Identification of co-existing cationic surfactants with preliminary separation on silica HPTLC plates using mixed aqueous sodium chloride-ethanol as eluent

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Ali Mohammad* and Rizwana Mobin†

Abstract: A new high-performance thin-layer chromatographic system comprising of silica gel 60 HPTLC plates as stationary phase and ethanol-5% aqueous sodium chloride (8:2) as mobile phase has been identified as most suitable for separation of quaternary mixture of cationic surfactants. Separation efficacy of developed method has been established by obtaining well-resolved densitogram of separated spots. To check the versatility, effects of presence of metal cations, inorganic anions, amino acids, vitamins and non-ionic surfactants as impurities were also examined. The chromatographic parameters like $\Delta R_F$, separation factor ($\alpha$), resolution ($R_s$) and limit of detection were also calculated. The proposed method is applicable for the identification of surfactants in eye drops and Colgate Plax mouthwash.

Subjects: Chemistry; Environment & Health; Physical Sciences; Analytical Chemistry; Chromatography

Keywords: High-performance thin-layer chromatography; separation; cationic surfactants; densitogram; eye drop; Colgate Plax mouthwash

ABOUT THE AUTHOR

Ali Mohammad is presently working as the UGC-BSR faculty fellow in the Department of Applied Chemistry, Faculty of Engineering and Technology, Aligarh Muslim University, Aligarh, India. His scientific interests include micellar thin-layer chromatography, surfactants analysis and green chromatography. The research group is actively engaged in searching of new methodologies involving green solvent systems for the analysis of inorganic ions and organic molecules. In this direction, several green chromatographic systems have been proposed for identification of targeted analyte in a variety of environmental and pharmaceutical samples. In addition to the identification and separation of surfactants belonging to the same group by green eluents on modified silica adsorbent layer, surfactants have also been utilised as eluents in the form of electrolyte as well as micelles for obtaining analytically important separations of biomolecules. The research group has provided a new direction for utilising the analytical potential of surfactants in chemical analysis.

PUBLIC INTEREST STATEMENT

Surfactants are surface active agents, generally organic compounds and are amphiphilic in nature. Based on the charge of the head group, surfactants are classified as—Anionic (negative charge), cationic (positive charge), non-ionic (no charge) and zwitterionic (two oppositely charge). Surfactants are important component of cosmetics, drugs and household cleaning products owing to their emulsifying, foaming and cleaning properties. Thin-layer chromatography is the simplest analytical tool for identification and separation of organic compounds and inorganic ions. With the help of thin-layer chromatography, surfactants are easily identified in pharmaceutical and commercial products. To ensure the level of surfactants in household products is therefore of public interest. The procedure proposed here is simple for routine analysis of surfactants in detergents, pharmaceutical and commercial products.
1. Introduction
Cationic surfactants are quaternary ammonium compounds mainly used as emulsifier, thickening agents and foaming agents in cosmetics (Rieger & Rhein, 1997), cleaning products (Schramm, Stasiukb, & Marangoni, 2003) as well as in pharmaceutical products (Mishra et al., 2009). Benzalkonium chloride (BZK) used as cationic surfactant, biocide and phase-transfer catalyst (Benzalkonium chloride, 2014). Cetylpyridinium chloride (CPC) used in mouthwashes, toothpastes, nasal sprays, throat sprays and breath sprays also helpful in reducing dental plaque. Benzyltrimethylammonium chloride (BTMAC) has been mainly used as phase-transfer catalyst and in oil and gas industries for stabilisation of clay and shale (Halpern, 2013; http://www.starchemglobal.com/Market%20Cards/StarChemOil&GasMarketCard.pdf). Tetramethylammonium hydroxide (TMAH) has numerous applications in industries as it commonly used in thermochemolysis (Filley, Minard, & Hatcher, 1999), photolithographic process (Levinson, 1999) and in the synthesis of ferrofluid (Maceira, Liz-Marzán, & Farle, 2004). However, TMAH adversely affect the human health causing muscular paralysis and in some cases causes death (Lin, Yang, Ger, Deng, & Hung, 2010). Surfactants are discharged into the natural waters through household, laundries and industrial sources. Surfactants are organic compounds, soluble in water and reached to river and other water bodies through wastewater discharge. Surfactants-containing water is easily absorbed by soil and thus adversely affects the plants and other aquatic organism (Ying, 2006). Hence, analysis of surfactants is very important because of their commercial importance as well as adverse effect on eco system.

Thin-layer chromatography (TLC)/high-performance thin-layer chromatography (HPTLC) has been the most popular, simplest and cost-effective analytical method for rapid and efficient analysis of mixture of surfactants. In this method, separations depend on the interaction between stationary and mobile phases with the analyte. Though, different types of stationary phases are available but silica gel has been most popular. The silanol groups (Si-OH) of silica gel play unique role in resolving the mixture of analytes on silica surface. As regards to mobile phase systems in TLC, the emphasis has been presently shifted to use solvents which are either green or less toxic in order to replace the traditional aqueous-organic systems containing volatile organic solvents. Therefore, over last 10 years, chromatographers are involved in search of greener and alternative solvents (Mohammad, Inamuddin, Siddiq, Naushad, & El-Desoky, 2012; Mohammad, Siddiq, & El-Desoky, 2013). Green solvents are environmentally friendly as these solvents are prepared from agricultural products. In the present study, combination of ethanol and 5% aqueous sodium chloride (8:2) as green eluent has been used which provide excellent environment for the separation of quaternary mixture of cationic surfactants (benzalkonium chloride; BZK—cetylpyridinium chloride; CPC—benzyltrimethylammonium chloride; BTMAC—tetramethylammonium hydroxide; TMAH) on silica HPTLC. The proposed method has been successfully applied for the identification of BZK in eye drop and CPC in Colgate Plax.

2. Experimental
All experiments were performed at room temperature 25 ± 2°C.

2.1. Apparatus
Chromatography was performed in 24.0 × 6.0 cm glass jars. Micropipette (Tripette, Germany) was used for spotting of analytes. A glass sprayer was used to spray reagent on the plates to locate the position of the spot of analytes.

2.2. Chemicals and reagents
Ethanol (Fischer Scientific, Mumbai, India), sodium chloride, copper (II) chloride, thorium (IV) nitrate, nickel (II) chloride, mercuric (II) chloride, sodium chloride, sodium bromide, sodium nitrate, sodium acetate, lysine, valine, glycine, phenylalanine, pyridoxine, thymine and ascorbic acid (CDH, New Delhi, India) were used. All the reagents used were of analytical grade.
2.3. Surfactants studied
Benzalkonium chloride (BZK), N-cetyl-N,N,N-trimethylammonium bromide (CTAB) and cetylpyridinium chloride (CPC) were purchased from CDH (New Delhi, India). CETRIMIDE and hexadecyltrimethylammonium chloride (HTAC) were purchased from Merck (Mumbai, India). Benzyltrimethylammonium chloride (BTMAC) and tetramethylammonium hydroxide (TMAH) were purchased from (Sigma-Aldrich, USA). All the surfactants were used as received.

2.4. Test solutions
Solutions of surfactants prepared in methanol were 0.5% (w/v). Solution of interfering chemicals were 1% (w/v) prepared in double distilled water (DDW).

2.5. Stationary phase
Pre-coated silica gel 60 HPTLC glass plates (Merck, Germany) were used as stationary phase for the entire study.

2.6. Mobile phases
Mobile phases used are listed in Table 1.

2.7. Detection reagent
Modified Dragendorff reagent prepared as described below was used to detect all the surfactants. Dragendorff reagent consists of two solutions A (5 ml) and B (5 ml) plus 20 ml of acetic acid and 70 ml of water. Solution A and B was prepared as described below:

Solution A: Prepared by dissolving 1.7 g of solid bismuth subnitrate (BiONO3·H2O) in a solvent composed of water and acetic acid in 4:1 ratio.

Solution B: Prepared by dissolving 40 g of potassium iodide (KI) in 100 ml of water.

Table 1. Following solvent systems were used as mobile phase

| Symbol | Composition                        |
|--------|-----------------------------------|
| M1     | Ethanol                           |
| M2     | 5% aq. sodium chloride            |
| M3     | Ethanol-5% aq. sodium chloride (2:8) |
| M4     | Ethanol-5% aq. sodium chloride (3:7) |
| M5     | Ethanol-5% aq. sodium chloride (5:5) |
| M6     | Ethanol-5% aq. sodium chloride (7:3) |
| M7     | Ethanol-5% aq. sodium chloride (8:2) |
| M8     | Ethanol-double distilled water (8:2) |
| M9     | Ethanol-1% aq. sodium chloride (8:2) |
| M10    | Ethanol-2% aq. sodium chloride (8:2) |
| M11    | Ethanol-10% aq. sodium chloride (8:2) |
| M12    | Methanol-5% aq. sodium chloride (8:2) |
| M13    | Propanol-5% aq. sodium chloride (8:2) |
| M14    | Ethanol-5% aq. lithium chloride (8:2) |
| M15    | Ethanol-5% aq. potassium chloride (8:2) |
| M16    | Ethanol-5% aq. calcium chloride (8:2) |
| M17    | Ethanol-5% aq. magnesium chloride (8:2) |
| M18    | Ethanol-5% aq. sodium bromide (8:2) |
| M19    | Ethanol-5% aq. sodium carbonate (8:2) |
| M20    | Ethanol-5% aq. sodium acetate (8:2) |
| M21    | Ethanol-5% aq. sodium nitrate (8:2) |
2.8. Composition of Pregent-D eye drop and Colgate Plax

Pregent-D eye drop (SGS pharmaceutical private limited, Roorkee) consists of ofloxacin (0.3% w/v), dexamethasone sodium phosphate (0.1% w/v), H. P. M. C. (0.25% w/v), benzalkonium chloride (0.02% v/v) in sterile aqueous base. Colgate Plax (Colgate-Palmolive India Limited, Mumbai) contains glycine, ethyl alcohol, sorbitol, propylene glycol, polysorbate 20, sodium benzoate, sodium fluoride, cetylpolyriodinium chloride, sodium saccharine, brilliant blue, FCF (CI 42090), flavour in aqueous base.

2.9. Procedure

Silica gel 60 HPTLC plates were activated at 60 ± 1°C in an electrically controlled oven for 30 min and stored in closed chamber until used. Spotting of 0.10 μL of analyte using micropipette was done at 2.0 cm from the lower edge of HPTLC plates. Spots were air dried and developed in a closed pre-saturated glass chamber with desired mobile phases by ascending technique up to 10.0 cm from the point of application. After development, the position of surfactants on HPTLC plates was located using Dragendorff reagent as detector. All cationic and non-ionic surfactants were visualised as orange coloured spots.

Surfactants under study belong to the group of quaternary ammonium compounds which can be protonated in the presence of acetic acid. The protonated surfactant tends to form ion pair with Dragendorff reagent that consists of \([\text{BiI}_4]^–\) and \([\text{HNR}_3]^+\). This complexation of ion pair with cationic surfactants results in the formation of orange-coloured spots on TLC plates (Scheme 1).

For the separation of surfactants mixture, equal volume of each surfactant was mixed and 0.1μL of the resultant mixture was applied onto activated silica gel 60 HPTLC plate. The plate was developed with \(M_7\) (ethanol-5% aq. sodium chloride, 8:2 by volume), the spots were detected and \(R_f\) values of the separated spots of the surfactants were calculated.

Scanning densitometry for identification of surfactants in four-component mixture consisting of BZK, CPC, BTMAC and TMAH has been carried out by spotting 0.10 μL of analytes mixture on silica HPTLC glass plates (10.0 cm × 10.0 cm). The plates were developed with chosen mobile phase (\(M_7\)). To locate the exact position of analyte spots, trial plates for individual samples were also run simultaneously. After chromatographic development, the spots were detected and the plates were subjected to densitometry scanning.

To examine the trend of mobility of surfactants at different concentration levels of sodium chloride in the mobile phase, 5% of sodium chloride in \(M_7\) was replaced with double distilled water (DDW), 1, 2 and 10% solutions of sodium chloride. The resultant mobile phase systems (\(M_{8-11}\)) were used for the chromatography of the surfactants. \(R_f\) values obtained with the use of these solvent systems were compared with those obtained with \(M_7\).

In order to examine the effect of alcohol on the mobility of the surfactants, ethanol in \(M_7\) was substituted with methanol (\(M_{12}\)) and n-propanol (\(M_{13}\)). These mobile phase systems were used for the chromatography of the surfactants.

To examine the ageing effect (stability) of mobile phase (\(M_7\)) on the separation of surfactants, the sample mixture was spotted on the activated silica gel 60 HPTLC plates, developed with freshly prepared mobile phase (\(M_7\)) and \(R_f\) values were calculated. The same process was repeated using previously prepared mobile phase (\(M_7\)) at different intervals of time for 24 h. Afterwards, the \(R_f\) values were calculated and compared with the values obtained with the use of freshly prepared mobile phase.
Scheme 1. Reaction scheme predicting the general behaviour of Dragendorff reagent's towards cationic surfactants.
For investigating the interference of the presence of metal cations, inorganic anions, vitamins and amino acids as impurities on the resolution of mixture of surfactants, 0.1μL of standard test mixtures of surfactants solutions were spotted on the plate followed by spotting of 0.1μL of the metal cations, inorganic anions, vitamins or amino acids being considered as impurities. The plates were developed with $M_7$, detected and $R_f$ values of the separated surfactants were calculated.

The limits of detection of separated surfactants were determined by spotting 0.1 μL solution of BZK, CPC, BTMAC and TMAH of different concentrations (0.1–0.01%) on the silica gel 60 HPTLC plates which were developed with selected mobile phase ($M_7$) and the spots were visualised using Dragendorff reagent as detector. This process of successive reduction in the concentration of surfactant was carried out until no detection of surfactant was possible. The amount of surfactant just detectable was taken as the detection limit.

3. Results and discussion

Results of the present study have been summarised in Tables 2–7 and Figures 1–9 and discussed below. Migration behaviour of seven cationic surfactants under study was examined on HPTLC 60 silica gel plates using ethanol, 5% aqueous sodium chloride and their mixtures as solvent systems. When ethanol ($M_1$) and 5% aq. sodium chloride solution ($M_2$) were used separately as eluents, all the surfactants remain near the point of application ($R_f$ in the range of 0.02–0.11). It shows stronger interaction of surfactants with silanol group of silica gel. But, with the mixture of ethanol-5% aq.

### Table 2. Mobility of surfactants in terms of $R_f$ values on silica gel 60 HPTLC as stationary phase with various mobile phases

| Surfactants | $M_1$ | $M_2$ | $M_3$ | $M_4$ | $M_5$ | $M_6$ | $M_7$ |
|-------------|------|------|------|------|------|------|------|
| Cetrimide   | 0.02 | 0.01 | 0.53 | 0.46 | 0.85 | 0.66 | 0.75 |
| CTAB        | 0.03 | 0.01 | 0.55 | 0.42 | 0.86 | 0.66 | 0.76 |
| BZK         | 0.11 | 0.03 | 0.59 | 0.46 | 0.89 | 0.80 | 0.86 |
| CPC         | 0.03 | 0.01 | 0.44 | 0.64 | 0.85 | 0.47 | 0.74 |
| BTMAC       | 0.06 | 0.11 | 0.46 | 0.66 | 0.85 | 0.52 | 0.50 |
| TMAH        | 0.03 | 0.01 | 0.15 | 0.20 | 0.49 | 0.16 | 0.28 |
| HTAC        | 0.04 | 0.01 | 0.50 | 0.46 | 0.83 | 0.66 | 0.73 |

Notes: CTAB: N-cetyl-N,N,N-trimethylammonium bromide; BZK: Benzalkonium chloride; CPC: Cetylpyridinium chloride; BTMAC: Benzyltrimethylammonium chloride; TMAH: Tetramethylammonium hydroxide; HTAC: Hexadecyltrimethylammonium chloride.

### Table 3. Effect of concentration of sodium chloride on the $R_f$ value

| Surfactants | $M_8$ | $M_9$ | $M_{10}$ | $M_{11}$ |
|-------------|------|------|----------|----------|
| Cetrimide   | 0.20 | 0.22 | 0.28     | 0.36     |
| CTAB        | 0.28 | 0.38 | 0.45     | 0.39     |
| BZK         | 0.32 | 0.46 | 0.51     | 0.51     |
| CPC         | 0.32 | 0.46 | 0.50     | 0.36     |
| BTMAC       | 0.17 | 0.25 | 0.30     | 0.18     |
| TMAH        | 0.17 | 0.10 | 0.08     | 0.04     |
| HTAC        | 0.17 | 0.28 | 0.32     | 0.41     |

Notes: CTAB: N-cetyl-N,N,N-trimethylammonium bromide; BZK: Benzalkonium chloride; CPC: Cetylpyridinium chloride; BTMAC: Benzyltrimethylammonium chloride; TMAH: Tetramethylammonium hydroxide; HTAC: Hexadecyltrimethylammonium chloride.
Table 4. Binary, ternary and quaternary separations achieved with different mobile phase in terms of $\Delta R_F$ and separation factor ($\alpha$)

| Concentration of sodium chloride | Binary separation HTAC-TMAH | Ternary separation CTAB-BTMAC-TMAH | Quaternary separation BZK-CPC-BTMAC-TMAH |
|---------------------------------|-----------------------------|------------------------------------|------------------------------------------|
|                                 | $\Delta R_F$ | $\alpha$ | $\Delta R_F$ | $\alpha$ | $\Delta R_F$ | $\alpha$ | $\Delta R_F$ | $\alpha$ |
| Ethanol-DDW ($M_8$, 8:2)        | 0.00         | 0.00     | 0.11         | 1.51     | 0.00         | 0.00     | 0.15         | 1.86     | 0.00         | 0.00     |
| Ethanol-1% NaCl ($M_8$, 8:2)   | 0.18         | 2.05     | 0.13         | 1.63     | 0.00         | 0.00     | 0.21         | 2.26     | 0.00         | 0.00     |
| Ethanol-2% NaCl ($M_8$, 8:2)   | 0.24         | 5.62     | 0.15         | 1.86     | 0.18         | 2.09     | 0.20         | 2.30     | 0.22         | 3.98     |
| Ethanol-5% NaCl ($M_8$, 8:2)   | 0.42         | 6.28     | 0.24         | 2.71     | 0.24         | 2.47     | 0.20         | 2.79     | 0.16         | 2.27     |
| Ethanol-10% NaCl ($M_8$, 8:2)  | 0.37         | 5.39     | 0.16         | 1.91     | 0.21         | 2.12     | 0.15         | 1.95     | 0.18         | 2.17     | 0.14         | 2.07     |

Table 5. Stability of mobile phase $M_8$, on the separation in terms of $\Delta R_F$ value

| Time interval (Hours) | BZK-CPC (0.20) | CPC-BTMAC (0.18) | BTMAC-TMAH (0.16) |
|-----------------------|----------------|------------------|--------------------|
| 2                     | 0.16           | 0.16             | 0.13               |
| 4                     | 0.21           | 0.15             | 0.12               |
| 6                     | 0.18           | 0.18             | 0.14               |
| 8                     | 0.15           | 0.16             | 0.17               |
| 24                    | 0.22           | 0.17             | 0.15               |

Note: Values of $\Delta R_F$ in bracket refers to the values obtained with freshly prepared mobile phase.

Table 6. Effect of various factors and interferences on $\Delta R_F$ and separation factor ($\alpha$) values of separated surfactants

| Foreign substances | Quaternary Separation (BZK-CPC-BTMAC-TMAH) |
|-------------------|--------------------------------------------|
|                   | $\Delta R_F$ (0.12) | $\alpha$ (2.40) | $\Delta R_F$ (0.16) | $\alpha$ (2.09) | $\Delta R_F$ (0.23) | $\alpha$ (2.04) |
| Cations           |                            |                |                            |                |                            |                |
| Cu$^{2+}$         | 0.13                        | 2.50           | 0.16                        | 2.05           | 0.24                        | 2.69           |
| Cr$^{3+}$         | 0.11                        | 2.05           | 0.15                        | 1.97           | 0.24                        | 2.68           |
| Ni$^{2+}$         | 0.13                        | 2.50           | 0.17                        | 2.14           | 0.23                        | 2.58           |
| Th$^{4+}$         | 0.19                        | 2.85           | 0.15                        | 1.95           | 0.23                        | 2.60           |
| Hg$^{2+}$         | 0.21                        | 2.57           | 0.16                        | 2.08           | 0.22                        | 2.46           |
| Anions            |                            |                |                            |                |                            |                |
| Br$^-$            | 0.13                        | 2.25           | 0.16                        | 2.08           | 0.22                        | 2.46           |
| Cl$^-$            | 0.12                        | 2.11           | 0.15                        | 2.00           | 0.23                        | 2.56           |
| CO$_3^{2-}$       | 0.13                        | 2.23           | 0.16                        | 2.05           | 0.21                        | 2.37           |
| NO$_3^-$          | 0.15                        | 2.50           | 0.13                        | 1.80           | 0.23                        | 2.56           |
| CH$_3$COO$^-$     | 0.13                        | 2.25           | 0.15                        | 2.00           | 0.23                        | 2.56           |
| Amino Acids       |                            |                |                            |                |                            |                |
| Valine            | 0.12                        | 2.17           | 0.15                        | 1.94           | 0.22                        | 2.46           |
| Glutamic acid     | 0.13                        | 2.31           | 0.16                        | 2.03           | 0.22                        | 2.66           |
| Histidine         | 0.13                        | 2.31           | 0.15                        | 1.94           | 0.25                        | 2.83           |
| Phenylalanine     | 0.12                        | 2.05           | 0.15                        | 2.06           | 0.23                        | 2.60           |
| Vitamins          |                            |                |                            |                |                            |                |
| Pyridoxine        | 0.13                        | 2.18           | 0.16                        | 2.08           | 0.21                        | 2.56           |
| Thymine           | 0.12                        | 2.38           | 0.15                        | 1.97           | 0.23                        | 2.38           |
| Ascorbic acid     | 0.15                        | 2.09           | 0.15                        | 2.14           | 0.23                        | 2.38           |
sodium chloride solution all the surfactants show differential mobility. Hence, mixtures of these solvent systems were used to examine the mobility trend of surfactants in order to resolve the surfactants from their mixture.

The results obtained with the use of ethanol and 5% aq. sodium chloride mixed in different ratio as mobile phases \(M_1-M_7\) are listed in Table 2. At lower volume ratio of ethanol \(M_3\) and \(M_4\), all the surfactants show almost mid \(RF\) values \(0.44-0.59\) except TMAH \(RF = 0.15\). Therefore, these solvent systems can be utilised for selective separation of TMAH from all other surfactants used. At equal volume ratio of ethanol and 5% aq. sodium chloride \(5:5, M_5\), all the surfactants show strong interaction with mobile phase. With increasing volume ratio of ethanol \(M_6\) and \(M_7\) differential mobility pattern of surfactants was achieved. Out of \(M_6\) and \(M_7\) mobile phases, \(M_7\) (ethanol-5% aq. sodium chloride, 8:2) was found most favourable for the resolution of four-component mixture of surfactant (BZK–CPC–BTMAC–TMAH). It was selected for the detailed study for the useful separation of BZK–CPC–BTMAC–TMAH.

Table 7. Various chromatographic parameters, separation factor \((\alpha)\) resolution \((R_s)\) and calculated for the separation of cationic surfactants in mixed (eye drop + Colgate Plax mouthwash) samples

| Sample                        | Resolved surfactants | \(\alpha\) | \(R_s\) |
|-------------------------------|----------------------|------------|---------|
| Eye drop—Colgate mouth wash   | BZK-CPC              | 1.95       | 18.4    |
Figure 2. Densitographic presentation of quaternary separation of cationic surfactants.

Figure 3. Effect of replacement of ethanol in M₇ with other alcohols.
Notes: A: Methanol-5% aq. NaCl (8:2); B: Ethanol-5% aq. NaCl (8:2); C: Propanol-5% aq. NaCl (8:2).

Figure 4. Effect of interference of foreign substances on separation in terms of $R_f$ value.
3.1. Separation of co-existing BZK–CPC–BTMAC–TMAH

The possibility of separation of co-existing BZK, CPC, BTMAC and TMAH in mobile phase (M7) arises due to their differential migration on HPTLC 60 silica plate because of selective interactions of analyte components in the mixture with stationary as well as mobile phases. In this mobile phase, Na+ ions present in aq. solution of sodium chloride react with ethanol forming sodium ethoxide (CH3CH2ONa) to provide CH3CH2O− sites. On the other hand, in stationary phase, silanol groups (SiO−) are the active sites for interaction with analyte. Thus, competitive interactions of surfactants with SiO− and CH3CH2O− decide their resolution from the mixture. The mobility trend of surfactants was in
the order: BZK > CPC > BTMAC > TMAH. Compared to TMAH (non-aromatic surfactant), higher mobilities of BZK, CPC and BTMAC can be accounted to the presence of benzene ring in their molecule. Thus, the develop method is useful for distinguishing between benzene-containing surfactants and aliphatic surfactants (TMAH). Amongst BZK, CPC and BTMAC, the mobility trend is probably controlled by the length of alkyl chain attached in the molecule of respective surfactants. BZK with long alkyl chain (C8–C18) and two methyl groups exhibits higher mobility compared to CPC which contains shorter alkyl chain (C16) and BTMAC that contains only methyl groups attached to nitrogen atom as shown in Figure 1. The lowest mobility of TMAH is due to the absence of benzene ring as it contains only four methyl groups as a result, it strongly interact with silanol groups of silica.
The representative densitogram of the separated surfactants from their mixture is presented in Figure 2.

3.2. Effect of sodium chloride concentration
To understand the effect of concentration of sodium chloride on separation, mobile phase systems $M_8$–$M_{11}$ were used. With $M_8$ and $M_9$, binary separation of HTAC-TMAH, CTAB-BTMAC and CPC-BTMAC was obtained. Ternary separation was achieved with $M_{10}$. Quaternary separation of surfactants was achieved with 5% ($M_7$) and 10% ($M_{11}$) concentration of sodium chloride. With $M_7$, better quaternary separation of surfactants was achieved as compared to $M_{11}$ (Tables 3 & 4).

3.3. Effect of alcohol
To examine the effect of the nature of alcohol on separation efficiency of surfactants, ethanol in mobile phase $M_7$ was replaced with methanol ($M_{12}$) and propanol ($M_{13}$). Butanol could not be used, as it was not completely miscible with 5% aqueous sodium chloride solution. Ternary separation of surfactants was achieved with methanol, while quaternary separation of surfactants was obtained with both ethanol and propanol. However, better separation was with ethanol (Figure 3).

3.4. Stability of mobile phase
Ageing effect of mobile phase ($M_7$) on the separation was insignificant since slight variation in their $R_f$ values was observed during the separation of surfactants from their quaternary mixture with the use of freshly prepared mobile phase and after storing it to 24 h. Hence, it can be concluded that the composition of eluent ($M_7$) remains unaltered for several hours and suitable for chromatographic analysis Table 5.

3.5. Effect of interference
Effect of metal cations, inorganic anions, vitamins and amino acids on the magnitude of separation factor ($\alpha$) and resolution parameter ($R_s$) for separation of four-component mixture consisting of BZK, CPC, BTMAC and TMAH has been examined and the results are presented in Table 6 and Figure 4. 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 is due to the slight broadening in spot size of the analyte because of certain interactions of surfactants with these foreign substances.
3.6. Replacement of silica gel 60 HPTLC plates with other stationary phases
Silica gel 60 HPTLC plates were replaced with other stationary phases (kieselguhr F254 TLC plates, aluminium sheet NH3 F254S, silica gel 60 RP-2 and cellulose). In case of aluminium sheet NH3 F254S, silica gel 60 RP-2 and cellulose TLC plates, binary separations (BZK-BTMAC, BZK-TMAH, CPC-BTMAC and CPC-TMAH) were obtained. On the other hand, kieselguhr F254 TLC was unsuitable for separation purpose as all the surfactants were moved to equal distance ($R_f$ in the range of 0.92–0.94) Figure 5. Separation efficiency of different adsorbents was in the order: silica gel 60 HPTLC > silica gel 60 RP-2 ~ aluminium sheet NH3 F254S ~ cellulose TLC > kieselguhr TLC.

3.7. Replacement of sodium chloride with other salts
Figure 6 shows the effect of replacement of sodium chloride in $M_7$ with chlorides of lithium, potassium, calcium and magnesium and the other salts of sodium (bromide, carbonate, acetate and nitrate). As evident from Figure 6 (A & B), the resulting mobile phase systems ($M_{15}$–$M_{21}$) have significant influence on the quaternary separation of cationic surfactants. With $M_{15}$ and $M_{21}$ binary separation was achieved and with the use of $M_{16}$–$M_{20}$ ternary separation of cationic surfactants was obtained. Separation was not possible with $M_{14}$ mobile phase.

3.8. Limit of detection
The lowest possible detectable amounts (μg/spot) of BZK, CPC, BTMAC and TMAH on silica gel 60 HPTLC plates developed with $M_7$ (ethanol-5%aq. sodium chloride, 8:2) were 0.01, 0.06, 0.08 and 0.09, respectively, showing reasonably good sensitivity for the detection of surfactants at trace level.

4. Application
The proposed method is applicable for identification and separation of BZK and CPC in Pregent-D eye drop and Colgate Plax mouthwash. Both BZK and CPC were easily identified and separated in pure and spiked samples with chosen TLC system (Table 7 and Figures 7–9).

5. Conclusion
It can be concluded from the present study that TLC system composed of silica gel 60 HPTLC as stationary phase and ethanol-5%aq. sodium chloride solution as mobile phase provides most favourable environment for the separation of cationic surfactants from their quaternary mixture. BZK in eye drop and CPC in mouthwash are easily identified with the chosen TLC system.

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