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EFFICACY OF IONIC LIQUIDS AS GREEN MOBILE PHASE SYSTEM IN THIN LAYER CHROMATOGRAPHY OF AMINO ACIDS

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ABSTRACT

A green thin layer chromatography (TLC) has been developed for the identification and separation of amino acids on silica gel (SG) and cellulose: SG static phases in combination with aqueous solutions of ionic liquids as mobile phase. Better separation efficiency was observed with silica gel as compared to the mixed stationary phase consisting of cellulose with SG. The resolution of three-component mixture (L-lysine + L-glutamic acid + DL-isoleucine) was successfully achieved on silica gel layer using 1% aqueous hexadecyl-trimethyl ammonium-chloride as mobile phase. The proposed method has been successfully applied for identification of L-lysine and L-glutamic acid in Ferseng-vit* syrup, and DL-isoleucine in Zisscovit* syrup.

KEYWORDS

Amino acid, Ionic liquid, Densitometry, Pharmaceutical syrup and Thin layer chromatography.

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INTRODUCTON

Presently, society needs the development of new analytical methods where the good characteristics as selectivity and sensitivity are not sufficient and these methods need to be 'Green', in which amount of hazardous reagents used and chemical waste generated during analysis should be minimal. Solvents are important components of nature providing one or more liquid phases for chemical reactions and processes. 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 containing organic

volatile organic solvent as one of the components[1-12].

Amino acids are important to life and are required by the body for performing different functions. They play a key role as building blocks of proteins and as intermediates in metabolic processes[13,14]. Their deficiency may cause a number of diseases. So their study is important. Amino acids are also the important precursor of biogenic amines hence their determination may be beneficial for the control of biogenic amines, particularly of known toxicity. The estimation of the quantity of amino acids in food items is of great interest because some of these have potential toxicity for human being when their concentration levels are above the acceptable daily intake limit[15,16].

Various chromatographic techniques have been employed for the separation and identification of amino acids and related substances of pharmaceutical importance[17–21]. Among these, thin layer chromatography (TLC) has enjoyed much popularity due to certain advantageous features such as a) open and disposable nature of TLC plates, b) wider choice of stationary and mobile phases, c) minimal sample clean up, d) low solvent consumption, and e) reduced need of modern laboratory facilities.

The work performed on TLC analysis of amino acids has been well described in literature[22,23]. The exchanged metal cations bring about the change in character of active centres on silica gel surface because their free orbitals are capable of forming coordination complexes with solvent molecules and separated compounds during chromatographic process. Because of this unique feature of silica surface, it has been most widely used as static phase in TLC of amino acids. The other sorbents used as stationary phase in TLC analysis of amino acids include, alumina, chitin and chitosan, Silica gel and RP-18, Talc, starch, silica gel, and alumina, Soil, Silica and kieselguhr[24-30].

Chemistry researches regarding the use of green solvents or biosolvents which are environment-friendly has grown enormously over the last 10 years after the publication by R. A. Sheldon in 2005[31].

The aim of this study was to search new environmental friendly TLC system for the analysis of amino acids and selection of the best possible combination of stationary and mobile phases for the resolution of analytes from their multi-component mixtures. In continuation of our previous work on developing environmentally friendly eluents for thin layer chromatography using aqueous solutions of surfactants[32,33] and ethylene glycol[34] for the analysis of organic compounds belonging to different groups. The present study was taken up to examine the mobility pattern of some amino acids through a silica static flat phase in combination with ionic liquids as mobile phase. The silica gel and silica gel: cellulose (4:1) ratio were examined as static phase using aqueous ionic liquids as mobile phase for thin layer chromatographic analysis of 22 amino acids. For reliable separation of three-component mixture of amino acids consisting of L-lysine, L-glutamic acid and DL-isoleucine, silica gel as static phase and 1 % aqueous hexadecyl-trimethyl ammonium-chloride (HTA) as mobile phase was identified as the most favourable TLC system. The use of aqueous ionic liquids as green mobile phase is a novel approach to developed environment friendly green TLC method. The silica gel and cellulose static phases were characterized by Fourier transform infrared (FTIR), X-ray diffraction (XRD) and Scanning electron microscopy (SEM) studies. The present study is advantageous because it protects scientists and chemists from the exposure of volatile and corrosive organic solvents during experimentation.

EXPERIMENTAL

All the experiments were performed at 22±2 ºC.

Apparatus

Glass plates 20 cm × 3.5 cm coated with silica gel and silica gel modified with cellulose using TLC applicator (Toshniwal, India) were used as stationary phase. The micropipette (Tripette, Germany) was used for spotting of amino acids and 24 cm × 6 cm glass jars were used to perform TLC.

Chemicals and Instrumentation

Silica gel (Fischer Scientific, India); cellulose (Central Drug House; CDH, India); copper sulphate, zinc sulphate, manganese sulphate, nickel nitrate, HCl and sodium salts of bromide, chloride, carbonate, acetate and nitrate; magnesium hydroxide (CDH, India); 1-methyl-imidazolium chloride; 1, 2, 3-trimethylimidazolium methyl sulphate; 1-ethyl 3-methyl-imidazolium tetra fluoroborate (Sigma-Aldrich); hexadecyl-trimethyl ammonium-chloride (Merck); and aniline (E-Merck India Ltd.) were used as received. All chemicals were of analytical reagent grade. The water used in these experiments was double distilled.

The Fourier transform infrared spectra (FTIR) were recorded using Perkin-Elmer 1725 spectrometer operating in the 400-4000 cm^{-1} range. X-ray diffraction (XRD) data were recorded by using Bruker D8 diffracto meter with Cu Kα radiation at 1.540 Å in the range of $5^o \leq 2\theta \leq 70^o$ at 40 kV. The morphology was observed by a JSM-6510LV system with a JEOL scanning electron microscope (SEM).

Amino Acids Studied

L- histidine (A1), arginine (A2), glycine (A3), L-lysine (A4), leucine (A5), DL-valine (A6), L-tyrosine (A7), L-cystine (A8), L-proline (A9), L-cysteine hydrochloride (A10), L-ornithine monohydrochloride (A11), DL-alanine(A12), L-glutamic acid (A13), DL-tryptophan (A14), DL-methionine (A15), DL-aspartic acid (16), DL-isoleucine (A17), DL-nor isoleucine (A18), DL-phenylalanine (A19), DL-threonine (A20), DL-serine (A21) were used and DL-2,amino n-butyric acid (A22) procured from (Central Drug House; CDH, India).

Composition of Ferseng-vit syrup and Zisscovit syrup

The Ferseng-vit syrup, Fern Biotech unit were used to identify L-lysine and L-glutamic acid and DL-isoleucine in Zisscovit syrup respectively. The pharmaceutical formulation of Ferseng-vit syrup contain multivitamin contains Iron, L-lysine and L-glutamin acid. The pharmaceutical formulation of Zisscovit Syrup contains vit A2500 IU; cholecalciferol 400 IU; vitamin E (15 IU); vitamin C (50 mg); niacin (17 mg); thiamine (1.5 mg); riboflavin (1.5 mg); pyridoxine (1.7 mg); D-pantotheno (l4 mg); Biotin (20 mg); potassium iodide (150mg); magnasenese chloride(1.25 mg); folic acid 150 (mg); Lysine HCl (25 mg); DL-isoleucine (5.9 mg); L-trypthophan (5mg); L-phenyalamine (5 mg); L-threonine 4.2 mg/15 ml.

Test solutions

Solutions of amino acids (1% w/v) were prepared in double distilled water.

Detecting reagents

Ninhydrin solution (0.3% w/v) in acetone was used to detect all amino acids.

Stationary phase

Silica gel G (S_1) and silica gel:cellulose ratio 4:1 (S_2) plates were used during the whole experiment.

Mobile phases

Solvent systems (v/v) used as mobile phase are listed in Table No.1.

Preparation of TLC Plates

The TLC plates were prepared by applying the homogenous slurry, obtained by mixing sorbent with demineralized water (ratio, 1:3), onto the clean glass plates with the help of an applicator to obtain a 0.25mm thick layer. The plates were dried in air at room temperature and then activated by heating for 1 h at 90 ± 1 ºC in an electrically controlled oven. The activated plates were stored in a close chamber at room temperature until used.

Procedure

An aliquot (0.10 µL) of amino acids (the test solution) was applied on TLC plates (S_1 and S_2) with the help of a micropipette at about 1.0 cm above the lower edge. The spots were dried in air. After drying of spots, the TLC plates were developed to a distance of 5 cm with different mobile phases (M_1-M_8). After development, the plates were taken out and dried in air. The spots of amino acids were detected by spraying ninhydrin solution. All amino acids except proline (yellow) appeared as purple spots and R_F values of amino acids were calculated the R_L (R_F of leading front) and R_T (R_F of trailing front) values of the spot as given below.

$$R_F = 0.5(R_L - R_T)$$

For separation, equal volumes of amino acids to be separated were mixed and an aliquot (0.10 µL) of the resultant mixture was loaded onto the activated TLC plate (S_1). The plates were developed with selected mobile phase M_8 (HTA) and static phase the spots were detected and the R_F values of the separated amino acids were determined.

Effect of Different Ionic Liquids on Separation

To understand the separation behaviour of amino acids in mobile phases having different concentrations of aqueous ionic liquids (1-ethyl-3-methylimidazolium tetrafluoroborate; 1-methylimidazolium chloride; 1, 2, 3 trimethylimidazolium methyl sulphate and hexadecyl-trimethyl ammonium chloride, silica gel (S_1) and mixture of silica gel and cellulose (4:1) ratio (S_2) were used for the chromatography of amino acids. The concentration of each ionic liquid was varied from 1.0 to 5.0%. The R_F values obtained by using these stationary phases were compared with those obtained with S_1 stationary phase in M_8 mobile phase on which better separation was obtained.

Effect of Interference

For investigating the interference of metal cations and inorganic anions as impurities on the separation of the mixture, 0.1 µL of the standard test mixture of amino acid solutions were spotted on the silica gel (S_1) TLC plate followed by spotting of 0.1 µL of the metal cations or inorganic anions being considered as impurities. The plates were developed with M_8, detected and the R_F values of the separated dyes were calculated.

Ageing Effect of Mobile Phase

To examine the stability (ageing effect) of the mobile phase (M_8) on the separation of amino acids, the sample mixture was spotted on the activated TLC plates and developed with freshly prepared mobile phase (M_8) and R_F values were calculated. The same process was repeated using the previously prepared mobile phase M_8 at different time intervals of two hours for 24 h and then R_F values were calculated and compared with the values obtained from freshly prepared eluent.

Limit of Detection

The detection limits of separated amino acids were determined by spotting different amounts of L-lysine (A4), L-glutamic acid (A13) and DL-isoleucine (A17) on the silica gel (S_1) plates and the chromatography was performed with selected mobile phase M_8. The plates were detected as described above. The method was repeated with successive lowering of the amount of amino acids. The lowest amount that could be detected was taken as the limit of detection.

RESULTS AND DISCUSSION

Separations of three-component mixtures of amino acids under different experimental conditions were efficiently achieved on silica gel instead of cellulose: SG composite. The oxygen atom present on the surface of silica gel particle bound to protons and presence of hydroxyl groups make silica gel surface extremely polar. Silanol groups present on the surface of silica gel are capable of forming hydrogen bond with electron rich species. The analyte molecules of polar functionality can bind to the SG in two ways i.e. through dipole-dipole interactions as well as hydrogen bonds. The overall strength of the interaction is the sum of these two components. The shape of organic analyte is also important factor in predicting of its interaction with silica gel. Analyte that shows multiple polar groups in position to interact with surface of stationary phase is more strongly retained by the stationary phase.

In present study, silica gel has been used as stationary phase where some of its hydroxyl groups form inter-hydrogen bond, leaving some hydroxyl groups free for interacting with other polar analyte species. Both positive and negative centers are developed on the static matrix which provides unique selectivity for the separation of polar molecules involving dipole-dipole interaction. The positive charged ammonium ion and negative charge carboxyl group of amino acids interact with positive center of Si via electrostatic interaction, giving rise to a new organo-functional group with both positive and negative centers Figure No.1. Hence, amino acids in aqueous media may exist in

zwitter ionic form consisting of both positively charged ammonium and negatively charged carboxyl groups, and thus may interact with the organo-functional group of the stationary phase via coulombic interaction[35]. However, distinct amino acid interacts differently due to steric hindrance experienced by an amino acid.

Thin layer chromatography (TLC) of 22 amino acids was performed on two stationary phases (a) pure silica static phase S_1 and (b) Silica gel plus cellulose (4:1) S_2 using eight mobile phases (M_1-M_8) in order to select a novel TLC system for achieving separation of amino acids from their multi-component mixtures. The results obtained have been presented in Tables No.2-6 and Figures No.1-7 which are discussed below.

Silica gel as stationary phase (S_1)

The mobility trends of amino acids on silica gel layers developed with different aqueous solutions of ionic liquids are discussed below.

1, 2, 3-Trimethylimidazolium methyl sulphate [M1-M2]

In M_1-M_2 mobile phase systems, on pure silica gel static phase (S_1), most of the amino acids reached close to the maximum allowed distance for development, showing R_F in the range of 0.70-0.97. In case of M_1 mobile phase having 5% aqueous solution of 1, 2, 3-Trimethylimidazolium methyl sulphate, binary separation of A1 (R_F=0.5) amino acid occurs from all other amino acids except six amino acids, out of which four amino acids (A10, A17, A18, A19) show R_F values (R_F=0.65, 0.64, 0.68, 0.68) almost equal to the R_F values of A1 and remaining two amino acids (A4, A5) were not detected on S_1 stationary phase. The R_F value of A3, A12-A22 amino acids with M_2 mobile phase are nearly equal to R_F value of all other amino acids as in M_1 mobile phase showing maximum mobility. The binary separation of A1, A2, A4, A5, A11 and A21 also occur in M_2 mobile phase from other amino acids except A7, A8 and A9 which are not detected.

1-Methylimidazolium chloride [M_3-M_4]

In M_3-M_4 mobile phase systems, on pure silica gel static phase (S_1), in case of M_3 mobile phase having 5% aqueous solution of 1-Methylimidazolium chloride, maximum amino acids shows R_F range from (R_F=0.65-0.94) reached near higher distance for development, except A7 and A8 which are not detected while A11 can be separated from other amino acids having higher R_F values(0.72- 0.97) like A1, A2, A3, A5, A6, A10, A12, A13 and A15-A22. In case of M_4 mobile phase A1, A2, A4 and A11 (R_F =0.45, 0.47, 0.41, 0.44) can be separated from A3, A5, A6, A9, A10, A12, A13 and A18-A22 which have R_F values in the range (0.75 – 0.90) of amino acids. However A14-A17 produced tailed spot and A7-A8 not detected as shown in Table No.2.

Aqueous 1-Ethyl 3-methyl-imidazolium tetra fluoroborate [M_5-M_6]

In mobile phase M_5, all amino acids that are detecting as coloured spots on TLC plates exhibit higher R_F values (R_F > 0.7). However, A3, A5 and A7-A11 amino acids could not be detected. With 1 % EMITF mobile phase (M_6), separation of A1, A2, A4 and A11 amino acids demonstrates R_F.

Hexadecyl-trimethyl ammonium-chloride [M_7-M_8]

The R_F value of M_7 mobile phase of A1, A2, A4 and A11 amino acids with lower R_F values (R_F=0.25-0.38) can be separated from amino acids (A3 A5, A6, A9, A10, A12, A13 and A15-A22) showing higher R_F values (R_F = 0.95, 0.78, 0.81, 0.75, 0.94, 0.92,0.93 , 0.78,0.94, 0.76, 0.75, 0.76, 0.95, 0.95 and 0.94) . Two amino acids (A7, A8) could not detect and A14 produced tailed spot. In M_8 mobile phase having lower (1%) concentration of HTA occur good ternary separation of A4 (R_F=0.07, A13 (R_F=0.97) and A17 (R_F=0.55). Because of getting differential mobility pattern of amino acids and maximum possibilities of separations, M_8 was selected as the best mobile phase for resolving amino acids from their ternary and binary mixtures. The amino acids present in each mixture are easily separable as shown in Table No.2.

Silica gel with cellulose mixed stationary phase (S2)

In order to assess the effect of cellulose (organic sorbent) in combination with silica gel (an inorganic sorbent) in respect of mobility of amino

acids , silica gel mixed with cellulose was used as stationary phase and different solvents of ionic liquids were used as mobile phase. From following it is clear that the presence of cellulose in silica gel hampered the separation efficiency of silica gel. It is therefore concluded that plain silica gel is better sorbent for analysis of amino acids compared to biphasic silica plus cellulose sorbent.

1, 2, 3-Trimethylimidazolium methyl sulphate [M1-M2]

In M_1-M_2 mobile phase systems, selective separation of A13 (R_F=0.21) from other amino acids (A1, A3- A6, A9-A12, A14-A22) showing R_F in the range of 0.65-0.90 can be separated. However, A7 and A8 amino acids were not detected and A2 amino acid produced tailed spot as evident from Table No.3.

1-Methylimidazolium chloride [M_3-M_4]

In M_3-M_4 mobile phase systems, all amino acids except A7 on both mobile phase (M_3-M_4) which were not detected showing R_F in the range of 0.67-0.86 while none of the amino acids leaves the point of application. It may be probably due to competitive interactions of amino acids with HTA ionic liquid hence no separation possibility was achieved.

Aqueous 1-Ethyl 3-methyl-imidazolium tetra fluoroborate [M_5-M_6]

In mobile phase M_5 higher concentration of EMITF all amino acid shows higher mobility having R_F value (0.72-0.92) except A7 and A 13.

Hexadecyl-trimethyl ammonium-chloride [M_7-M_8]

With TLC system (S2- M7), amino acids such as A6, A7, A8, A9, A15 and A16 were not detected on S_2. All other amino acids showed higher mobility having R_F value (0.71-0.87). The similar results were obtained with S_2- M_8 TLC system indicating that none of these systems is useful for achieving separation of amino acids as shown in Table No.3.

An important separation of three-component mixture of amino acid consisting of L-lysine (A4) glutamic acid (A13) and DL-isoleucine (A17) has been achieved on S_1 with M_8. Hence, TLC system comprising of silica gel (S_1) as stationary phase and 1% aqueous hexadecyl-trimethyl ammonium-chloride (M_8) as mobile phase has been most favourable for resolution of multi-component mixtures of amino acids (two- or three-component mixtures). The list of possible separations that can be achieved with different combinations of stationary and mobile phases is provided in Table No.4.

FTIR Spectroscopic Studies

The FTIR spectra of silica gel and cellulose are shown in Figure No.2. The strong absorption band at 1092 cm^{-1} due to the asymmetric stretching vibration of Si–O–Si bond because of the formation of SiO_2 network, the absorption peak at 807 cm^{-1} represents Si-O-Si bending vibrations and the band at 468 cm^{-1} is due to the deformation vibration of Si-O-Si[36-38].

The main characteristic peaks of silica gel are broad absorption at around 3431 cm^{-1} may be attributed to vibration bands of hydrogen bonded hydroxyl groups.

In case of cellulose The FTIR spectra have shown a wide band in the region between 3151 and 3446 cm^{-1} that specifies the free O–H stretching vibration of the OH groups in cellulose molecules[39]. In addition, the vibration peak detected at 1427 cm^{-1} is related to the bending vibration of the C– H and C–O bonds in the polysaccharide aromatic rings[40]. The most important absorption band are nanocellulose is at 933 cm^{-1} associated with the glycosidic linkages between glucose units in cellulose.

X-ray Diffraction (XRD) Studies

The XRD patterns of silica gel and cellulose are shown in Figure No.3. The XRD peak of silica gel are broad angle ranging around 2θ (18.10-29.45°) corresponding to the amorphous nature with low intensity centred at an angle of around 2θ=22.4[41,42]. However, cellulose exhibited peak around 2θ= 15.8, 22.6 and 34.9 ° which shows crystal structure exhibit characteristic assignments of 110, 200, and 004 planes, respectively[43-46]. These XRD results of cellulose indicate that the crystal pattern probably due to the crystalline structures of the polysaccharides. These results of crystallinity show similar trend as calculated by crystallinity analysis from FTIR spectra.

Scanning Electron Micrograph (SEM) Studies

The shape and surface morphologies of silica gel and cellulose have been demonstrated in Figure No.4 (a-b) at two different magnifications. SEM images of silica nanoparticles shown in Figure No.4 (a) are indicative of porous, irregular, and spherical shape with relatively smooth surface. In Figure No.4 (b), cellulose show that size and diameter of fibrils of cellulose was semi-crystalline cellulose consist nano-scale rod-like pasty material[47]. However, the surface morphology of these two samples is different shows that the surface of the SiO_2 was very slick, and a small aggregate was recognized on the surface of silica gel supported.

Effect of Addition of Cellulose in Silica Gel

Cellulose- silica gel composite was also used in place of silica gel with the hope of getting different separations. However, no improved separations were achieved as majority of the amino acids moved with the mobile phase on the TLC plate revealing the R_F values in the range, 0.65–0.92. L-glutamic acid was the only amino acid which showed lower R_F in M_1 and M_2. Thus, the better chromatographic performance of silica gel is probably due to large surface area of silica gel as compared to other stationary phases as shown in Figure No.5.

Stability of the Mobile Phase

Stability (aging effect) of mobile phase (M_8) was found to remain unaltered for several hours as insignificant variation in their R_F values was observed during the separation of a three-component mixture of amino acids irrespective of the use of a freshly prepared mobile phase or its use after 24 h. It is therefore assumed that the selected mobile phase (M_8) was stable for several hours and suitable for chromatographic analysis.

Effect of Interference

The separation efficiency of three-component mixture of L-lysine (A4),
L-glutamic acid (A13) and DL- isoleucine (A17) in the presence of metal cations and anions on the magnitude of separation factor (α) and resolution parameter (Rs) and ΔR_F values for separation has been examined and the results are presented in Table No.5. The result of mutual interactions of these foreign substances with the amino acids, a slight change in spot sizes of the amino acids takes place and hence the values of chromatographic parameters slightly increased or decreased but separation was always possible in each case. The spot size of the analyte slight increase or decrease due to interactions of amino acids with these foreign substances.

Limit of Detection

The lowest possible detectable amounts (µg $spot^{-1}$) given in parenthesis were for L-lysine (1.3), DL-isoleucine (1.3) and L-glutamic acid (2.7) showing reasonable sensitivity of proposed method for on spot detection of these amino acids. L-lysine (A4), DL-isoleucine (A17), L-glutamic acid (A13) of different concentrations on the silica gel (S_1) TLC plates which were developed with the selected mobile phase M_8 and the spots were visualized. This process was repeated by successive reduction of the concentration of amino acids until the detection of amino acid was not possible anymore.

Application

The proposed thin layer chromatographic method comprising of silica gel S_1 as static phase and 1% (HTA) as mobile phase is applicable for the identification of L-lysine and L-glutamic acid in Ferseng-vit syrup and DL-isoleucine in Zisscovit Syrup (Table No.6 and Figure No.6-7).

Table No.1: List of mobile phases used during whole study

Mobile Phase Code	Mobile Phase
M_1	5% Aqueous 1,2,3-trimethylimidazolium methyl sulphate (TIMS)
M_2	1% Aqueous 1,2,3-trimethylimidazolium methyl sulphate (TIMS)
M_3	5% Aqueous 1-methylimidazolium chloride (MIC)
M_4	1% Aqueous 1-methylimidazolium chloride (MIC)
M_5	5% Aqueous 1-ethyl 3-methylimidazolium tetrafluoroborate (EMITF)
M_6	1% Aqueous 1-ethyl 3-methylimidazolium tetrafluoroborate (EMITF)
M_7	5% Aqueous hexadecyltrimethyl ammonium chloride (HTA)
M_8	1% Aqueous hexadecyltrimethyl ammonium chloride (HTA)

Table No.2: Mobility of amino acids in terms of R_F values on S_1 (silica gel) stationary phase with different mobile phases

Amino Acid	R_F Value							
	Mobile Phase							
	M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8
A1	0.50	0.45	0.75	0.45	0.91	0.53	0.38	0.30
A2	0.88	0.45	0.72	0.47	0.88	0.54	0.25	0.13
A3	0.81	0.95	0.94	0.90	ND	0.96	0.95	0.92
A4	ND	0.40	0.69	0.41	.085	0.54	0.27	0.07
A5	ND	0.45	0.73	0.78	ND	0.70	0.78	0.77
A6	0.82	0.65	0.84	0.79	0.86	0.80	0.81	0.84
A7	0.81	ND	ND	ND	ND	ND	N.D	N.D
A8	0.75	ND	ND	ND	ND	ND	N.D	N.D
A9	0.90	ND	0.67	0.75	ND	0.72	0.75	0.75
A10	0.65	0.93	0.85	0.87	ND	0.96	0.94	0.95
A11	0.75	0.41	0.49	0.44	ND	0.56	0.26	0.11
A12	0.92	0.94	0.96	0.77	0.91	0.94	0.92	0.92
A13	0.94	0.93	0.97	0.88	0.86	0.95	0.93	0.97
A14	0.93	0.94	0.65	0.35T	0.80	0.45T	0.41T	0.40T
A15	0.88	0.82	0.78	0.35T	0.82	0.91	0.78	0.95
A16	0.96	0.94	0.95	0.45T	0.81	0.89	0.94	0.95
A17	0.64	0.78	0.75	0.32T	0.80	0.64	0.76	0.55
A18	0.68	0.70	0.75	0.75	0.75	0.72	0.75	0.66
A19	0.68	0.76	0.76	0.76	0.81	ND	0.76	0.68
A20	0.94	0.97	0.90	0.90	0.92	ND	0.95	0.90
A21	0.95	0.96	0.90	0.90	0.91	0.90	0.95	0.91
A22	0.89	0.91	0.86	0.86	0.91	0.90	0.94	0.89

ND = Not Detected, T = Tailed Spot, $R_L - R_T \geq 0.3$, A1 = L- Histidine, A2 = Arginine , A3 = Glycine, A4 = L-Lysine, A5 = Leucine, A6 = DL-Valine, A7 = L-Tyrosine, A8 = L-Cystine, A9 = L-Proline, A10 = L-Cysteine hydrochloride, A11 = L-Ornithine monohydrochloride, A12 = DL-Alanine, A13 = L-Glutamic acid, A14 = DL-Tryptophan, A15 = DL-Methionine, A16 = DL-Aspartic acid, A17 = DL-Isoleucine, A18 = DL-Nor isoleucine, A19 = DL-Phenylalanine, A20 = DL-Threonine, A21 = DL-Serine and A22 = DL-2 Amino n-butyric acid.

Value = 0.53-0.56 can be separated from A3 A6, A10, A12, A13, A15, A16, A21 and A22. While A7, A8, A19 and A20 not clearly detected, A14 produced tailed spot.

Table No.3: Mobility of amino acids in terms of R_F values on S_2 (Silica gel: cellulose, 4:1) stationary phase with different mobile phases

Amino Acid	R_F Value							
	Mobile Phase							
	M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8
A1	0.87	0.80	0.87	0.70	0.72	0.88	0.78	0.70
A2	0.39T	0.81	0.87	0.78	0.77	0.86	0.84	0.78
A3	0.87	0.82	0.83	0.70	0.80	0.88	0.87	0.87
A4	0.88	0.84	0.86	0.70	0.79	0.90	0.87	0.77
A5	0.90	0.87	0.81	0.72	0.84	0.86	0.81	0.84
A6	0.80	0.86	0.84	0.71	0.84	0.88	ND	0.79
A7	ND	0.75	ND	ND	ND	0.89	ND	N.D
A8	ND	ND	0.82	0.75	0.84	ND	ND	084
A9	0.88	0.77	0.87	0.68	0.87	0.89	ND	ND
A10	0.89	ND	0.86	0.78	0.86	0.91	0.71	0.84
A11	0.88	0.86	0.88	0.69	0.83	0.86	0.77	0.86
A12	0.65	0.74	0.88	0.81	0.83	0.82	0.78	0.84
A13	0.21	0.48	0.85	0.76	ND	ND	0.75	0.80
A14	0.76	0.67	0.67	0.80	0.87	ND	0.72	0.88
A15	0.72	0.66	0.68	0.74	0.86	0.89	ND	0.93
A16	0.73	0.69	0.71	0.77	0.85	0.81	ND	0.85
A17	0.71	0.69	0.64	0.82	0.81	0.90	0.87	0.86
A18	0.86	0.65	0.81	0.81	0.84	0.92	0.86	0.85
A19	0.89	0.83	0.84	0.81	0.88	0.88	0.87	0.89
A20	0.86	0.84	0.85	0.86	0.91	0.89	0.87	0.87
A21	0.88	0.86	0.87	0.82	0.92	0.91	0.81	0.88
A22	0.90	0.84	0.88	0.80	0.92	0.90	0.86	0.88
List of abbreviation are mentioned in Table 2								

Table No.4: List of possible separations of amino acids on S1 and S2 layers developed with aqueous ionic liquids of different concentration levels

Mobile Phase	Stationary Phase	Separation
M_1	**S_1**	Selective separation of L-histidine from all amino acids except A4, A5 A10 and A17-A19.
M_2	**S_1**	Binary separation of L- histidine, arginine, L-lysine, leucine and ornithine from all other amino acids except A7-A9.
M_3	**S_1**	Selective separation of ornithine from all amino acids except A7 and A8.
M_4	**S_1**	Binary separation of L- histidine, arginine, L-lysine, and ornithine from all other amino acids except A14-A17.
M_6	**S_1**	Binary separation of L- histidine, arginine, L-lysine and ornithine from all other amino acids except A7, A8, A14, A19 and A20.
M_7	**S_1**	Binary separation of L- histidine, arginine, L-lysine, and ornithine from all other amino acids except A7, A8, and A14.
M_8	**S_1**	Selective separation of DL- isoleucine from all other amino acids except A7, A8 and A14.

M_1	**S_2**	Selective separation of L- glutamic acid from all other amino acids except A2, A7 andA8.
M_2	**S_2**	Selective separation of L- glutamic acid from all other amino acids except A8, A10 and A14-A18.

Table No.5: Effect of interference on ΔR_F, separation (α) and resolution (R_S) factors of the separated ternary mixtures of amino acids

S.No	Ionic Impurities	Ternary Separations					
		L-lysine (A4), DL- isoleucine (A17), L-glutamic acid (A13)					
		A4 and A17			A17 and A13		
		ΔR_F (0.48)	α (16.238)	R_S (120.0)	ΔR_F (0.40)	α (15.544)	R_S (100.0)
Cations							
1	Cu^{2+}	0.49	16.908	140.0	0.39	14.934	111.428
2	Zn^{2+}	0.46	13.513	153.33	0.40	13.338	100.0
3	Mn^{2+}	0.51	18.349	145.714	0.37	13.756	105.714
4	Ni^{+}	0.47	17.681	104.444	0.39	10.195	86.666
5	Mg^{2+}	0.50	17.619	100.0	0.37	11.818	105.71
Anions							
6	CO_3^{2-}	0.46	12.360	115.0	0.41	19.632	91.111
7	NO_3^{-}	0.52	21.60	130.0	0.36	11.348	90.0
8	Br^{-}	0.48	16.238	120.0	0.38	10.878	95.0
9	Cl^{-}	0.45	14.393	100.0	0.43	17.537	95.55
10	CH_2COO^{-}	0.49	13.965	89.09	0.34	8.331	113.33

Table No.6: Identification of L-lysine and L-glutamic acid (in Ferseng-vit* syrup) and Isoleucine (in Zisscovit Syrup) according to their R_F values

S.No	Amino Acid	R_F Value	
		Standard Sample	Drug Sample
1	L-lysine	0.07	0.05
2	L-glutamic acid	0.97	0.91
3	DL-isoleucine	0.55	0.53

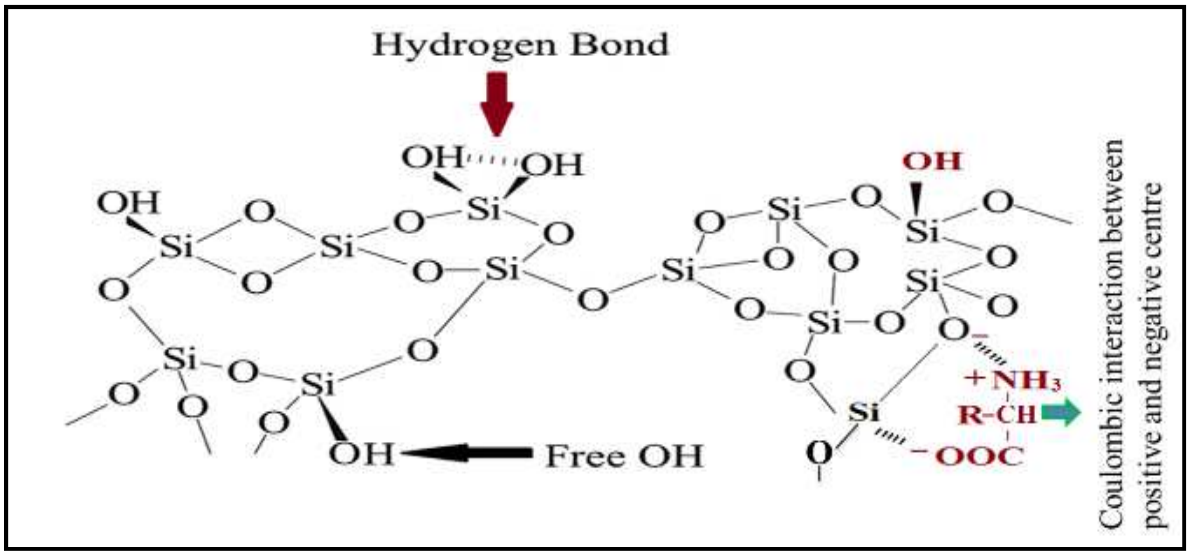

Figure No.1: Scheme showing Coulombic interaction between silica gel and amino acid during interaction, polarity and number of additional functional groups. Thus the differential motilities of amino acid promote amino acids separation

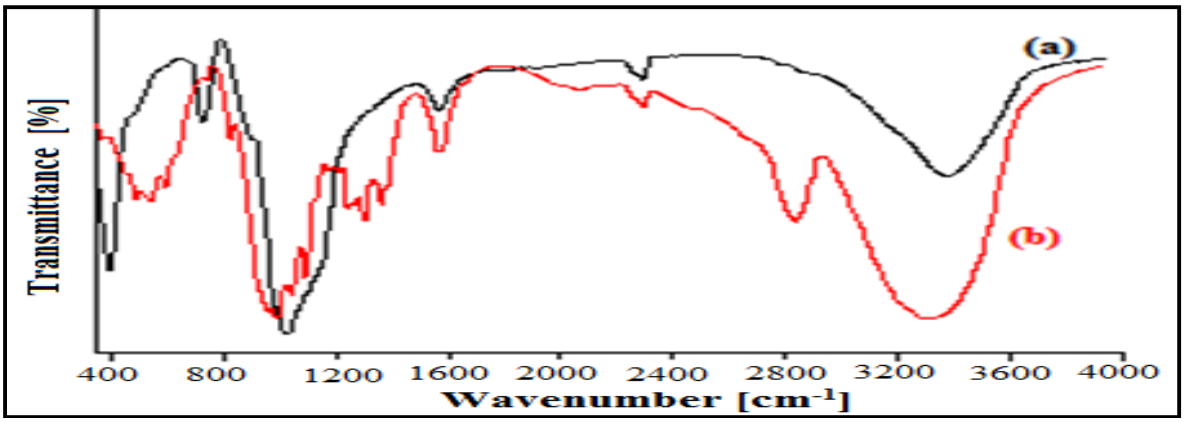

Figure No.2: FTIR spectra of: (a) Cellulose (S_2) and (b) Silica gel (S_1) stationary phases

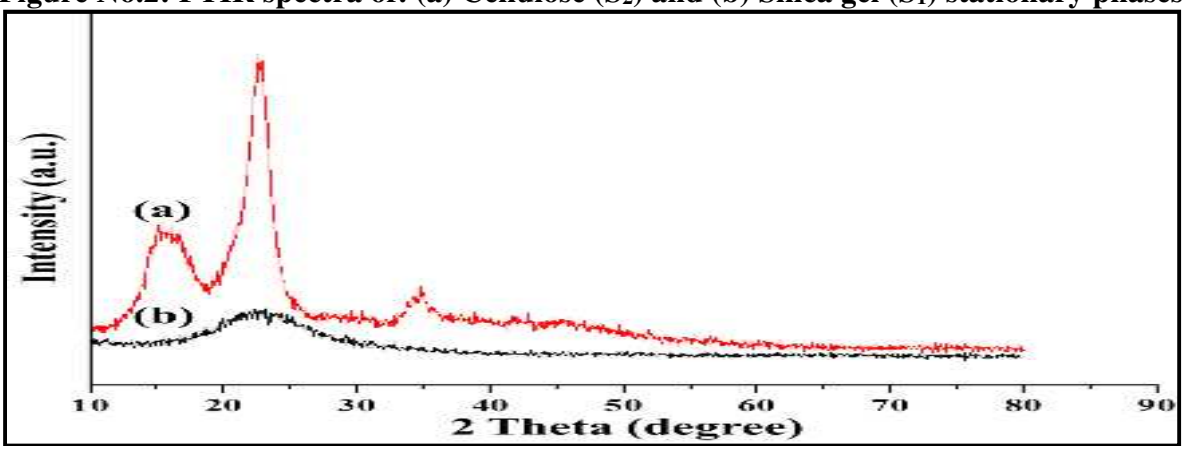

Figure No.3: XRD spectra of: (a) cellulose (S_2) and (b) silica gel (S_2) stationary phases

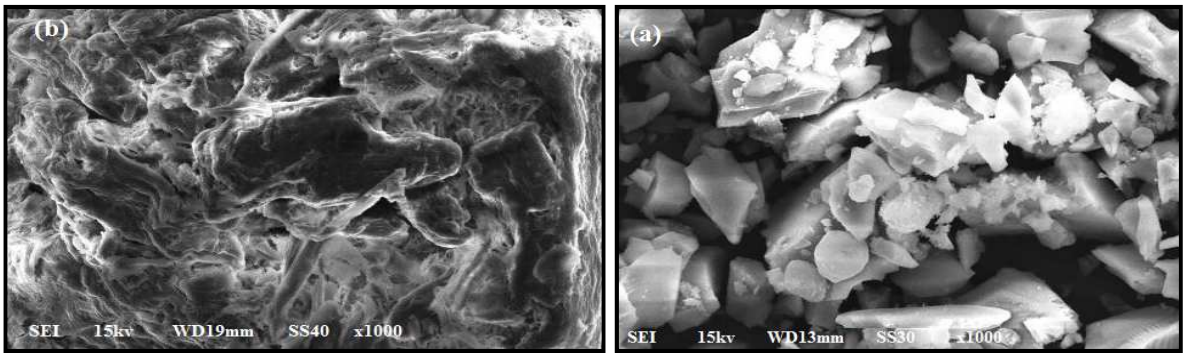

Figure No.4: SEM micrographs of (a) silica gel (S_1) and (b) Cellulose (S_2) at different magnifications

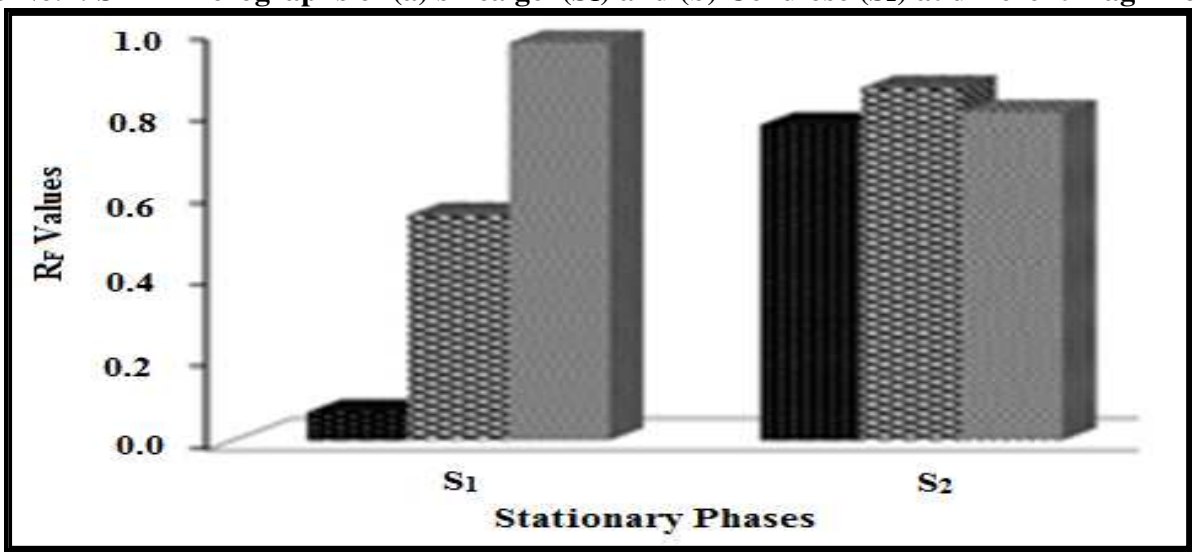

Figure No.5: Dependence of R_F values of separated amino acid on the nature of stationary phases developed with M_8 as the mobile phase

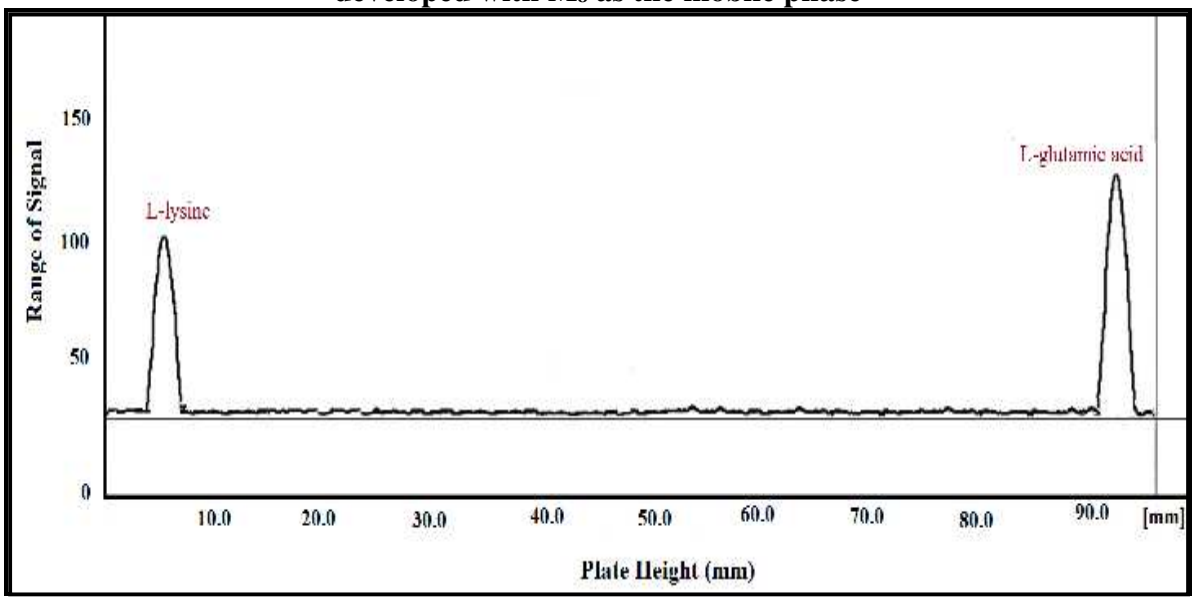

Figure No.6: Densitographic illustration of identification of L-lysine and L-glutamic acid in Ferseng-vit* syrup on S_1 with M_8

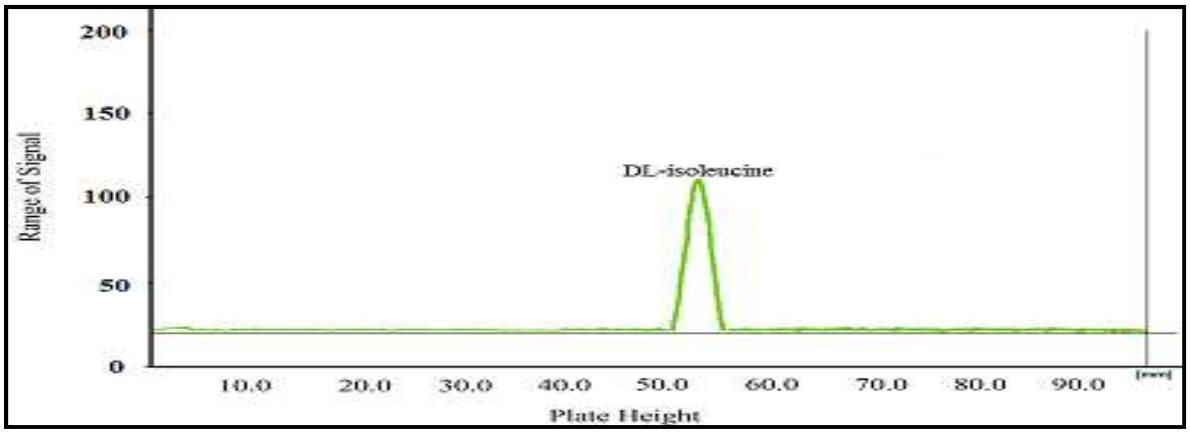

Figure No.7: Densitographic illustration of identification of DL-isoleucine in Zisscovit syrup on S_1 with M_8

CONCLUSION

Thin layer chromatographic system comprising of silica gel (S_1) as static flat phase with 1% hexadecyl-trimethyl ammonium-chloride (HTA) as eco-friendly mobile phase is most favourable for the identification and separation of three-component mixtures of L-lysin, L-glutamic acid and DL-isoleucine. The proposed system is also applicable for the identification of these amino acids in pharmaceutical products. L-lysine and L-glutamic acid have been successfully identified in Ferseng-vit syrup in addition to the identification of DL-isoleucine in Zisscovit Syrup. The use of aqueous solutions of cationic ionic liquids as green mobile phase system paves the way for developing green chromatographic systems.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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