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Research Article

DLLME Extraction of Amino Acids from Blood Sample and its Profiling with Special References to Essential and Non-Essential Amino Acid by Thin Layer Chromatography.

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Abstract

Micro extraction technique, Dispersive Liquid-Liquid Micro Extraction (DLLME) is used for extracting amino acids from blood sample. Solubility of amino acids are checked in various solvent and tertiary mixture of mobile phase (1butanol: acetic acid: water) in ratio 8:2:2 is optimized and used for separating amino acid. Distinct different resolved R_f values were recorded for both blood samples and standard amino acids. By comparing the R_f values of the standard amino acids, phenyl alanine, valine and Aspartic acid were confirmed to be present in the blood samples. DLLME is a simple, rapid; environment friendly method is coupled with Thin Layer Chromatography (TLC). TLC is a simple chromatographic technique for separation, when combined with DLLME, the recovery and reproducibility of the method is found to be increased. Ninhydrin spray is used for chemically visualizing the amino acids. In this study better extraction efficiency that is 95% is achieved in comparison to conventional liquid-liquid micro extraction.

Keywords: Dispersive Liquid-Liquid Micro Extraction (DLLME); Thin Layer Chromatography (TLC); Non-Essential Amino Acid; Blood Sample; R_f Values

Introduction

Amino acid is the building block of protein, which are organic compound containing amine and carboxyl functional group along with a side chain for specific each amino acid. There are 20 amino acid classified into two groups based on their requirement, among twenty-nine amino acids are essential amino acids which cannot be synthesized in the body and comes through food and nutritional supplement in few cases. There are eleven amino acids are non-essential which are synthesized in the body of an individual. When a person consumes any toxic or poisonous substances there occurs a change in metabolism of amino acids. Sometime it is observed that metabolism of amino acid come to a halt and many proteins are found to be denatured. Essential amino acids are used in various therapeutic purposes as nutritional supplement, when consumed in excess leads to poisoning. An attempt is made to identify the change in metabolism of amino acid and for profiling purpose; blood sample of those individuals and control samples were collected and analyzed. A simple thin layer Chromatography has been performed coupled with micro extraction technique DLLME. An identification factor in TLC is Retardation Factor or R_f Value. Rf value is defined as distance moved by the substance from origin divided by distance moved by the solvent from origin.

In TLC the separation depends on several factor

a. Solubility: The more soluble a compound the faster it will move up the plate.

b. Attraction between the compound and silica: The more the compound interacts with silica the lesser it moves.

c. Size of the compound: Larger the compound slower it moves it up to plate. The solubility of amino acid was checked and optimized mobile phase in TLC for better separation of amino acids. This method being a simple, rapid and inexpensive can be used in toxicology laboratory with minimum infrastructure.

Out of many techniques available for micro extraction, Dispersive Liquid-Liquid Micro Extraction has attracted the interest of forensic toxicology in recent year. It is the micro extraction procedure, used for extraction of amino acids from the post mortem blood samples. Blood samples are subjected for amino acids profiling by TLC. DLLME is simple, fast, inexpensive solvent micro extraction technique when compared with other methods and it consume a less amount of organic solvent. This DLMME extraction method is coupled with TLC is very much useful for rapid screening; the method is less time consuming and fast and thereby minimizing experiment time.

Methods and Materials

All the reagents were of analytical grade and standard reference material were procured from sigma Aldrich pvt ltd. Silica gel 60F-254 pre-coated TLC aluminum plates (20×20 cm, 0.25mm layer thickness) were procured from Merck, Germany. Acetonitrile, acetone, methanol, butanol, acetic acid, Millipore water were used in the extraction and thin layer chromatography method as a mobile phase was procured from Merck and qualigens. The standard solutions of each amino acid were prepared in Millipore water in 1ppm to 10 ppm range for LOD and LOQ measurement. Mini centrifuge (Tommy) is used for centrifugation. Cyclomixer (Remi) is used for mixing the sample.

Extraction of Blood Sample

A new simple micro extraction technique was developed for determination of amino acid from the blood samples and control samples. The amino acids were extracted by DLLME based on separation of blood plasma and deposition of the cell extracts.

Procedure

A mixture of 1ml blood sample, 1ml of methanol is added and shaken well. Then 1ml of Acetonitrile is added to the tube. Then the blood sample was centrifuged at 3000 rpm for 5 to 6 minutes. After centrifugation pellets that is the cell extracts are found to be settle at the bottom, and the supernatant is found floating at the top of the reaction mixture. After that supernatant is separated and air dried and reconstituted with methanol and spotted on the TLC plates along with the amino acid standard.

Thin Layer Chromatography (TLC) Procedure

We used Aluminum support pre-coated silica coated TLC plates 20*20 cm. To activate the silica, plates are heated at 1100c on the TLC activator (camag) for 30 minutes. Then the chamber is washed and rinsed with the newly prepared solvent that is (1 butanol, acetic acid, dist, water) in the ratio of 8:2:2 that is 4:1:1. Then the chamber is saturation with mobile phase and it is set to equilibrate with mobile phase for 30 minutes. Then on a TLC plates, 5µl of the extracted samples and standard amino acids were loaded equidistantly using capillary tubes and the plate is then kept inside the chamber and allow running for 10 cm. And when the solvent front reaches 10cm the plates are taken out and allowed to air dry at room temperature. Then the newly prepared .2% ninhydrin spray reagent is sprayed on the TLC plates in the spraying chamber TLC (Fume wood), Camag and the wet plate is air dried in a room temperature and slightly warmed in a TLC Activator, Camag and the spots appears on the plate and Rf values were recorded .

Results and Discussion

The R_f values of the amino acid standards were measured and the extracted samples amino acids Rf were also measured. The amino acids phenyl alanine, Valine and Aspartic acid were found to be more clear in the samples extracted blood samples and matches with the standard amino acid by their unique R_f value confirms the presence in the blood. This method is very rapid and sensitive and the LOD and LOQ were found to be 1-5mg and 5-10mg respectively was tabled in (Table 3).

Serial No.	Name of the amino acids	LOD	LOQ
1.	Valine	2-3mg	8-10mg
2.	Aspartic acid	2-3mg	8-10mg

Table 3: LOD and LOQ value.

Other amino acids were not detected because those amino acids were extracted in the form of different metabolites. According to the R_f values mentioned in the (Table 1), R_f value of phenyl alanine .63 is found to be as that of the spot 6 of sample 1 mentioned in (Table 2). In the same way the RF values of the valine and aspartic acid are .53 and .21 respectively are found to be same as that of the fourth spot of the sample 1, second spot of the same sample mentioned in (Table 4).

Name of Amino Acid	Classification	Spraying reagent	Rf value
Alanine	Non- essential	Ninhydrin	.33
Aspartic acid	Non-essential	Ninhydrin	.21
Glutamic acid	Non-essential	Ninhydrin	.32
Ornithine Mono hydrochloride	Non-essential	Ninhydrin	.10
Serine	Non-essential	Ninhydrin	.23
Glycine	Non-essential	Ninhydrin	.20
Proline	Non-essential	Ninhydrin	.30

D-L nor leucine	Proteinogenic	Ninhydrin	.65
D-L amino butyric acid	-	Ninhydrin	.42
Arginine	Non-essential	Ninhydrin	.15
Threonine	Essential	Ninhydrin	.27
Tryptophan	Essential	Ninhydrin	.70
Valine	Essential	Ninhydrin	.53
Lysine	Essential	Ninhydrin	.08
Histidine	Essential	Ninhydrin	.11
Isoleucine	Essential	Ninhydrin	.65
Leucine	Essential	Ninhydrin	.10
Methionine	Essential	Ninhydrin	.55
Phenyl alanine	Essential	Ninhydrin	.63

Table 1: R_f values of standard amino acid.

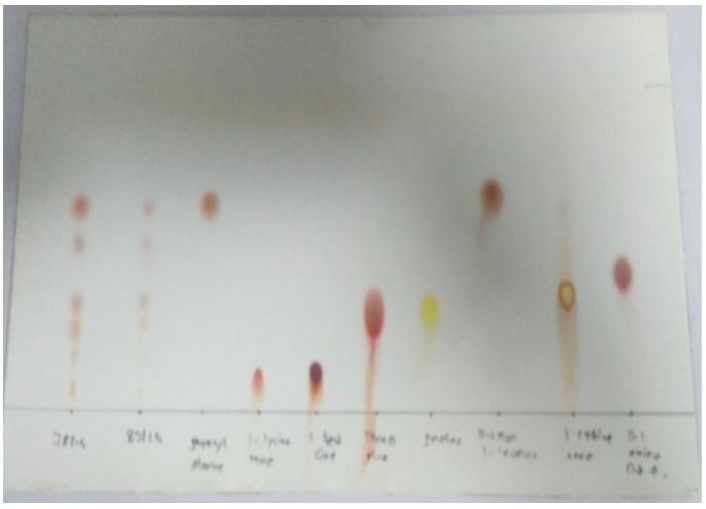
Sample1	Distance travel by solvent	R_f value
Spot1	6.7 cm	.04
Spot2	6.7cm	.15
Spot3	6.7cm	.23
Spot4	6.7cm	.32
Spot5	6.7cm	.50
Spot6	6.7cm	.59
Sample2		
Spot1	6.7 cm	.04
Spot2	6.7 cm	.16
Spot3	6.7 cm	.25
Spot4	6.7 cm	.33
Spot5	6.7 cm	.49
Spot	6.7 cm	.59
Sample1	Distance travel by solvent	R_f value
Spot1	7.5cm	.08
Spot2	7.5cm	.22
Spot3	7.5cm	.29
Spot4	7.5cm	.37
Spot5	7.5cm	.56
Spot6	7.5cm	.63
Sample2		
Spot1	7.5cm	.07
Spot2	7.5cm	.22
Spot3	7.5cm	.30
Spot4	7.5cm	.37
Spot5	7.5cm	.57
Spot6	7.5cm	.66

Table 2: R_f value of unknown blood sample.

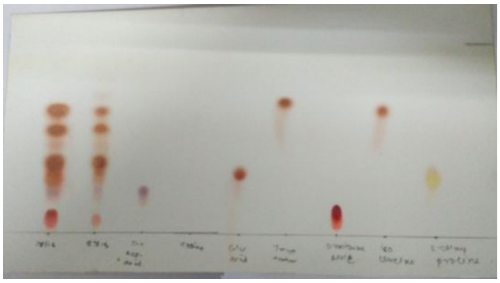
Sample1	Distance travel by solvent	R_f value
Spot1	6cm	.08
Spot2	6cm	.21
Spot3	6cm	.26
Spot3	6cm	.35
Spot4	6cm	.53
Spot5	6cm	.65
Sample2		
Spot1	6cm	.08
Spot2	6cm	.16
Spot3	6cm	.25
Spot4	6cm	.33
Spot5	6cm	.51
Spot6	6cm	.65

Table 4: The solubility of amino acids were checked in various solvents and the mobile phase that is the tertiary mixture of solvent (1 butanol: acetic acid: water) in the ratio of 8:2:2 were tried and optimized for separation of amino acids by the thin layer chromatography coupled with DLLME. DLLME is a very advanced process compared to the liquid-liquid extraction because DLLME is an environment friendly, less time taking and uses very little amount of solvent. The known amounts of amino acids were spotted with known loading capacity and 98% repeatability and 95% recovery were achieved in this method. The excessive intake of nine essential amino acids provided through nutritional supplements or diet leads to poisoning only two essential amino acid such as phenyl alanine and valine and non-essential amino acid, Aspartic acid could be extracted from the blood sample from the above mentioned procedure. In this study control blood samples from donors who are healthy human beings were also collected and subjected for extraction and identification. This method can be adopted in any forensic science laboratory for extraction and identification of amino acids.

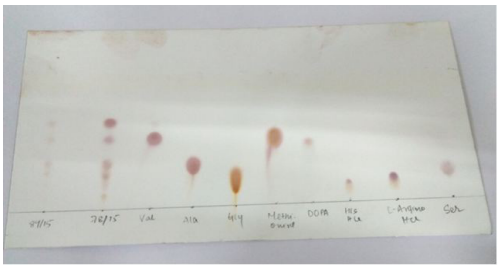
Picture of plate1



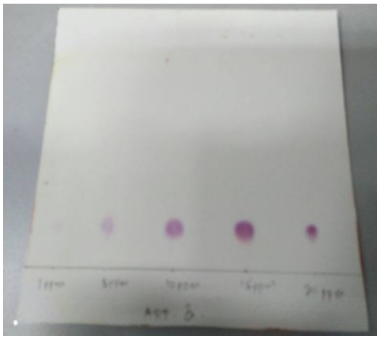
Picture of plate2



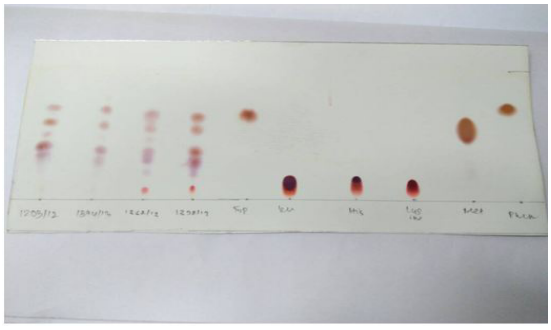
Picture of plate3



Picture of LOD and LOQ



Pictures of reference blood sample



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References

1. Bhushan R, Reddy GP (1989) Thin-Layer Chromatography of Dansyl and Dinitrophenyl Derivatives of Amino Acids. A review. Biomed Chromatogr 3: 233-240.
2. Bhushan R (1991) Amino Acids and Their Derivatives. Chromatograph Sci 55: 353-387.
3. Jain R (1996) Thin-Layer Chromatography in Clinical Chemistry. In: Fried B, Sherma J (1996) Practical Thin-Layer Chromatography-A Multi disciplinary Approach. CRC Press: Boca Raton, FL 131-152.
4. Bhushan R, Martens J (1996) Amino Acids and their derivatives. In: Sherma J, Fried B (1996) Handbook of Thin-Layer Chromatography. Marcel Dekker, Inc, New York 389-426.
5. Gołkiewicz W, Polak B (2007) Chiral Separation of Amino Acid Enantiomers. In: Kowalska T, Sherma J (2007) TLC in Chiral Separations and Analysis. Taylor & Francis Group, LLC 305-322.
6. Hodisan T, Culea M, Cimpoiu C, Cot A (1998) Separation, Identification and Quantitative Determination of Free Amino Acids from Plant Extracts. J Pharm Biomed Anal 18: 319-323.
7. Male Z, Plazibat M, Vundac VB, Zuntar I, Pilepic KH (2004) Thin-Layer Chromatographic Analysis of Flavonoids, Phenolic Acids, and Amino Acids in Some Croatian Hypericum Taxa. J Planar Chromatogr 17: 280-285.
8. Gui M, Rutan SC, Agbodjan A (1995) Kinetic Detection of Overlapped Amino Acids in Thin-Layer Chromatography with a Direct Trilinear Decomposition Method. Anal Chem 67: 3293-3299.
9. Siddiqi ZM, Rani S (1995) Studies of Tin (IV) Seleno arsenate III: Thin-Layer Chromatography of AminoAcids. J Planar Chromatogr 8: 141-143.
10. Petrovic M, Macon KM (1995) Validation of Quantitative Chromatographic Analysis on Laboratory Prepared Layers. J Chromatogr 704: 173-178.
11. Degterev E, Degtiar WG, Polanuer BM, Tyaglov BV, Kralov V, et al. (1996) Quantitative Analysis of L-tryptophan in Fermentation Broths. J Planar Chromatogr 9: 35-38.
12. Thin-Layer Chromatography of Amino Acids (2013) Malaysia.
13. Lei G, Cao Y (1996) Study on the Constituents of Bosgrunnienslinnaeus Horn by Thin-Layer Chromatography (Chinese). Chinese J Chromatogr 14: 158-160.
14. Ai Y, Lin H, Fu S, Xing M, Inoue Y (1995) Direct Thin-Layer Chromatographic Separation of Enantiomers of Six Selected Amino Acids Using 2-o-[(R)-2-hydroxypropyl]-CD as the Mobile PhaseAdditive. Anal Lett 28: 2041-2048.
15. Bhushan R, Ali I, Sharma SA (1996) Comparative Study of HPLC and TLC Separation of Amino Acids Using Cu(II) ion. Biomed Chromatogr 10: 37-39.
16. Malakhova II, Tyaglov BV, Degterev EV, Krasikov VD, Degtiar WG (1996) Quantitative Thin-Layer Chromatography of Industrial Amino Acids. J Planar Chromatogr 9: 375-378.

17. Bhushan R, Parshad V (1996) Thin-Layer Chromatographic Separation of Enantiomeric Dansylamino Acids Using a Macrocyclic Antibiotic as a chiral Selector. J Chromatogr A 736: 235-238.

18. Matysik G (1996) Separation of DABS Derivatives of Amino Acids by Multiple Gradient Development(MGD) in Thin-Layer Chromatography. Chromatographia 43: 301-303.

19. Demeglio DC, Svanberg GK (1996) A Thin Layer Chromatographic Procedure for the Separation of Proline and Hydroxyproline from Biological Samples. J Liq Chrom RT 19: 969-975.

20. Bhushan R, Martens J, Wallbaum S, Joshi S, Parshad V (1997) TLC Resolution of Enantiomers of Amino Acids and Dansyl Derivatives Using (1 R,3 R, 5 R)-2-azabicyclo [3,3,0]octan-3-carboxylic Acid as Impregnating Reagent. Biomed Chromatogr 11: 286-288.

21. Yuesong W, Leming L, Jun Z (1998) Prediction of the Thin-Layer Chromatographic Retention of Amino Acids. J Planar Chromatogr 11: 300-304.

22. Wang Y, Yu B, Wang T, Zhang X, Xu Z (1998) In situ Thin-Layer Chromatography - Fourier Transform- Surface - Enhanced Raman Scattering Spectroscopy of Amino Acids. (Chinese). Chin Anal Chem (Fenxi Huaxue) 26: 1047-1051.

23. Steiner RA, Fried B, Sherma J (1998) HPTLC Determination of Amino Acids in Snail-Conditioned Water from Biomphalaria glabrata, Two Strains of Helisomatrivolvis, and Lymnaeaelodes. J Liq Chrom RT 21: 427-432.

24. Bhushan R, Thuku Thiong'o G (2000) Separation of the Enantiomers of Dansyl-DL-amino Acids by Normal-Phase TLC on Plates Impregnated with a Macrocyclic Antibiotic. J Planar Chromatogr 13: 33-36.

25. Degterev EV, Degtiar WG, Tyaglov BV, Tarasov AP, Krylov VM, et al. (2000) Quantitative Analysis of L-tryptophan in Fermentation Broth. J Planar Chromatogr 13L: 191-194.

26. LeFevre JW, Gublo EJ, Botting C, Wall R, Nigro A (2000) Qualitative Reversed-Phase Thin-Layer Chromatographic Analysis of the Stereochemistry of D- and L-aminoAcids in Small Peptides. J Planar Chromatogr 13: 160-165.

27. Mohammad A, Agrawal V (2000) Micellar Thin-Layer Chromatographic Separation and Identification of Amino Acids: Separation of L-proline from Other Aliphatic and Aromatic Amino Acids. J Planar Chromatogr 13: 365-374.

28. Besant PG, Lasker MV, Bui CD, Turck CW (2000) Phosphohistidine Analysis Using Reversed-Phase Thin-Layer Chromatography. Anal Biochem 282: 149-153.

29. Malakhova II, Degterev EV, Tyaglov BV, Makarova SV, Krasikov VD (2001) Quantitative Determination of Industrial Essential Amino Acids in Fermentation Solution. Proc. Intern. Symp. on Planar Separations. Planar Chromatography 23-25 and Lillafurd, Hungary 2001:355-362.

30. Kowalski WJ, Nowak J (2001) Molecular Modeling of Planar Chromatographic Enantio separation of Amino Acids On Sorbents Containing Copper Complexes of l-hydroxyproline Derivatives: on Planar Separations. Planar Chromatography 2001: 45-54.

31. Pachuski J, Fried B, Sherma J (2002) HPTLC Analysis of Amino Acids in Biomphalaria glabrata Infected with Schistosoma mansoni. J Liq Chrom RT 25: 2345-2349.

32. Sharma SD, Sharma H, Sharma SC (2002) Normal- and Reversed-Phase Thin-Layer Chromatography of Amino Acids on Unconventional Supports with Mixed Mobile Phases Containing Dimethyl sulfoxide. J Planar Chromatogr 15: 371-376.

33. Zakrzewski R, Ciesielski W, Kazmierczak D (2002) Application of the Iodine-Azide Procedure for the Detection of Glycine, Alanine, and Aspartic Acid in Planar Chromatography. J Liq Chrom RT 25: 1599-1614.

34. Nowakowska J, Marszall M (2003) Retention Properties of Rosmarinic and Valerenic Acids on an Amino-Modified Hydrophilic Layer. J Planar Chromatogr 16: 369-373.

35. Ponder EL, Fried, B, Sherma J (2003) Thin-Layer Chromatographic Analysis of Free Pool Amino Acids in Cercariae, Rediae, Encysted Metacercariae, and Excysted Metacercariae of Echinostoma caproni. J Liq Chrom RT 26: 2697-2702.

36. Mohammad A, Agrawal V, Kumar S (2003) Use of Water-in-Oil Micro-Emulsion as Mobile Phase in Complexation TLC of Amino Acids on Silica Layers Impregnated with Metal Cations. J Planar Chromatogr 16: 220-226.

37. Nabi SA, Khan MA (2003) Selective TLC Separation of Lysine and Threonine in Pharmaceutical Preparations. Acta Chromatogr 13: 161-171.

38. Krasikov VD, Malakhova II, Degterev EV, Tyaglov BV (2004) Planar Chromatography of Free Industrial Amino Acids. J Planar Chromatogr 17: 113-122.

39. Khawas S, Panja D, Laskar SA (2004) New Reagent for Identification of Amino Acids on Thin-Layer Chromatography Plates. J Planar Chromatogr 17: 314-315.

40. Hess B, Sherma J (2004) Quantification of Arginine in Dietary Supplement Tablets and Capsules by Silica Gel High-Performance Thin-Layer Chromatography with Visible Mode Densitometry. Acta Chromatogr 14: 60-69.

41. Mohammad A, Hena S (2004) Use of Sodium Bis (2-ethylhexyl)sulfosuccinate) Anionic Surfactant Mobile Phase Systems in Thin-Layer Chromatography of Amino Acids: Simultaneous Separation of Thioamino Acids. Chromatography 25: 111-118.

42. Basak B, Bandyopadhyay D, Banerji A, Chatterjee A (2005) Use of Ninhydrin for Detection of Silylated Amino Acids. J Planar Chromatogr 18: 251-252.

43. Kazmierczak D, Ciesielski W, Zakrzewski R (2005) Detection and Separation of Amino Acids as Butyl-thio-carbamyl Derivatives by Thin-Layer Chromatography with the Iodine-Azide Detection System. J Liq Chromatogr RT 28: 2261-2271.

44. Flieger J, Tatarczak M, Szumilo H (2006) Multiple Development HPTLC Analysis of Amino Acids on Cellulose Layers. J Planar Chromatogr 19: 161-166.

45. Samanta TD, Laskar S (2006) New Reagent for Detection of Amino Acids on TLC Plates. J Planar Chromatogr 19: 252-254.

46. Mohammad A, Hena S, Bhawani SA (2006) TLC Separation of L-tryptophan Using Micro-emulsion Mobile Phase and Its Spectrophotometric Determination. Indian J Chem 45A: 1663-1666.

47. Bhushan R, Bruckner H, Kumar V, Gupta D (2007) Indirect TLC Resolution of Amino Acid Enantiomers after Derivatization with Marfey's Reagent and Its Chiral Variants. J Planar Chromatogr 20: 165-171.

48. Rezic I, Rezic T, Bokic L (2007) Optimization of the TLC Separation of Seven Amino Acids. J Planar Chromatogr 20: 173-177.

49. Mohammad A, Laeeq S (2007) Mixed Surfactants Enable Separation of Lysine from Other Essential Amino Acids in TLC on Silica Gel. J Planar Chromatogr 20: 423-427.

50. Tian D, Xie HQ (2008) Influence of Micro-emulsion Conditions on the Thin Layer Chromatographic Behaviour of Amino Acids. J Liq Chromatogr RT 31: 763-771.

51. Baranowska I, Markowski P, Wilczek A, Szostek M, Stadniczuk M (2009) Normal and Reversed-Phase Thin-Layer Chromatography in the Analysis of L-Arginine, its Metabolites, and Selected Drugs. J Planar Chromatogr 22: 89-96.

52. Vasta JD, Cicchi M, Sherma J, Fried B (2009) Evaluation of Thin-Layer Chromatography Systems for Analysis of Amino Acids in Complex Mixtures. Acta Chromatogr 21: 29-38.

53. Mohammad A, Haq N (2010) TLC separation of amino acids with a green mobile phase. J Planar Chromatogr 23: 260-264.

54. Mohammad A, Haq N, Siddiq A (2010) Resolution of Multi component Mixture of Amino Acids Using Environmentally Benign Eluents: A Green Chromatographic Approach. J Sep Sci 33: 3619-3626.

55. Adam C, Kinga K, Tadeusz HD (2011) Two-Dimensional Separation of Some Amino Acids by HPTLC and Pressurized Planar Electro chromatography. J Planar Chromatogr 24: 6-9.

56. Tomislav R, Iva R (2011) Use of Genetic Algorithms and Artificial Neural Networks to Predict the Resolution of Amino Acids in Thin-Layer Chromatography. J Planar Chromatogr 24: 16-22.