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Ionic liquid as separation enhancer in thin-layer chromatography of biosurfactants: mutual separation of sodium cholate, sodium deoxycholate and sodium taurocholate

Ali Mohammad* and Rizwana Mobin

Abstract

Background: Physiological biosurfactants plays an important role in digestion of lipids and cholesterol also emulsifies fat-soluble vitamins.

Methods: A new green thin-layer chromatographic system comprising ionic liquid (1-methylimidazolium chloride) impregnated silica gel G and 2-methyl tetrahydrofuran as stationary and mobile phases was found to be most suitable for the separation of ternary mixture of biosurfactants (sodium cholate, sodium deoxycholate and sodium taurocholate). The surface structure and chemical composition of silica gel G modified by impregnation with 1-methylimidazolium chloride were examined with the aid of scanning electron microscopy and energy-dispersive X-ray spectrophotometry, respectively.

Results: Compared to plain silica gel, enhanced separation efficiency of ionic liquid impregnated silica gel was observed for the resolution of biosurfactants from their mixture. Chromatographic parameters such as ΔR_F, separation factor (α) and resolution (R_S) for the separation were calculated. Effect of foreign substances (metal cations, inorganic anions, vitamins, amino acids and non-ionic surfactants as impurities) on the separation of surfactants was also examined. Effects of concentration level of 1-methylimidazolium chloride as impregnant and its substitution by other ionic liquids (1,2,3-trimethylimidazoliummethyl sulphate, 1-ethyl-3-methylimidazolium tetrafluoroborate) were also studied to decide the optimum experimental conditions for better separation possibilities. The limits of detection of sodium cholate, sodium deoxycholate and sodium taurocholate were calculated.

Conclusion: A new green thin-layer chromatographic system comprising ionic liquid (1-methylimidazolium chloride) impregnated silica gel G and 2-methyl tetrahydrofuran as stationary and mobile phases provide the most suitable environment for the separation of ternary mixture of biosurfactants.

Keyword: Thin-layer chromatography; Ionic liquid; Biosurfactants; SEM; EDX

Background

Bile salts commonly occurring as sodium salts of bile acid and produced in the liver of mammals are known as primary bile acids, and those produced by the bacteria present in the colon are called secondary bile acids. Because of micelles' forming properties, bile salts [sodium cholate (SC), sodium deoxycholate (SDC) and sodium taurocholate (STC)] have been popular as physiological biosurfactants (Santhanalakshmi et al. 2001; Matsuoka and Moroni 2002; Hofmann and Mysels 1987; Selvam et al. 2009). These biosurfactants play an important role in (a) digestion of lipids and cholesterol, (b) emulsifying fat-soluble vitamins to enable their absorption in the intestine and (c) excretion of bilirubin. Biosurfactants have found use in medicines and cosmetics as emulsifying and dispersing agents (Santhanalakshmi et al. 2001; Selvam et al. 2009). SC and SDC being water-soluble mild ionic detergents are used

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for the isolation of membrane protein and lipids. In affinity chromatography SC is used for preventing non-specific binding. It is also used in cell culture media supplements Sodium Cholate, Pierce Biotechnology, USA. SDC is used as a non-surgical cosmetic medicinal treatment in mesotherapy injection and acts as a photo-resistant component in microlithography (Nanda 2011; Matarasso and Pfeifer 2009; Kim et al. 2000). Thus, analysis of these biosurfactants has been important because of their physiological importance.

Various analytical methods have been used in separation science, but thin-layer chromatography (TLC) is the most popular analytical tool used for separation because it is simple, rapid, versatile and cost-effective (Mohammad and Hina 2005; Mohammad and Bhawani 2008; Mohammad et al. 2010; Mohammad et al. 2013). Several inorganic, organic and aqueous-organic mobile phases and different stationary phases have been used in TLC analysis of surfactants (Mohammad and Agrawal 2001; Mohammad et al. 2002; Touchstone 1992; Gocan 2002). Most of mixed aqueous-organic and organic solvents previously used in chromatographic analysis need to be replaced by environmentally benign solvent systems in order to reduce their adverse impact on the environment.

Our aim of the present study was to achieve important separations of biosurfactants making TLC green by using environmental friendly mobile phase and silica gel modified with ionic liquid as stationary phase. Ionic liquids (ILs) are organic compounds, which exist in liquid form at room temperature, and have received much attention for their use in chemical industries due to various salient features such as good thermal stability, non-volatility and non-generating harmful volatile organic compounds (VOCs) as products. ILs provide good medium to solubilise H_2, O_2, CO and CO_2 gases and easily interact with other compounds via hydrogen bonding and electrostatic, dipolar, dispersive, pi-pi and n-pi bonding (Mallakpour and Dinari 2012).

In the present study, 1-methylimidazolium chloride ionic liquid is introduced in silica gel stationary phase which modifies the surface structure and provides better resolution of biosurfactants (SC, SDC and STC) in the presence of 2-methyltetrahydrofuran, a green alternative solvent for tetrahydrofuran as mobile phase. The use of green solvents in mobile as well as in stationary phases is a novel approach to develop environmental friendly green TLC method. It fulfils the growing demand of making analytical process greener. The proposed method was successful to identify SDC in formulated sample.

Methods

All experiments were performed at 25 ± 2°C.

Apparatus

A TLC applicator was used for coating silica gel on 20 × 3.2 cm glass plates and chromatography was performed in 24 × 6 cm glass jars. A glass sprayer was used to spray reagent on the plates to locate the position of the spot of the analyte.

Chemicals and reagents

Silica gel G (Thermo Fisher Scientific, Mumbai, India), alumina, kieselguhr, 2-methyltetrahydrofuran (CDH, New Delhi, India) and 1-methylimidazolium chloride, 1,2,3-trimethylimidazoliummethyl sulphate and 1-ethyl-3-methylimidazolium tetrafluoroborate (Sigma-Aldrich, St. Louis, MO, USA) were used. All the reagents used were of analytical grade.

Surfactants studied

Sodium cholate, sodium deoxycholate, sodium taurocholate, Tween 20, brij35, Tween 80, Triton X-100 and formaldehyde purchased from CDH (New Delhi, India) were used. All the surfactants were used as received.

Test solutions

Solution of biosurfactants was prepared in double-distilled water (DDW) to give a concentration of 5% (*w*/*v*).

Stationary phases

S_1 - Silica gel G
S_2-S_5 - Silica gel G impregnated with 0.1, 1.0, 5.0 and 10.0% 1-methylimidazolium chloride (aq), respectively.
S_6 - Silica gel G impregnated with 5.0% 1,2,3-trimethylimidazoliummethyl sulphate (aq)
S_7 - Silica gel G impregnated with 5.0% 1-ethyl-3-methylimidazolium tetrafluoroborate (aq)

Mobile phases

The mobile phases used were as follows:

M_1 - 5.0% 1-Methylimidazolium chloride (aq)
M_2 - Pure 2-methyltetrahydrofuran (purity, 99.0%)
M_3 - Pure acetone (purity, 99.0%)
M_4 - Pure dimethyl sulfoxide (DMSO; purity, 99.0%)

All the solvents were used as received.

Detection reagent

For detection of anionic surfactants, 100-mg pinacryptol yellow (Fluka, Sigma-Aldrich, Steinheim, Germany) was dissolved in 100-mL hot water and sprayed on TLC plates to visualise yellow to orange fluorescence spots under long wavelength UV light (366 nm).

Preparation of 1-methylimidazolium chloride impregnated TLC plates

Both pre-impregnation and post-impregnation methods described below as (a) and (b) were used for obtaining 1-methylimidazolium chloride impregnated silica TLC plates.

(a) In pre-impregnation methods, plates were prepared by mixing silica gel G and aqueous solution of 1-methylimidazolium chloride of desired concentration (0.1~10.0%) in 3:1 ratio. The resultant slurry was mechanically shaken for 15 min and then coated onto glass plates with the help of a TLC applicator to give a layer of 0.25-mm thickness. The plates were first air dried at room temperature and then activated by heating at 100 °C for 1 h. After activation, the plates were kept in an air-tight chamber until used.

(b) In post-impregnation method, activated silica gel plates were impregnated with desired concentration of aqueous solution of 1-methylimidazolium chloride (0.1~10.0%) by dipping silica gel plates in a solution of impregnate for a specific time period (20 min) followed by drying of plates at room temperature (25°C) and activation by heating for 1 h at 100°C. After activation, the plates were kept in an air-tight chamber until used.

In the present study, the TLC plates obtained by pre-impregnated method found better from a chromatographic point of view in terms of yielding more compact and well-resolved spots for biosurfactants. For example, the higher value (3.2 cm) of separated distance between the resolved spots of SDC and SC from their mixture on pre-impregnated TLC plates compared to the value of 2.1 cm on post-impregnated TLC plates is evident for better chromatographic performance of pre-impregnated method. Therefore, the detailed study was undertaken using pre-impregnating TLC plates.

Procedure

The biosurfactant solutions (0.1 μl) were spotted on non-impregnated and impregnated TLC plates with micropipette at approximately 2 cm above the lower edge of the non-impregnated and impregnated TLC plates. The spots were dried at room temperature (25 ± 2°C). The glass jars containing the mobile phase were covered with lids and left for 10 min for saturation before introducing the plates for development. The plates were developed with listed solvent systems (M_1-M_4) to a distance of 10 cm from the origin in all cases. After development, the plates were detected by a spraying solution of pinacryptol yellow and viewing the location of biosurfactants under UV light (366 nm).

For the separation of biosurfactant mixtures, equal volume of each biosurfactant was mixed and 0.1 μL of the resultant mixture was applied onto 5%(aq) 1-methylimidazolium chloride impregnated TLC plates (S_4). The plates were developed with M_2, the spots were detected and R_F values of the separated spots of the biosurfactants were calculated.

To understand the separation behaviour of biosurfactants in different impregnating liquids, 1-methylimidazolium chloride in S_4 was replaced with 1,2,3-trimethylimidazolium methyl sulphate and 1-ethyl-3-methylimidazolium tetrafluoroborate. The resultant stationary phase systems (S_6 and S_7) were used for the chromatography of biosurfactants. R_F values obtained with the use of these stationary phases were compared with those obtained with S_4.

Surface structure and chemical composition of pure silica gel and silica gel impregnated with 5%(aq) 1-methylimidazolium chloride were studied with scanning electron microscopy (SEM) and energy-dispersive X-ray spectrophotometer (EDX).

In order to examine the mobility pattern of biosurfactants on S_4, acetone (M_3) and dimethyl sulfoxide (M_4) were also used as eluents.

For investigating the interference of the presence of metal cations, metal anions, vitamins amino acids and non-ionic surfactants as impurities on the resolution of mixture, 0.1 μL of standard test mixture of biosurfactant solutions was spotted on the impregnated TLC plate followed by spotting of 0.1 μL of the metal cations, metal anions, vitamins and amino acids and non-ionic surfactants being considered as impurities. The plates were developed with M_2 and detected, and R_F values of the separated biosurfactants were calculated.

The limit of detection of separated biosurfactants were determined by spotting 0.1 μL of SC, SDC and STC of different concentrations on the impregnated TLC plates which were developed with selected mobile phase M_2, and the spots were visualised using pinacryptol yellow reagent under UV light. This process was repeated with successive reduction in concentration of biosurfactant until no detection of biosurfactant was possible. The amount of biosurfactant just detectable was taken as the detection limit.

Results and discussion

Thin-layer chromatography of three biosurfactants was performed on pure silica gel plate and silica gel G impregnated with different concentration levels (0.1~10%) of 1-methylimidazolium chloride using four mobile phases (M_1-M_4) in order to select a novel TLC system for achieving separation of three coexisting biosurfactants. The results obtained have been presented in Tables 1, 2, 3, 4, 5 and 6 and Figures 1, 2, 3, 4, 5 and 6 and are discussed below.

Table 1 Mobility of biosurfactants in terms of R_F values on different stationary phases

Biosurfactants	R_F value					
	S_1	S_2	S_3	S_4	S_5	
	M_1	M_2	M_2	M_2	M_2	M_2
Sodium cholate	ND	0.54	0.48	0.53	0.48	ND
Sodium deoxycholate	0.02	0.65	0.76	0.83	0.89	ND
Sodium taurocholate	ND	0.02	0.03	0.02	0.03	0.02

A. Unimpregnated silica gel TLC plates (S_1)

(a) On pure silica static flat phase (S_1) using 5%(aq) 1-methylimidazolium chloride (M_1) as mobile phase, SDC remains at the point of application (R_F~0.02) whereas SC and STC were not detected.

(b) SC (R_F = 0.54) and SDC (R_F = 0.65) exhibit high mobility and STC remains at the point of application (R_F~0.02) with 2-methyltetrahydrofuran (M_2) as mobile phase and S_1 as stationary phase. Binary separations of these biosurfactants were achieved with the use of S_1 and M_2 as stationary and mobile phases (Table 1).

B. Impregnated silica gel TLC plates (S_2-S_5)

In order to obtain improved separation possibilities, silica gel G was impregnated with different concentrations of ionic liquid (1-methylimidazolium chloride; 0.1~10%) and the resultant stationary phases (S_2-S_5) were used for chromatographic analysis of biosurfactants.

(a) SC and SDC showed differential migration (R_F = 0.48, 0.76) while STC remains at the point of application (R_F~0.03) on using S_2 (silica gel impregnated with 0.1%(aq) 1-methylimidazolium IL) as stationary phase and M_2 as mobile phase. Though the mutual separation of these SC, SDC and STC was possible with this TLC system, but the spots lack clarity and compactness; hence, concentration of impregnant was increased up to 10%.

Table 2 Effect of concentration of 1-methylimidazolium chloride on ΔR_F and separation factor (α) of biosurfactants

Concentration of 1-methylimidazolium chloride	SDC-SC-STC			
	SDC-SC		SC-STC	
	ΔR_F	α	ΔR_F	α
0.1% 1-Methylimidazolium chloride	0.30	3.48	0.45	29.9
1% 1-Methylimidazolium chloride	0.46	8.5	0.35	28.8
5% 1-Methylimidazolium chloride	0.31	4.15	0.45	29.9
10% 1-Methylimidazolium chloride	0.00	0.00	0.02	0.00

(b) At 1.0% concentration of impregnant (S_3), the mutual separation of three surfactants SC (R_F = 0.53), SDC (0.83) and STC (R_F~0.02) was also possible with slightly improved compactness of spots.

(c) The better resolved spots of surfactants (SC, SDC and STC; R_F = 0.48, 0.89 and 0.03) from their three-component mixture were realised with M_2 mobile phase and silica gel G impregnated with 5%(aq) 1-methylimidazolium chloride (S_4) stationary phase. Therefore, this TLC system was considered the best system for the resolution of coexisting SC, SDC and STC biosurfactants.

(d) The higher concentration of impregnant exceeding to 5.0% was not found suitable because SC and SDC could not be detected on the use of silica gel impregnated with 10%(aq) 1-methylimidazolium chloride (S_5) as stationary phase in combination with M_2 mobile phase (Table 2).

(e) Thus, the TLC system (S_4-M_2) was most favourable for the resolution of coexisting SC, SDC and STC biosurfactants (Figure 1).

C. Silica gel impregnated with other ionic liquids (S_6-S_7)

In order to examine the effect of nature of ionic liquid on the mobility of biosurfactants, 1,2,3-trimethylimidazoliummethyl sulphate and 1-ethyl-3-methylimidazolium tetrafluoroborate were used as impregnating liquid in place of 1-methylimidazoilum chloride and the resultant stationary phases S_6 and S_7, respectively, were used for chromatography of surfactants.

Table 3 Effect of substitution of 1-methylimidazolium chloride with different ionic liquids on R_F of separated biosurfactants

Different ionic liquids	R_F value		
	SDC	SC	STC
1-Methylimidazolium chloride	0.89	0.48	0.03
1, 2, 3-Trimethylimidazoliummethyl sulphate	0.53	0.36	0.02
1-Ethyl-3-methylimidazolium tetrafluoroborate	0.92	0.92	0.03

Table 4 Effect of substitution of 1-methylimidazolium chloride on ΔR_F and separation factor (α)

Different ionic liquids	SDC-SC-STC			
	SDC-SC		SC-STC	
	ΔR_F	α	ΔR_F	α
1-Methylimidazolium chloride	0.31	4.15	0.45	29.9
1, 2, 3-Trimethylimidazoliummethyl sulphate	0.17	2.01	0.34	27.6
1-Ethyl-3-methylimidazolium tetrafluoroborate	0.00	1.00	0.89	37.5

Table 5 Mobility of biosurfactants in terms of *RF* value on S_4 with different mobile phases

Biosurfactants	R_F value on S_4		
	M_2	M_3	M_4
Sodium cholate	0.48	0.81	0.93
Sodium deoxycholate	0.89	0.76	0.89
Sodium taurocholate	0.03	ND	ND

(a) Compared to S_4 ($R_F = 0.79$) (Tables 3), the lowering in R_F value of SDC on S_6 ($R_F = 0.53$) results in the inferior resolution of SDC from SC.
(b) Mutual separation of SC and SDC was hampered because both surfactants co-migrate on S_7 with M_2 as mobile phase (Tables 3 and 4).

D. SEM and EDX

From SEM and EDX images (Figures 2 and 3), it is evident that surface morphology as well as the chemical composition of silica gel are altered due to the incorporation of impregnant (5%(aq) 1-methylimidazolium chloride). The change in nature of stationary phase on impregnation provides unique opportunities for realising improved separation of biosurfactants.

E. Mobility of biosurfactants with other aprotic polar solvents (acetone and DMSO)

To examine the effect of other aprotic polar solvents on the mobility of these surfactants, acetone (M_3) and DMSO (M_4) were selected as commonly used solvents in chromatography. Both SC and SDC showed almost identical mobilities on S_4 stationary phase with M_3 and M_4 mobile phases while STC was not detected in any case (Table 5 and Figure 4). It is therefore concluded that cyclic aprotic polar solvent system is more useful as compared to non-cyclic solvents for chromatographic analysis of surfactants.

F. Effect of replacement of silica with other sorbents

In order to understand the separation pattern of biosurfactants on other adsorbents, 5%(aq) 1-methylimidazolium chloride impregnated silica gel G (S_4) was replaced with

Table 6 Effect of interference on ΔR_F, separation (α) and resolution (R_S) factors of the separated biosurfactants

Foreign substances	Ternary mixture SDC-SC-STC					
	SDC-SC			SC-STC		
	ΔR_F (0.52)	α (30.0)	R_S (48.0)	ΔR_F (0.36)	α (14.8)	R_S (57.7)
Metal cations						
Cu^{2+}	0.73	128.9	100	0.20	4.75	20.0
Ni^{2+}	0.66	71.8	94.4	0.21	4.09	23.3
Th^{4+}	0.73	148.4	102.4	0.18	4.71	20.0
Metal anions						
Br^-	0.73	148.4	97.3	0.14	2.75	16.4
Cl^-	0.61	84.4	122.0	0.26	4.83	32.5
CO_3^{2-}	0.62	61.0	82.6	0.21	3.31	22.1
Vitamins						
Pyridoxine	0.47	47.1	72.3	0.32	4.52	35.5
Thymine	0.43	40.1	61.4	0.31	3.93	34.4
Ascorbic acid	0.43	21.4	53.7	0.33	4.48	36.6
Amino acids						
Lysine	0.66	73.4	69.4	0.25	4.88	25.0
Glycine	0.69	122.5	115.0	0.19	3.63	22.3
Valine	0.69	122.5	86.2	0.21	4.44	22.1
Non-ionic surfactants						
Brij 35	0.49	51.0	65.3	0.31	4.36	34.4
Triton X 100	0.55	65.3	64.7	0.29	4.68	26.3
Tween 20	0.53	60.4	75.7	0.35	7.36	31.1

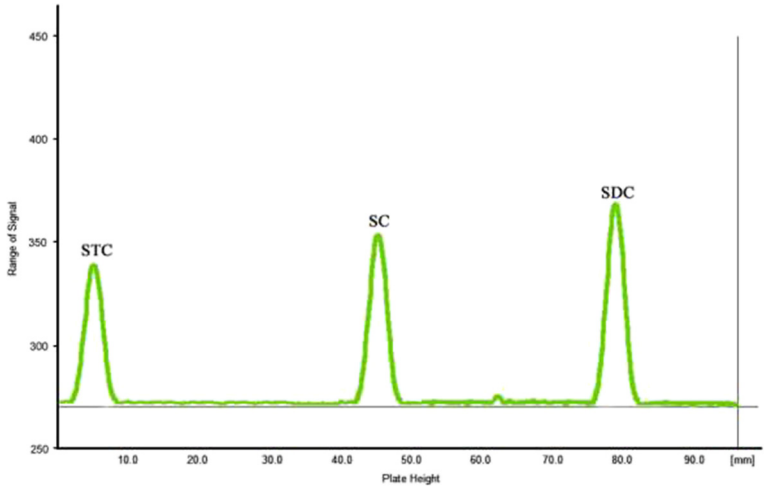


Figure 1 Densitographic presentation of representative ternary separations of anionic surfactants achieved with M_2 mobile phase on S_4 stationary phase.

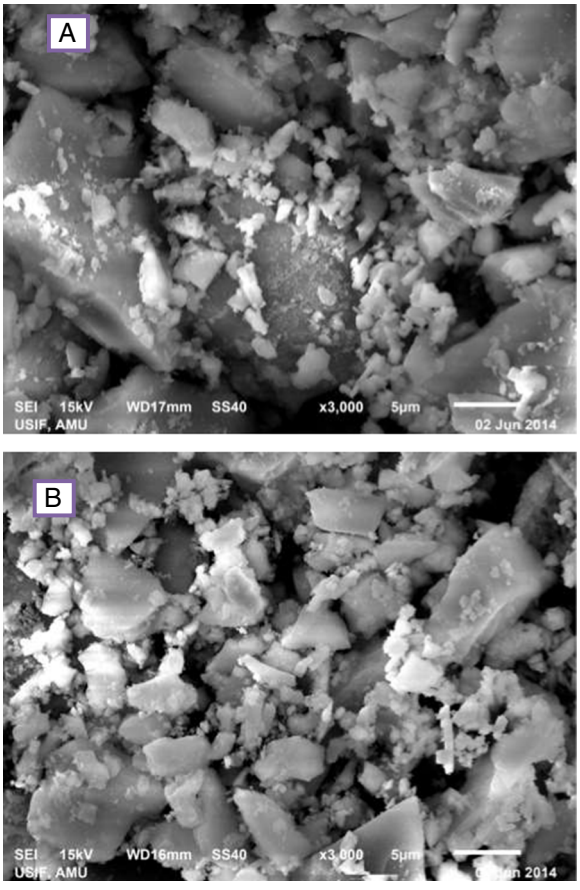


Figure 2 Scanning electron microscopy. SEM of **(A)** silica gel G and **(B)** silica gel G impregnated with 5% 1-methylimidazolium chloride.

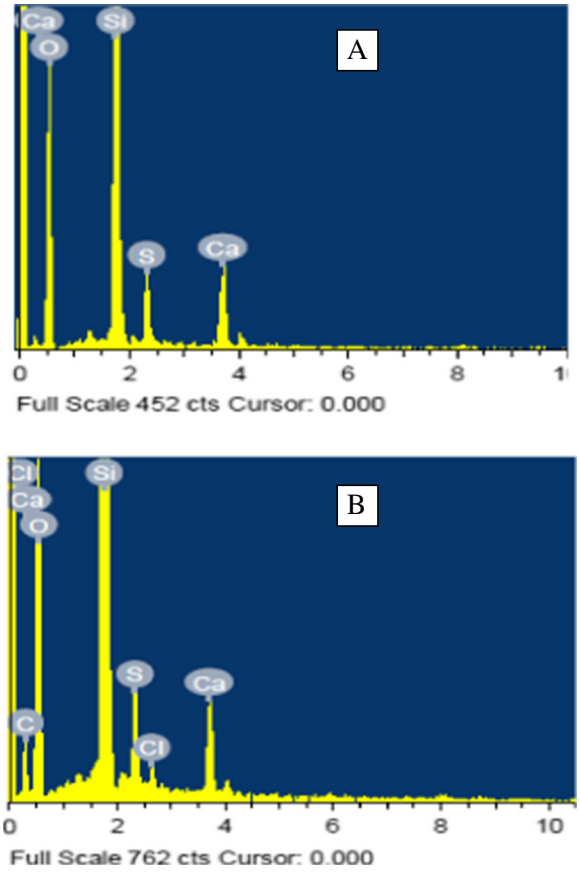


Figure 3 Energy-dispersive X-ray spectrophotometer (EDX) of **(A)** silica gel G and **(B)** silica gel G impregnated with 5% 1-methylimidazolium chloride.

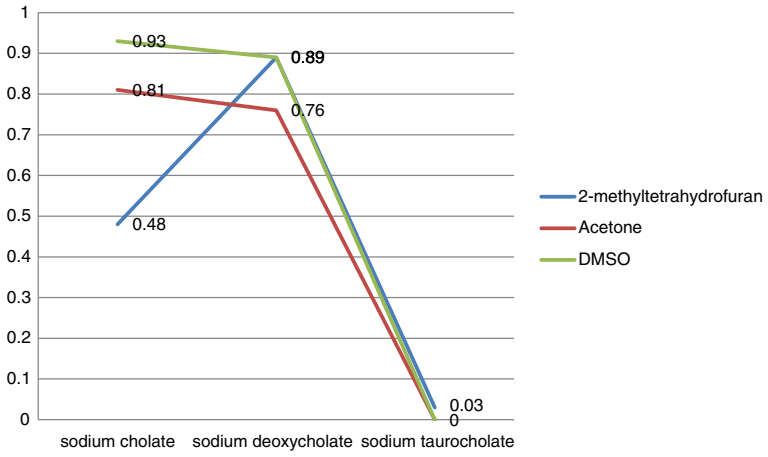


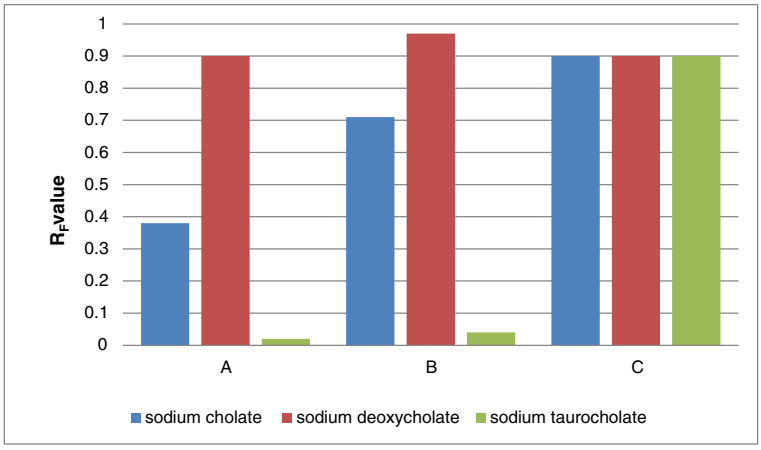
Figure 4 Mobility trend of biosurfactants on S_4 as stationary phase with different mobile phases.

alumina and kieselguhr impregnated with the same level of 1-methylimidazolium chloride (5%). The separation efficacy of different adsorbents towards biosurfactants was in the order silica gel G > alumina > kieselguhr as evident from Figure 5. It appears that the differences in surface area and activity level of these adsorbents influence the separation pattern. Compared to alumina and kieselguhr (neutral), silica gel (acidic) performs better as stationary phase in the separation of biosurfactants.

G. Effect of interference

Effect of metal cations, anions, vitamins and amino acids and non-ionic surfactants on the magnitude of separation factor (α) and resolution parameter (R_s) and ΔR_F for separation of three-component mixture consisting of SC, SDC and STC has been examined and the results are presented in Tables 6. From the results, it is clear that magnitude of these parameters is marginally influenced (increases or decreases) in the presence of these foreign substances, but separation was always possible in each case. The minor change in the value of these parameters was due to the slight increase in spot size of the analyte because of certain interactions of biosurfactants with these foreign substances.

H. Limit of detection



A: Silica gel impregnated with 5% aq. 1-methylimidazolium chloride

B: Alumina impregnated with 5% aq. 1-methylimidazolium chloride

C: Kieselguhr impregnated with 5% aq. 1-methylimidazolium chloride

Figure 5 Effect of stationary phases on R_F values of separated surfactants using M_2 as mobile phase.

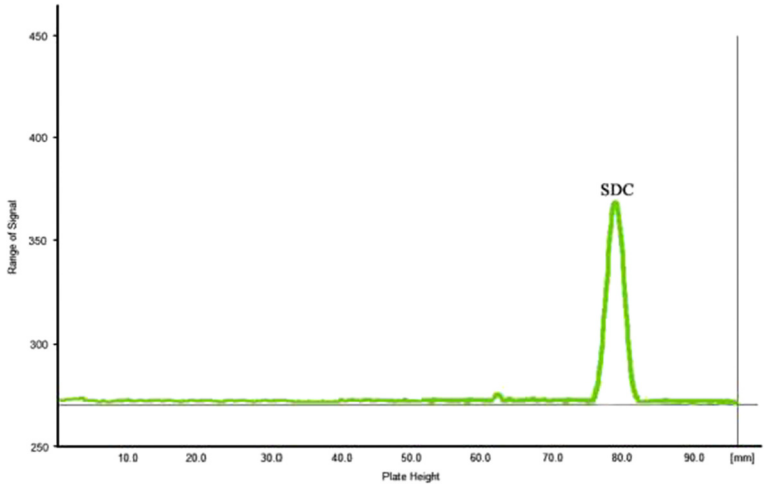


Figure 6 Densitographic presentation for identification of sodium deoxycholate in the mixture of formaldehyde, Triton X-100, Tween 80, sodium deoxycholate.

The lowest possible detectable amounts (μg/spot) of SC, SDC and STC on 5%(aq) 1-methylimidazolium chloride impregnated silica gel (S_4) plates developed with M_2 (2-methyltetrahydrofuran) were 0.7, 0.4 and 3.5, respectively, showing reasonably good sensitivity of the proposed method for the identification of surfactants at a trace level with preliminary separation from their mixture.

I. Application

Composition of Fluarix influenza vaccine

Each 0.5-mL dose of "Fluarix Influenza Vaccine" contain formaldehyde (0.05 μg), Triton X-100 (0.085 μg), α-tocopheryl hydrogen succinate (0.1 mg), Tween 80 (0.415 mg), hydrocortisone (0.0016 mg), gentamicin sulphate (0.15 μg), ovalbumin (0.05 μg) and sodium deoxycholate (50 μg). In order to check the applicability of the proposed method, formaldehyde, Triton X-100, Tween 80 and sodium deoxycholate are mixed; all these are the constituent of this vaccine. Sodium deoxycholate was easily identified in this mixture with the chosen TLC system (Figure 6).

Conclusion

It can be conclude that the use of 1-methylimidazolium chloride in stationary phase as impregnant and 2-methyltetrahydrofuran as mobile phase provides favourable environment for better separation possibilities of biosurfactants. The chosen TLC system was successfully applicable for the identification of sodium deoxycholate in a formulated sample containing the components of influenza vaccine. Another important aspect of this study is the use of green solvent as impregnant in stationary phase in addition to the use of green mobile phase making the proposed TLC system environment friendly.

Abbreviations

SC: sodium cholate; SDC: sodium deoxycholate; STC: sodium taurocholate; TLC: thin-layer chromatography; ILs: ionic liquids; VOCs: volatile organic compounds; DDW: double-distilled water; DMSO: dimethyl sulfoxide; SEM: scanning electron microscopy; EDX: energy-dispersive X-ray spectrophotometer.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

Experimental was done by RM. Both authors equally contributed in experimental design, framing, writing, proofing and approval of the manuscript.

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