in 1mL aqueous 30% iso-propanol (v:v). Maless *et al*. [7] have extracted amino acids from air-dried, powdered flowering tops of Hypericum taxa (1.0g) by heating under refluxing with water (10.0mL) for 60min and then filtered. The filtrate was concentrated under reduced pressure and the residue was dissolved in water (5.0mL) [8].

Chromatographic Separation of Amino Acids

The present experiment employs the technique of thin layer chromatography to separate the amino acids in a given

mixture.All 20 of the common amino acids [standard amino acids] are a-amino acids. They have a carboxyl group and an amino group bonded to the same carbon atom (the α- carbon). They differ from each other in their side chains, or R groups, which vary in structure, size, and electric charge. The interaction of the amino acids with the stationary phase like silica varies depending on their 'R' groups. The amino acid that interacts strongly with silica will be carried by the solvent to a small distance, whereas the one with less interaction will be moved further. By running controls [known compounds] alongside, it is possible to identify the components of the mixture [9].

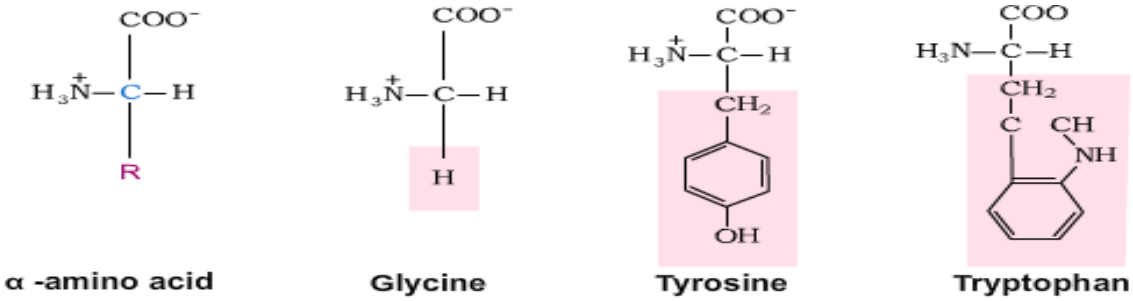

Fig. 2. Structure of Amino Acid

Since amino acids are colourless compounds, ninhydrin is used for detecting them. To identify this, after development, the TLC plate is sprayed with ninhydrin reagent and dried in an oven, at 105°C for about 5 minutes. Ninhydrin reacts with α- amino acids that results in purple coloured spots. Rf values can be calculated and compared with the reference values to identify the amino acids.

MATERIALS REQUIRED

Reagents

1) 2% solution of individual amino acids.
2) Solvent mixture of normal butanol, acetic acid and water in the ratio 12:3:5 by volume.
3) Ninhydrin reagent.

Requirements

1) TLC plate
2) TLC chamber
3) Capillary tubes
4) Reagent spray bottle
5) Conical flasks
6) Beakers

PROCEDURE

1) Pour the solvent mixture in to the TLC chamber and close the chamber.
2) The chamber should not be disturbed for about 30 minutes so that the atmosphere in the jar becomes saturated with the solvent.
3) Cut the plate to the correct size and using a pencil (never ever use a pen) gently draws a straight line across the plate approximately 2 cm from the bottom.
4) Using a capillary tube, a minute drop of amino acid is spotted on the line.

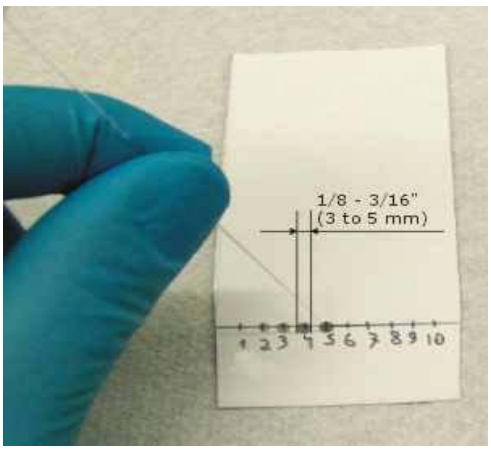

Fig. 3. Capillary Tube Method

1) Allow the spot to dry.
2) Spot the second amino acid on the plate [enough space should be provided between the spots].
3) Repeat the above step for spotting the unknown acid.
4) Place the plate in the TLC chamber as evenly as possible and lean it against the side (immerse the plate such that the line is above the solvent). Allow capillary action to draw the solvent up the plate until it is approximately 1 cm from the end.
5) Remove the plate and immediately draw a pencil line across the solvent top.
6) Under a hood dry the plate with the aid of a blow dryer.
7) Spray the dry plate with ninhydrin reagent.
8) Dry the plates in hot air oven at 105°C for 5 min. [Ninhydrin will react with the faded spots of amino acids and make them visible as purple coloured spots.]

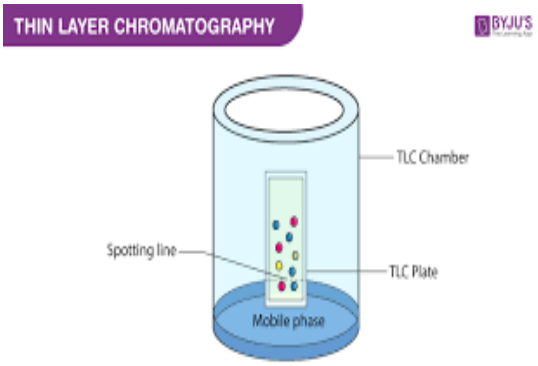

Fig. 4. Thin Layer Chromatography

After some time, mark the center of the spots, then measure the distance of the center of the spots from the origin and calculate the Rf values.

Amino acids	Cold condition		After final heating		Detection limit for ninhydrin (µg) (R_F)
	Observed colors	Detection limit (µg)	Observed colors	Detection limit (µg)	
Glycine	Deep orange	0.5	Deep pink	0.1	0.01 (0.03)
Alanine	Pinkish violet	0.1	Light pink/milky pink	0.1	0.009 (0.22)
Valine	Reddish pink	0.1	Reddish pink	0.05	0.01 (0.14)
Leucine	Bluish violet	0.5	Violetish pink	0.1	0.01 (0.09)
Isoleucine	Very light violet	1.0	Light violet	0.1	0.20 (0.32)
Serine	Deep pink	0.1	Deep bluish pink	0.1	0.008 (0.38)
Threonine	Yellowish orange/ivory	0.5	Yellowish pink/candy	0.1	0.05 (0.28)
Aspartic acid	Yellowish violet	0.2	Greyish violet	0.1	0.10 (0.12)
Aspargine	Light yellow/pale cream	1.0	Greyish yellow	0.1	0.10 (0.45)
Glutamic acid	Light violet	0.5	Light violet	0.1	0.04 (0.33)
Glutamine	Light violet	0.5	Light violet	0.2	0.10 (0.38)
Lysine	Reddish violet	0.2	Brick red	0.1	0.005 (0.42)
Histidine	Yellowish violet	0.1	Yellowish pink/petal	0.1	0.05 (0.18)
Arginine	Light pink/mauve	0.5	Pink	0.1	0.01 (0.05)
Phenyl alanine	Orangish violet	1.0	Greyish pink	0.2	0.05 (0.58)
Tyrosine	Light violet	1.0	Light pink	0.1	0.03 (0.51)
Tryptophan	Greyish violet	0.5	Pinkish violet	0.1	0.05 (0.55)
Cysteine	Yellowish violet	2.0	Pinkish violet	1.0	0.02 (0.41)
Cystine	Very light pink	2.0	Light pink	1.0	0.01 (0.35)
Methionine	Lilac/bluish violet	0.5	Bluish violet	0.2	0.01 (0.48)
Proline	Light yellow/off white	1.0	Grey/beige	0.2	0.10 (0.22)
Hydroxy proline	Pinkish violet	0.2	Yellowish brown	0.1	0.05 (0.34)

Analyte	***Stationary Phase***	***Mobile Phase***	***Remark***
Aminoacids	*Silica*	*1-butanol-acetone-acetic acid-water (7:7:2:4)*	*Analysis of the resulting third-order data using a direct tri-linear decomposition (TLD). Monitoring the kinetic profile of the reaction with a charge-coupled device camera by taking sequential images of the separation medium after the reaction starts. Detection by dipping into a solution of 0.05% o-phthalaldehydeinmethanol, containing 0.2%2-mercaptoethanol and 0.09% Brij-35, and under UV366 nm.(4)*
Aminoacids	*Tin(IV)selenoarsenatelayers, silica-tin(IV)selenium arsenate(1:1)layersand silica-calciumsulfatelayers*	*Dimethylsulfoxide*	*Thin-layer chromatography of a-amino acids was studied on different layers. Detection by spraying within hydrin.(5)*
Amino acids	*Zeolite-cellulose (1:1)*	*Water-saturated phenol ethanol-water-acetic acid (12:4:4:1)*	*Investigation of the precision, detection limit and limit of quantification for each method was carried out on laboratory prepared plates. Detection by spraying with 1% ninhydrin solution in I-butanol and heating at 80℃ for 20min. Determination by visible reflectance spectrometry, image analysis and densitometry(6)*
L-tryptophan	*Silica*	*2-propanol-NH3(25%) (7:3)*	*Quantitative analysis of L-tryptophan was performed by densitometry at 625 or 276nm. Tryptophan was detected by spraying or dipping with=into 4- (dimethyl amino) benzaldehyde solution. (6)*
Amino acids	*cellulose-silica (5:2)*	*Isopropanol-ethylacetateacetone-methanolisopentanol-NH3-water (9:3:3:1:1:3:3)and butanol-acetone isopropanol-formic acid-water(18:8:8:3:6)*	*Study of 17 amino acids in Bos grunniens Linnaeus horn hydrolysate was performed by Two-dimensional TLC. Detection by spraying with 0.5%ninhydrininacetone. (7)*
Amino acids (enantiomer)	*Silica*	*Acetonitrile-water(2:5) containing2-o-[(R)2-hydroxypropyl]-CD as additives.*	*Separation of enantiomers of six selected amino acids using 2-o-[(R)-2-hydroxypropyl]—CD as the mobile phase additive. Detection by spraying with a solution of 1.5 g salicyl aldehyde in 100mL toluene and heating at 50℃ for 10 min. (8)*
Amino acids	*Silica gel*	*1, Chloroform-methanol-NH3 conc.(2:2:1)2,phenol-water (3:1).*	*Identification of tryptophan and histidine by FT-SERS. Detection under UV 254nm. Detection limit was 8 mg.*

The data presented in this review indicates that amino acids have been extensively analyzed by thin-layer chromatography. Further, we have divided this section into the subsections to make it more informative for the researchers and readers.

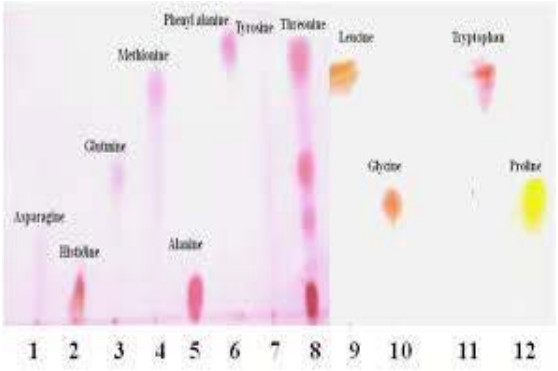

Fig. 5. Analysis of Amino Acids using Thin Layer Chromatography

Detection of Amino Acids: Various Derivatization Methods

A great variety of detection reagents (Table) are used in the detection of amino acids. Among them, the most frequently used is ninhydrin. Despite its common use, ninhydrin indicates decreased sensitivity toward some amino acids such as proline and hydroxyl proline. In order to enhance the sensitivity in detection, an iodine azide reaction has been proposed for the detection of amino acids.[60] This method is based on the visual observation of the plate after spraying it with a freshly prepared solution of sodium azide and then exposure to iodine vapors. The sulfur compounds can only induce this reaction. The sulfur was introduced into the amino acids by derivatization with the phenyl isothiocyanate. The derivatization reaction with phenyl isothiocyanate (PITC) was carried out directly on the plate before the developing step. Subsequently, the plates were sprayed with a mixture of sodium azide and starch solution in NP-TLC, and, in the case of RP-TLC, a sodium azide solution with starch was incorporated into the mobile phase and then exposed to iodine vapor. The spots became visible as white spots on violet-grey background. The iodine-azide system has been proven to be the most favorable and enabled the detection of quantities per spot in the range of 1–60pmol (HPTLC) and 3–100pmol (TLC). Wawrzycki *et al*. [61] have synthesized 4-diethylaminodiazabenzene-4-isothiocyanate (DEA-BITC) and then used it to obtain thiohydantoin derivatives of 15 a-amino acids (DEABTH-AMK). The colored derivatives were separated by means of TLC on silica gel using various solvent systems. The effects of the reagent structure on the chromatographic separation of the selected protein amino acids in the form of their thiohydantoin derivatives, and the selectivity of the chromatographic separation with regard to free amino acids was studied.

On the basis of color selectivity, it is reported that ninhydrin is used widely because of its high sensitivity, but produces same colors, purple-violet, for all amino acids except proline and hydroxylproline. To overcome this problem, Samanta *et al*.[44] have introduced 2,3- dichloro-1,4-naphthaquinone and isatin asa new reagent that produces distinguishable and stable colors (more or less stable for 24 to 48hr) with many amino acids. The chromatography was performed on silica gel plates with n-propanol–water, 70þ30 (v=v), as the mobile phase.

After development, plates were dried and sprayed with 0.25% 2,3-dichloro-1,4-naphthaquinone in ethanol (Reagent 1), kept at room temperature in air until the ethanol had evaporated completely, heated in an oven at 110C for 10min, cooled, and sprayed with 0.4% is at in ethanol (Reagent 2). The plates were again dried in air and the colors were noted. The plates were then heated at 110C for 10min in an oven and the colors were recorded again. This method enables easy detection with

high sensitivity and the detection limits were between 0.01 and 0.3mg.(1)

Chromatographic Systems (Layers and Eluents)

The reported findings indicate that thin-layer chromatography greatly contributes in the analysis of amino acids from various natural as well as manmade products. From Table 1, it is clear that a variety of stationary phases have been used for the analysis of amino acids. Both polar as well as non-polar static phases have been utilized for the identification and separation of amino acids. Various impregnated layers have also been used for the analysis. The layers were impregnated mainly with metal ions and also with few chiral agents. On the other hand, a large number of mobile phases have been used for the analysis of amino acids. It is obvious that most of the solvent systems used are harmful to the environment. Keeping in mind the hazardous natures of various chemicals used as solvent systems, chromatographers are leading toward green technology. The use of surfactants (less toxic) is the absolute solution for green technology. Several methods developed by the use of surfactants are mentioned in this review.

CONCLUSION

Thin-layer chromatography continues to be an important method for qualitative and quantitative analysis of amino acids because of its inherent advantages such as many samples can be analyzed simultaneously and quickly. Numerous chromatographic methods have been used, including high-performance liquid chromatography (HPLC) and gas chromatography (GC). These techniquesare, however, usually time-consuming and require expensive equipment. For these reasons TLC has a privileged position due to its simplicity, convenience, and cost-effectiveness for separation of amino acids. These methods do not meet the demands of high throughput detection of amino acids, because expensive equipment and tedious sample preparations have to be involved in the experiment. TLC can also be employed to detect amino acids and needs no expensive equipment or tedious sample pretreatments and allows a parallel separation of many samples. The absence of a need for UV activity (as in LC), paramagnetic properties (as in NMR), or volatility (as for GC), makes TLC one of the most powerful and general analytical tools for the analysis of amino acids. TLC is considered to be the most applicable technique for the separation of enantiomer amino acids as compared to HPLC, because the amino acids can be visualized directly onto layers after evaporation of the mobile phase; whereas, in the case of HPLC, the ultraviolet (UV) absorbance of chiral additives in the mobile phases causes detection problems.

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