# Mixed stationary phase for TLC: separation of components from Manjistha root extract

**Prakash B. Samnani*, Nisha V. Khandarkar, Jalpa N. Rajai and Yesha B. Patel**

Department of Chemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Sayajigunj 390 002, India

**Thin layer chromatography owes its popularity to several advantages, being faster, easy to perform and ability to use a variety of mobile phase compositions because detection is post mobile phase removal. Silica gel is the most commonly employed stationary phase. This poses certain limitations in terms of selectivity, hence suitable modifications are attempted. Selectivity can be improved using modified silica but the process of modification can be tedious and non reproducible. We report here use of mixed bed stationary phase for separation of components in extract of roots of *Rubia cordifolia* L. (family Rubiaceae), a common medicinal plant used in the preparation of various formulations in Ayurveda. Microcrystalline cellulose (MCC) was prepared and used as a stationary phase with silica in the ratio 10 : 90 and 5 : 95 w/w respectively. Mobile phase composition was optimized and an additional component was separated, which was not observed on silica TLC plate. Moreover, characterization studies of prepared MCC were performed. The FTIR spectra obtained resembled the commercial MCC spectra with all the characteristic peaks obtained well in the prepared MCC. The dynamic light scattering data of the prepared MCC showed the mean particle diameter of 0.715 μm. The TGA-DTA data showed no deviations in the plot, even when the analysis was carried out in the presence of air instead of nitrogen.**

**Keywords:** Microcrystalline cellulose, mixed stationary phase, *Rubia cordifolia*, thin layer chromatography, silica gel.

THIN layer chromatography (TLC) remains one of the undisputed chromatographic techniques due to several advantages, being faster, easier, economic, free from carryover since fresh stationary phase used every time and ability to use a variety of mobile phase compositions because detection is post mobile phase removal. Though several other materials were studied as TLC stationary phases during its evolution and development[1], silica gel is the most commonly employed stationary phase, as of today. This poses certain limitations in terms of selectivity, hence suitable modifications are attempted[1,2]. Thus, a comparative study is reported for the analysis of amino acids on silica gel (SIG) and polyaniline modified SIG as stationary phases using 1% ethanolic solution of boric acid as mobile phase[3]. There is a report of simple and efficient TLC method for separation of gold ($Au_{25}L_{38}$) metal clusters[4]. Newer developments in the area continue, for instance, micro-TLC approach comprising one and two dimensional separation to generate fingerprints for fast screening of environmental samples originated from sewage and ecosystem waters[5]. Cellulose in native form and as microcrystalline form has been used as stationary phase for several separations[6,7]. Cellulose has unique advantages of being relatively mild stationary phase, compared to silica gel and also, shows good separation of chiral compounds[1]. For moderately polar compounds, however, it does not yield good separations. For a mixture of two compounds of varying polarity, a mixture of silica gel and micro crystalline cellulose (MCC) has been reported to be useful[8]. Such mixed stationary phases (silica gel containing 25% MCC) are commercially available as precoated plates. As phytochemicals are typically a mixture of varying polarity and tend to contain some chiral compounds as well, we considered the option of using such *mixed* stationary phase for improving separation of extract of manjishta roots. This approach has shown promising results and we demonstrate here improved separation of components of Manjistha root extract, prepared in methanol and chloroform.

*Rubia cordifolia* L. (Rubiaceae), also known as Manjistha, has been used as a traditional herbal medicine in Ayurveda for centuries for many healthcare disorders[9]. It is an important medicinal plant used for treatment of various diseases such as tumours[10–12], inflammations, urinary disorders and as antimicrobial[13], hepatoprotective, hypoglycemic[14] and antipsychotic[15]. The roots of *R. cordifolia* are listed officially in *Chinese Pharmacopeia* for treatment of arthritis, chronic bronchitis, uterine hemorrhage and uteritis[16].

TLC fingerprinting studies have been reported on *R. cordifolia* root extracts and its commercial samples, the components of Manjistha on silica TLC plates were separated in a ternary solvent system of toluene : diethyl ether : acetic acid in the ratio of 1 : 1 : 1 for the methanolic extract of Manjistha. Also, TLC studies were undertaken for extract prepared in different solvents. Another combinations of solvents used for resolving the components of Manjistha were benzene, ethyl acetate for diethyl ether extract[17].

We have attempted to improve the separations selectivity and separation efficiency by making use of silica gel mixed with microcrystalline silica (MCC) as stationary phase (mixed stationary phase) and succeeded in separating an additional component. MCC was chosen because there is possibility of chiral interactions between MCC and components of Manjistha, which may also have chiral centre. Silica does not have this advantage.

For chromatography, TLC grade silica (Acme's Laboratory Chemicals Silica Gel G, particle size: max. 76 μm), Column grade silica gel (Acme's Laboratory, 60–120

*For correspondence. (e-mail: pbsam2015@gmail.com)

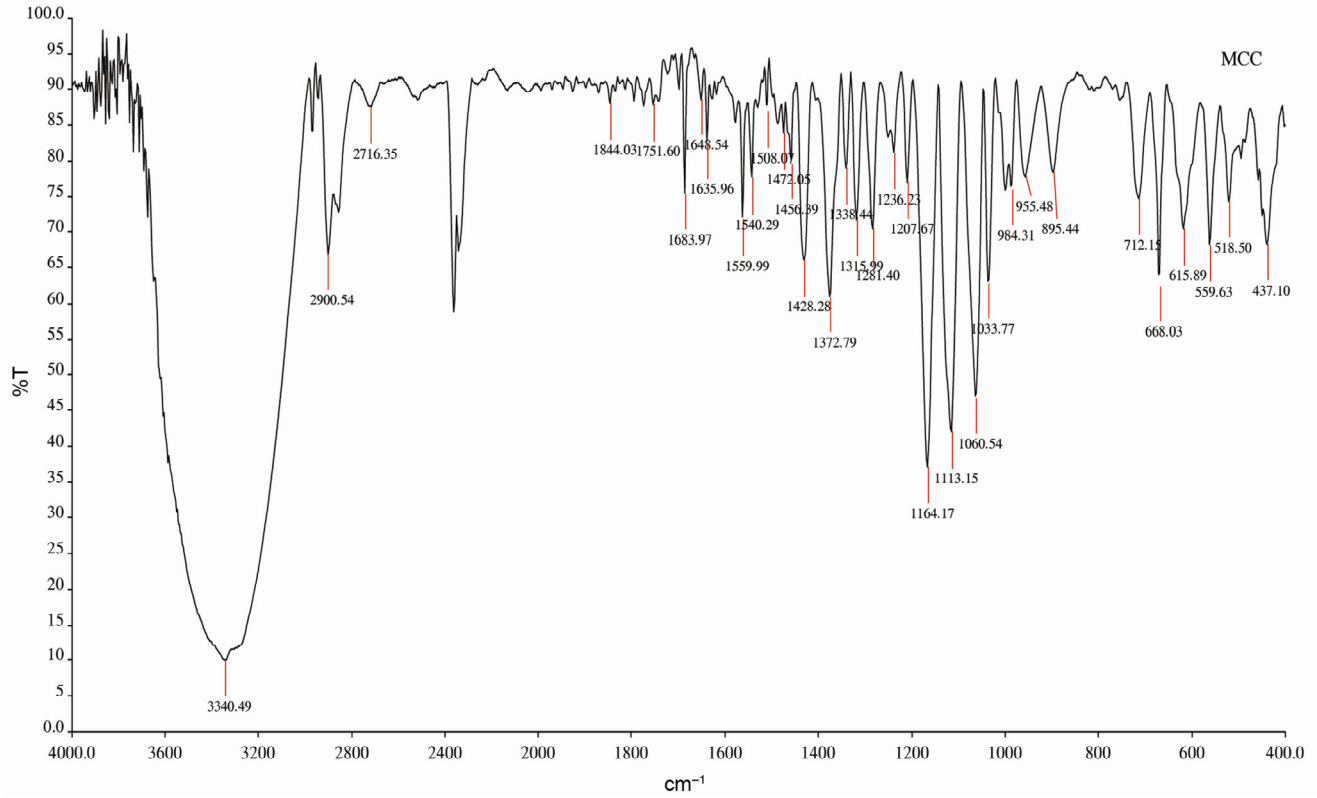
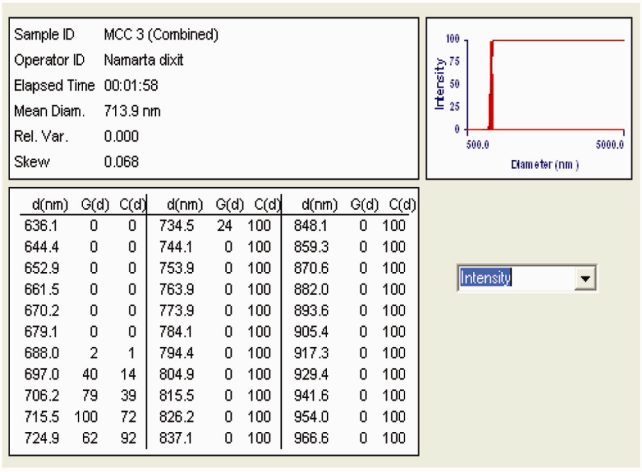



**Figure 1.** FT-IR spectrum of prepared MCC.



| | |
|---|---|
| Sample ID | MCC 3 (Combined) |
| Operator ID | Namarta dixit |
| Elapsed Time | 00:01:58 |
| Mean Diam. | 713.9 nm |
| Rel. Var. | 0.000 |
| Skew | 0.068 |



| d(nm) | G(d) | C(d) | d(nm) | G(d) | C(d) | d(nm) | G(d) | C(d) |
|---|---|---|---|---|---|---|---|---|
| 636.1 | 0 | 0 | 734.5 | 24 | 100 | 848.1 | 0 | 100 |
| 644.4 | 0 | 0 | 744.1 | 0 | 100 | 859.3 | 0 | 100 |
| 652.9 | 0 | 0 | 753.9 | 0 | 100 | 870.6 | 0 | 100 |
| 661.5 | 0 | 0 | 763.9 | 0 | 100 | 882.0 | 0 | 100 |
| 670.2 | 0 | 0 | 773.9 | 0 | 100 | 893.6 | 0 | 100 |
| 679.1 | 0 | 0 | 784.1 | 0 | 100 | 905.4 | 0 | 100 |
| 688.0 | 2 | 1 | 794.4 | 0 | 100 | 917.3 | 0 | 100 |
| 697.0 | 40 | 14 | 804.9 | 0 | 100 | 929.4 | 0 | 100 |
| 706.2 | 79 | 39 | 815.5 | 0 | 100 | 941.6 | 0 | 100 |
| 715.5 | 100 | 72 | 826.2 | 0 | 100 | 954.0 | 0 | 100 |
| 724.9 | 62 | 92 | 837.1 | 0 | 100 | 966.6 | 0 | 100 |

**Figure 2.** DLS data of prepared MCC.

mesh size) were used. All the other reagents and solvents used were of analytical grade. The solvents used were methanol, ethyl acetate, chloroform, pet ether (60–80°C), diethyl ether, toluene, glacialacetic acid, THF, hydrochloric acid (1 M to 6 M), ethanol, dichloromethane and acetonitrile. Technical grade acetone was used for cleaning glassware. Roots of Manjistha and commercial grade cotton were procured from local market. MCC used for comparison was from Sigma Aldrich. Glass slides with dimension 7.5 × 2.5 cm were used for preparing TLC plates in laboratory using pour and dry method.

Manjistha roots were vacuum dried for 4 h at 60°C followed by grinding in the domestic grinder. Dried powder of the roots (1 g) was taken in 150 ml conical flask, followed by 10 ml of extracting solvent (chloroform or methanol). The mixture was kept on a mechanical shaker for 24 h at 150 rpm. The extract was filtered using Whatman filter paper no. 1. The extracts of ME (methanol extract) and CE (chloroform extract) were subjected to TLC separation using numerous solvent systems on silica gel and mixed stationary phase.

All silica and mixed phase plates were prepared by using the slurry of stationary phase in ethyl acetate following pour and dry method. These plates were then allowed to dry at room temperature. The MCC TLC plates were prepared by making their slurry in distilled water and drying for 24 h at room temperature. Activation at elevated temperature was not performed and the plates were used as such.

In each case, 5 ml of the prepared solvent system was taken in the TLC chamber and was allowed to saturate for 5 min. The sample spot was placed on the dried plate and after half a min, the plate was kept in the TLC chamber, was taken out after about 7 min, allowed to dry in ambient conditions and observed in UV chamber.

MCC was prepared using the method reported earlier[18]. Keeping all the experimental conditions similar, MCC was prepared in more amounts and the yield obtained in each batch was around 80%.

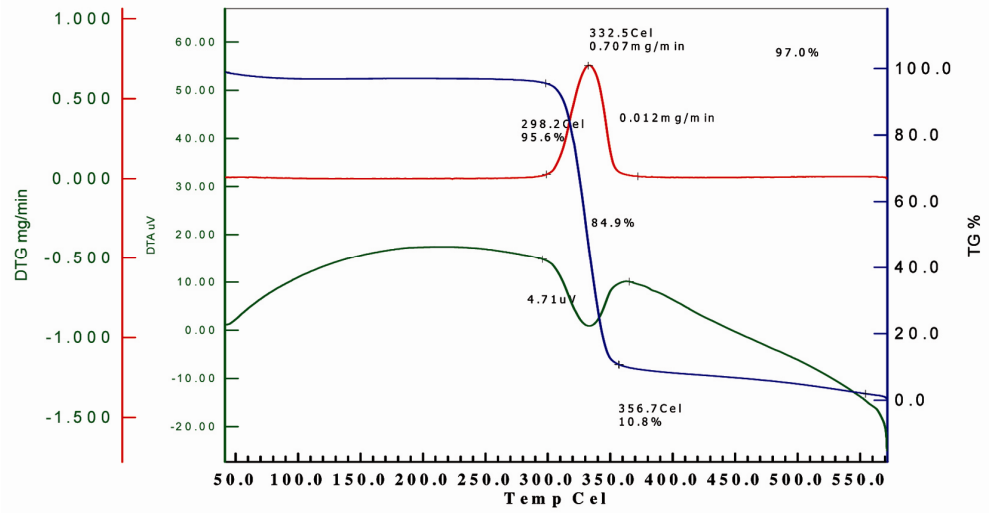



**Figure 3.** TGA data of prepared MCC.

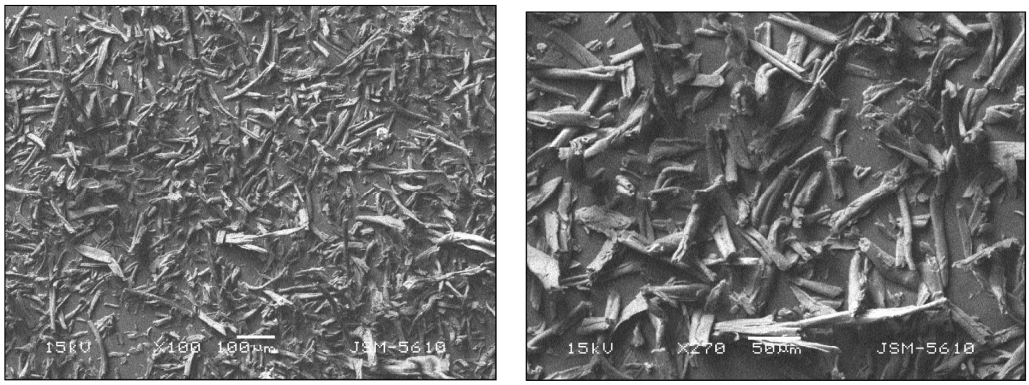



**Figure 4.** SEM image of MCC.

MCC sample was characterized by FTIR using Perkin Elmer FTIR, Fourier Transform Infrared Spectrometer, model RX1. For FTIR scan, KBr pellets of the sample were prepared. Spectra in the range 400–4000 $cm^{-1}$ were recorded. The analyses were done with all the prepared batches of MCC to check for batch-to-batch reproducibility which was satisfactory.

Thermo gravimetric analysis of MCC was done with the SII TG/DTA6300 EXSTAR. The analysis was carried out in nitrogen atmosphere or in presence of air. This was done to determine the thermal stability of prepared MCC in air as the active medium. The amount of MCC taken was 2.299 mg and was heated from 30°C to 550°C at a heating rate of 10°C $min^{-1}$. Derivative TG (DTG) curves expressed the weight-loss rate as a function of temperature.

Dynamic light scattering (DLS) analysis of MCC was done using A BIC 90 PLUS (BROOK HAVEN) instrument equipped with 35.0 mW solid state lasers operating at 660 nm with avalanche photo diode detector. Water was used as medium to obtain the mean particle diameter of prepared MCC.

The SEM analysis was carried out using JEOL JSM-5610 at an acceleration voltage of 15 kV.

The FTIR spectrum, DLS curve, thermogravimetric analysis (TGA) and DTG curve plot and SEM analysis for MCC are shown in the figures.

The FT-IR spectrum recorded for synthesized MCC showed resemblance to that of the commercial sample. Both the commercial and synthesized MCC exhibit the general characteristic spectrum of cellulose. The characteristic bands at 3300–3500, 2900, 1163 and 896 $cm^{-1}$ corresponding to $CH_2$ group, C–O–C stretching, C–H rock vibrations were observed. Absence of peaks located in the range 1509–1609 $cm^{-1}$, corresponding to C=C aromatic skeletal vibrations, indicated complete removal of lignin[19,20]. The absorption band which corresponds to either the acetyl or uronic ester groups of hemicelluloses normally appears in the region 1700–1740 $cm^{-1}$; this band is absent, indicating the removal of hemicelluloses[21] (Figure 1).

The DLS curve of the prepared MCC showed the mean particle diameter 0.715 μm as shown in Figure 2.

The analysis showed that there were no significant deviations from the reported data[18]. Also, no differences were observed when the analysis was carried out in air, rather than in nitrogen as reported in the literature. The

**Table 1.** Optimization of solvent system on silica gel

| Solvent system | $R_f$ values in UV | No. of spots observed |
|---|---|---|
| 100% EA | 0.1, 0.74, 0.84 | 3 |
| 100% $CHCl_3$ | 0.1, 0.46, 0.86 | 3 |
| 1.0 : 9.0 = EA : $CHCl_3$ | 0.86 | 1 |
| 1 : 1 = EA : $CHCl_3$ | 0.86 | 1 |
| 9.6 : 0.4 = PE : EA | 0.208, 0.354 (both with trail) | 2 |
| 8.0 : 2.0 = $CHCl_3$ : EA | 0.775 (trail) | 1 |
| 9.0 : 1.0 = $CHCl_3$ : EA | 0.86 | 1 |
| 9.5 : 0.5 = $CHCl_3$ : EA | 0.583 (trial), 0.75 | 2 |
| 9.5 : 0.5 = PE: EA | 0.26 (trail), 0.34, 0.40, 0.50, 0.80 | 4 |
| 9.2 : 0.8 = PE: EA | 0.36 (trail), 0.40, 0.56, 0.70 | 4 |
| 9.0 : 1.0 = PE: EA | 0.204 (trail), 0.306, 0.408, 0.571 | 4 |
| 9.4 : 0.6 = PE: EA | 0.208, 0.354 (both with trail) | 2 |
| 9.5 : 0.5 = PE: MeOH | 0.12, 0.18, 0.34 (trail), 0.50, 0.70, 0.90 | 3 |
| 9.1 : 0.9 = PE: MeOH | 0.14 (trail), 0.26, 0.42, 0.58 & 0.70 (trail), 0.84 | 4 |
| 9.1 : 0.9 = PE: EA | 0.22, 0.26 (trail), 0.36, 0.56, 0.76 | 3 |
| 8.5 : 1.5 = PE: EA | 0.20 (trail), 0.26, 0.34 and 0.50 (in trail), 0.82 | 3 |
| 100% $CHCl_3$ | 0.345 (trail), 0.418, 0.472 | 3 |
| 100% PE | 0.090, 0.20, 0.636 | 3 |
| 9.7 : 0.3 = PE: EA | 0.090 (trail) | 1 |
| 100% Acetone | 0.775 (trail) | 1 |

PE, Pet ether; EA, Ethyl acetate; ACN, Acetonitrile.

**Table 2.** Separations done on silica gel and mixed stationary phase TLC

| Sl. no. | Stationary phase | Solvent system | $R_f$ values in UV | No. of spots observed |
|---|---|---|---|---|
| 1. | MCC TLC plate | 9.3 : 0.7 = PE : MeOH | 0.30 (trail), 0.88 | 2 |
| 2. | 1 : 1 = MCC : Starch TLC plate | 9.3 : 0.7 = PE: MeOH | 0.30 (trail), 0.88 | 2 |
| 3. | MCC TLC plate | 9.3 : 0.7 = PE: EA | 0.775 (trail) | 1 |
| 4. | MCC TLC plate | 9.3 : 0.7 = PE: ACN (v/v) | 0.775 (trail) | 1 |
| 5. | 1 : 1 = MCC : Silica plate | 100% EtOH | 0.775 (trail) | 1 |
| 6. | 1 : 1 = MCC : Silica plate | 1 : 1 = MeOH : Acetone (v/v) | 0.775 (trail) | 1 |
| 7. | 1 : 1 = MCC : Silica plate | 100% MeOH | 0.775 (trail) | 1 |
| 8. | 100% MCC plate | 6.0:4.0 = MeOH : EA (v/v) | 0.775 (trail) | 1 |
| 9. | 2 : 8 = MCC : Silica plate | 9.3 : 0.7 = PE : EA (v/v) | 0.283 (trail), 0.349, 0.415, 0.622, 0.867 | 5 |
| 10. | 1 : 1 = MCC : Silica plate | 9.3 : 0.7 = PE: EA (v/v) | 0.775 (trail) | 1 |
| 11. | 1 : 9 = MCC : Silica plate | 9.3 : 0.7 = PE: EA (v/v) | 0.283 (trail), 0.349, 0.415, 0.622, 0.867 | 5 |
| 12. | 1.5 : 8.5 = MCC : Silica plate | 9.3 : 0.7 = PE: EA (v/v) | 0.30 (trail), 0.46, 0.58 | 3 |
| 13. | 100% MCC plate | 5.0 : 4.5 : 0.5 = DEE: EA : THF (v/v) | 0.86 | 1 |
| 14. | 100% MCC plate | 4.5 : 3.5 : 2.0 = DEE: EA : THF (v/v) | 0.775 (trail) | 1 |
| 15. | 1 : 9 = MCC : Silica plate | 10 ml 8.5 : 1.5 = PE: EA + 7 drops THF (v/v) | 0.30 (trail), 0.40, 0.47, 0.56, 0.82 | 5 |
| 16. | 1 : 9 = MCC : Silica plate | 8.0 : 2.0 = PE : EA + 7 drops THF (v/v) | 0.30 (trail), 0.46, 0.52, 0.60, 0.80 | 5 |
| 17. | 1 : 9 = MCC : Silica plate | 100% Acetone | 0.06 and 0.28 (trail), 0.82 | 3 |
| 18. | 1 : 9 = MCC : Silica plate | 9.0 : 1.0 = PE : EA (v/v) | 0.30 (trail), 0.38, 0.50, 0.62, 0.76 | 5 |
| 19. | Silica TLC plate | 10 ml 8.5 : 1.5 = PE : EA + 7 drops THF (v/v) | 0.207, 0.283, 0.377 (all 3 in trail), 0.716 | 4 |
| 20. | Silica TLC plate | 10 ml 8.0 : 2.0 = PE : EA + 7 drops THF | 0.1, 0.74, 0.84 | 3 |
| 21. | Silica TLC plate | Only acetone | 0.775 (trail) | 1 |
| 22. | Silica TLC plate | 9.0 : 1.0 = PE : EA (v/v) | 0.775 (trail) 0.306, 0.408, 0.571 | 4 |

PE, Pet ether; EA, Ethyl acetate; ACN, Acetonitrile; DEE, Diethyl ether; THF, Tetrahydrofuran.

first derivative peak is observed at 332.5°C which was close to the reported data (334°C) where the analysis was carried out in nitrogen (Figure 3).

SEM analysis for synthesized MCC shows changes in the morphology of the fibres in terms of size and level of smoothness after acid hydrolysis. It is however worth noticing, as seen in the figure, that acid hydrolysis altered the morphology of cellulose in MCC, which shows individualized and uniform fibres. This correlates with the spectroscopic evidence for the removal of cementing material around the fibre bundles; namely hemicelluloses and lignin. These studies thus establish the formation of MCC and its compliance with the literature report[22] (Figure 4).

The following classes of compounds were detected by standard tests for identification of ME and CE[23,24]. ME: Anthraquinones, carbohydrates, phenols, saponins, tannins, terpenoids, quinones, phlobatannins; CE: Anthraquinones, carbohydrates, phenols, tannins, glycosides. Alkaloids,

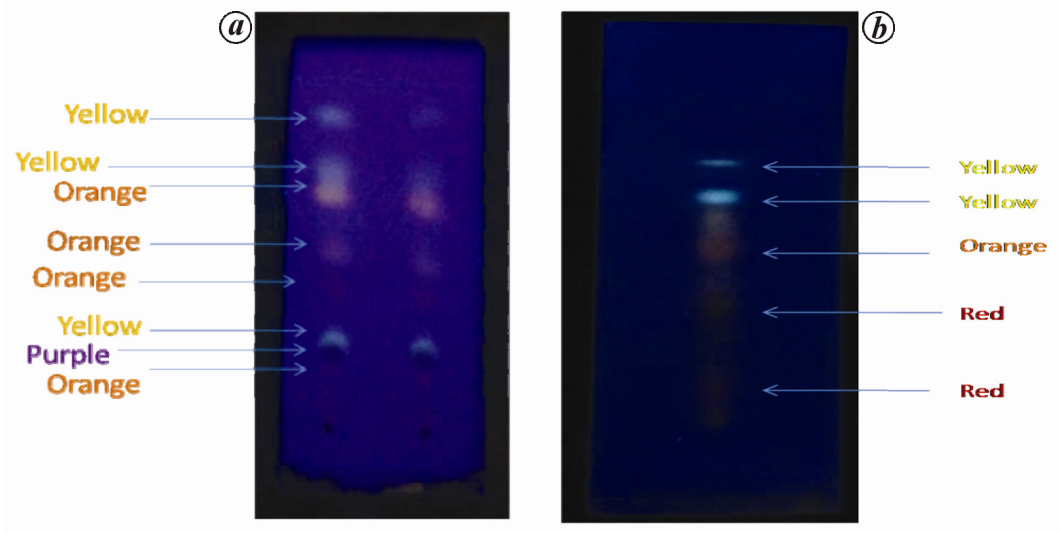



**Figure 5.** TLC separation of CE (***a***) and ME (***b***) on mixed stationary phase.

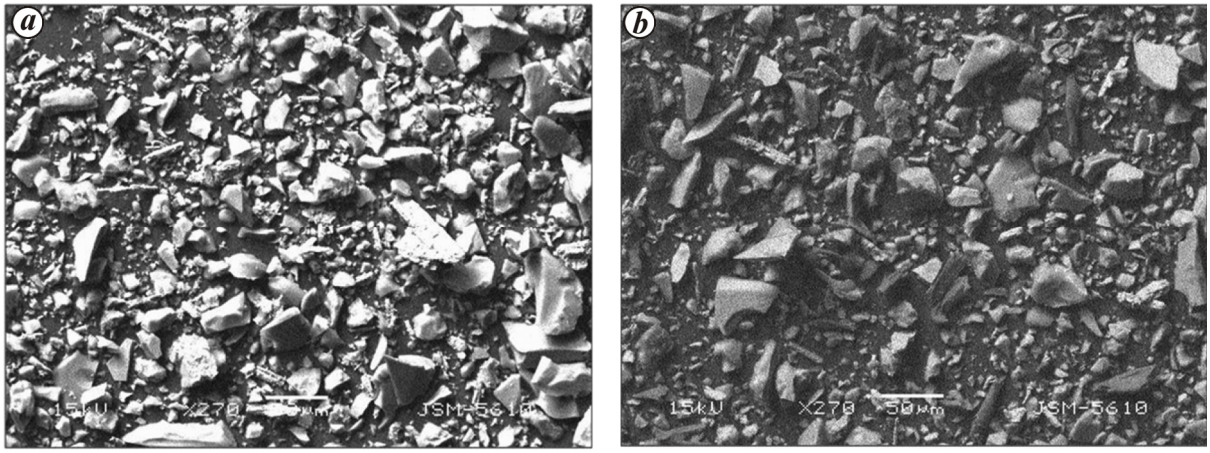



**Figure 6.** SEM data of silica gel (***a***) and mixed stationary phase (***b***).

saponins and steroids were also detected after removing the chloroform completely and reconstituting the residue with methanol.

Optimization of solvent systems for silica-based TLC separations was carried out and the $R_f$ values are shown in Table 1.

None of the solvent systems yielded good separation on silica gel. We also attempted improvement in the separation on non-silica-based single composition stationary phases and silica mixed with other components as mixed stationary phases along with the different solvent systems were employed for the fractionation of ME. For comparison separation was performed on filter paper (both ordinary and Whatman 1), but no separation was observed.

The different solvent systems employed for the fractionation of the methanolic extract on silica gel and other mixed stationary phase and their respective $R_f$ values are given in Table 2.

From Table 2 it is evident that 10% MCC in silica (cases 15 and 16) serves to be good stationary phase compared to only silica (cases 19 and 20). Also, tailing obtained on silica was significantly reduced on 10% MCC in silica, for instance in cases 16 and 19 respectively (Table 2). Also, in case of 9, 11 and 18 the number of components separated increased on mixed silica stationary phase compared to only silica. But as the composition of mobile phase becomes ternary, maintaining it for large scale separation as in for column chromatography may be tedious, hence we chose 9.0 : 1.0 = PE : EA as the mobile phase and performed column chromatography and the separated additional spot was analysed and identified as saponin using the standard identification tests. The new spot in the collected fraction was identified by comparing its TLC under the same conditions and matching its $R_f$.

For the chloroform extract, after optimization of the mobile phase system and the mixed stationary phase, the best separation was found in the composition of 73 : 23 = pet ether : methylene dichloride + 0.1 ml MeOH (v/v) and 5 : 95 (w/w) = MCC : silica respectively.

**Table 3.** Comparison of $R_f$ values of the separations of CE on 5% MCC in silica (w/w)

| Spots | $R_f$ value on 5% MCC in silica | $R_f$ value on 100% silica |
|---|---|---|
| Yellow | 0.983 | 0.982 |
| Yellow | 0.525 | 0.446 |
| Orange | 0.372 | 0.357 |
| Orange | 0.271 | 0.267 |
| Orange | 0.254 | – |
| Yellow | 0.169 | 0.178 |
| Purple (tailing observed) | 0.084 | 0.107 |
| Orange | 0.033 | 0.071 |

Table 3 shows the $R_f$ values and colour of spots for CE with the stationary phase as 5 : 95 = MCC : silica (w/w) and only silica. Figure 5 *a* shows the TLC plate under UV light for this separation (two same sample spots were run parallel). Figure 5 *b* shows the TLC plate under UV light for ME.

TLC plates using 100% starch and MCC : starch in 1 : 1 ratio were prepared by making the slurry in distilled water, but the layer breaks immediately on drying. Mixed TLC phase plate made from MCC and silica in ethyl acetate gives a mechanically stable and strong plate when dried at room temperature than the one which is dried in oven at 60°C for 2 h, as it breaks on drying producing many cracks on the plate. No binder is needed with MCC to make its TLC plates on glass slides with water.

The SEM analysis was performed to observe any changes in the morphology of silica due to MCC. It was observed that the mixed stationary phase does not show much morphological changes but the distribution of MCC in silica can be observed (Figure 6).

In this study, we have demonstrated potential of mixed phase stationary phase for TLC separation of phytochemical components. We could obtain an additional spot on the mixed stationary phase TLC plate in both the methanol extract and the chloroform extract which was not observed in only silica as the stationary phase. And in the case of methanol extract, it was identified as saponin by performing class test.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