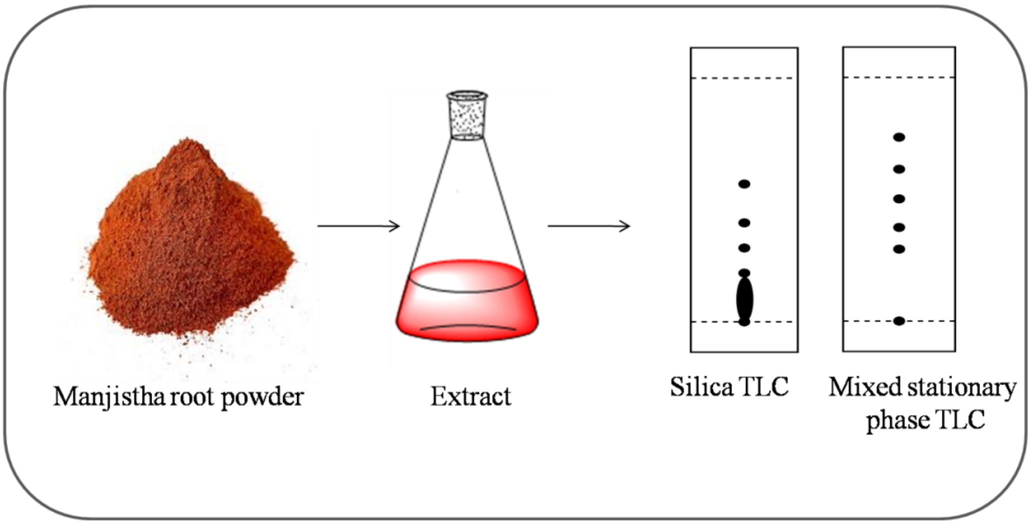
Chapter 2a

Development of Mixed Stationary Phase for TLC



This chapter describes development of mixed stationary phase for thin layer chromatography for the separation of chemical constituents of *Rubia cordifolia* L., from its extracts. Thin layer chromatography serves as the preliminary separation technique for complex mixture of constituents of medicinal plants. Medicinal plants are composed of not just one but a mixture of different classes of organic compounds. Their separation on TLC turns out as tail and not partitioned properly on polar silica gel TLC plates. With an aim to resolve these components efficiently on the TLC plates, addition of a moderately polar compound to silica has been made to develop mixed stationary phase TLC plates. One such material has been synthesized and characterized by analytical techniques.

Chapter 2a Development of Mixed Stationary Phase for TLC

2a. Introduction

2a.1 Importance of *Rubia cordifolia* L. as medicinal plant

Rubia cordifolia L., commonly known as Manjistha, belongs to the family Rubiaceae and has been used since ancient times for its medicinal value. It finds application in external use to heal wounds, used for glowing skin (used as face cleanser), and used in number of Ayurvedic preparations to treat skin, blood and urinary diseases [1]. It is a perennial, climbing plant that grows in the north western Himalayas and other hilly districts of India. All parts of the plant have been used as medicines with the roots being used predominantly. It has been a renowned blood purifier since the ancient times; and has been immensely used for problems related to skin, blood and urinary diseases [1]. Not only have this but the roots of Manjistha been used as dyes and pigments. The roots of *Rubia cordifolia* L. are known to have chemical constituents that have pharmacological properties and thus the extracts prepared from these roots have exhibited excellent pharmacological activities against cough, diabetes, tubercular conditions of skin, intermittent fevers and pharyngitis [1]. Plants (barks, leaves, roots, fruits) have been source of dyes and pigments since time immemorial, Apatani tribe who have traditionally settled in villages of Arunachal Pradesh, use Rubia cordifolia extensively [2]. *Rubia cordifolia* L. has not only been used in Ayurveda but it's been traditionally used as a medicinal plant in Chinese traditional medicine (TCM). It's been officially collated with the name Qian-cao in Chinese Pharmacopoeia (2015) [3]. Since ancient times, it has been used for neuroprotection and vascular disease in China. Medicinal plants contain different classes of bioactive compounds such as anthraquinones and their glycosides, bicyclic hexapeptides, naphthoquinones, terpenes, carboxylic acids, phenols and polysaccharides, coumarins, flavanoids, alkaloids, which are found to be the reasons for all the biological activity these medicinal plants exhibit. These secondary metabolites are compounds produced by plants which are indirectly involved in their normal growth, development or reproduction of the organism.

Secondary metabolites often play an important role in plant defence against herbivore and other interspecies defences. *Rubia cordifolia* L. is known for its colorant properties because of the large number of anthraquinones present in it. *Rubia cordifolia* L. has been used extensively for the pharmacological properties it exhibits. The root powder and its extract have been used to treat ailments in Ayurveda. It is known to be an astringent, thermogenic, antidysenteric, anti-inflammatory, antipyretic, analgesic, antiseptic, constipating, diuretic [1].

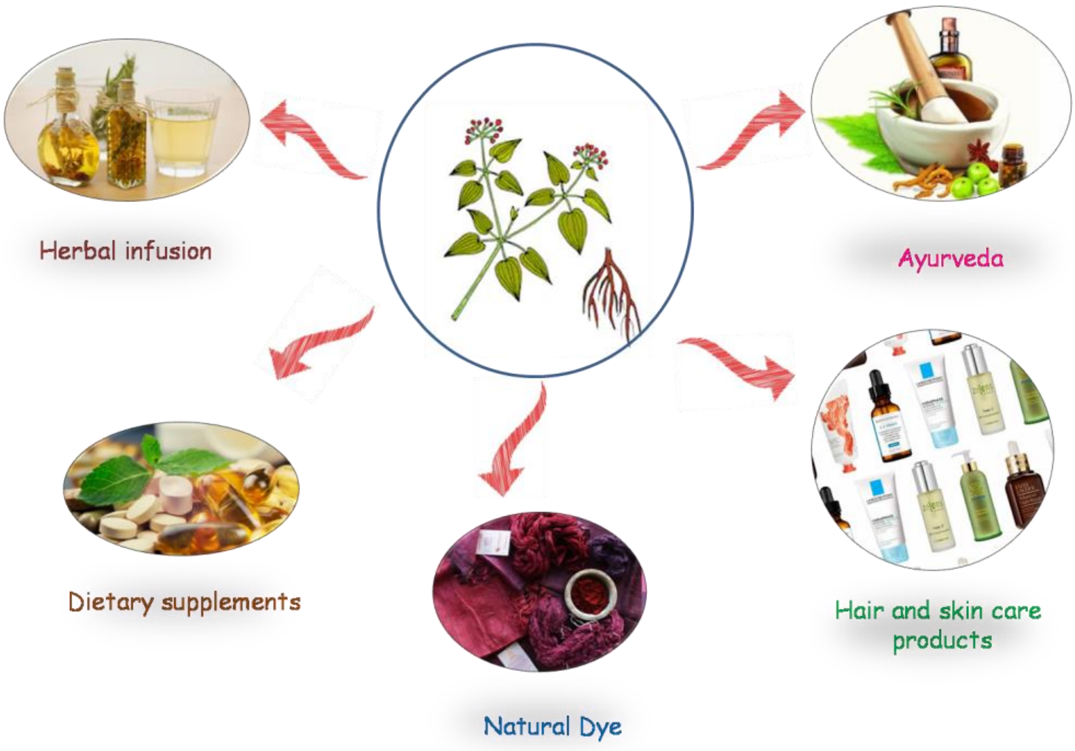


Figure 1 Applications of *Rubia cordifolia* L.

Dried roots of the plant are used as astringent and diuretic. Traditionally, they have been used for the treatment of dropsy, paralysis, jaundice, amenorrhea, and visceral obstructions [4]. Tripathi et al reported the alcohol extract of R. cordifolia possessed anti-PAF activity in 1993 [5] and potato-lipoxygenases inhibitory activity in 1995 [6]. Ojha and Dwivedi revealed the beneficial action of R. cordifolia roots against non-healing diabetic foot ulcer by oral administration anddipping the foot in R. cordifolia decoction healed 90% of the patients within 4–5 weeks of treatment by clinical trials. In 1999 it was proved that the ethanol extract of aerial parts of the plant has hypoglycemic activity in albino rats [7].

It has been used directly as root powder, as Churna, dietary supplements tablets, oil, face masks, etc. which are available commercially as exhibited in figure 2. In short, *Rubia cordifolia* L. has tremendous medicinal properties and therefore can be called a total healthcare herb.

Figure 2 Commercially available products of *Rubia cordifolia* L.

2a.2.Mixed stationary phase thin layer chromatography

Thin layer chromatography abbreviated and popularly called TLC has been used constantly in laboratories to monitor reactions. It was developed and routinely used by Kirchner and his group since 1951 and Stahl five years later developed standardized equipments and techniques [8]. Whether the reactions have reached completion or the starting material of the reaction is fully consumed to yield a product can be confirmed via a simple force of capillary action of the mobile phase which is partitioned on a stationary phase (thin layer). TLC owes its maximum use in laboratories (chemical, forensic, biomedical, pharmaceutical, biochemistry, etc) to the following reasons: cost effective, easy handling, fast determination and results, visual results (in case of compounds falling in visible range) or in UV chamber and observing the inactive ones either in UV chamber or by application of I_2 powder. These reasons are the main cause of the popularity of TLC. Thin layer chromatography is applicable for the preliminary determination of whether a chemical reaction has proceeded or not. The simplicity of the technique, its cost per sample and the application of number of spots on it at a time and giving results simultaneously has led to its application as the primary step for a chemical reaction. In short, TLC is an economical screening technique [9]. Not only this but it serves as the primary step or as a stepping stone for separation of the resolved constituents to a large scale that is achieved by column chromatography, the initial experiments performed on TLC are helpful in determining mobile phase for column chromatography. Co-spotting the starting material of the reaction with that of the reaction mixture taken out during the reaction helps understand whether the reaction has reached its completion as the starting material would be fully consumed.

The principle behind TLC is "adsorption" of the solute particles which are partitioned based on their polarity through mobile phase by capillary action. Silica has been the most widely and extensively used stationary phase, a universal stationary phase with its application to all types of compounds. The other stationary phases that are used are alumina, kieselguhr, magnesium oxide, florisil (magnesium silicate) and cellulose. These all help to resolve the chemical constituents from a mixture [10]. Up scaling TLC has led to the development of preparative TLC. Application of TLC are not limited to just monitoring chemical reactions but also to pesticide residue analysis. It was this planar chromatography (TLC) which served as the stepping stone for the development of high performance thin layer chromatography (HPTLC) in 1975 [11] which presented better resolution, the method took shorter development time and lower detection limit were achieved. TLC can be qualitative, semi-quantitative and quantitative. Thin-layer chromatography was used for the separation of lipophilic substances for the first time and then the adsorbent application was further extended to hydrophilic substances [12]. With the development in the technique over the years, new adsorbents are available but the most predominantly used being uncoated silica or biphasic TLC plate (two side by side stationary phase). Also, the modification involves developing the TLC plates in two mobile phases. Or developing the TLC plate initially in a mobile phase vertically such that the resolved spots are then subjected to be developed in the same or different mobile phase by placing the already developed TLC plate horizontally, which is termed as two dimensional (2D) - TLC development as shown in figure 3.

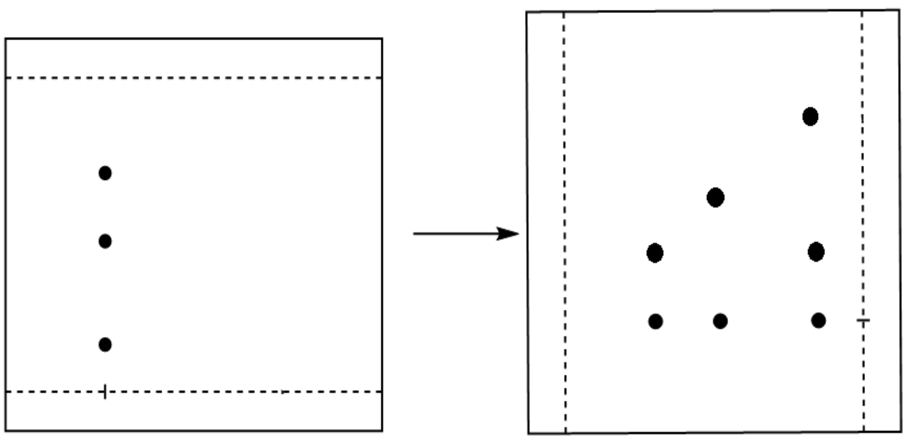


Figure 3 Two Dimensional (2D) TLC development

Two side by side stationary phases was developed in 1978. It was developed to achieve two different selectivity phase by dipping a pre-coated silica gel plate up to half its height in a silylating agent. And this plate was used for the separation of mixture of oxidation products of cholesterol which were developed using 2D dimensional thin layer chromatography (figure 3). The advantage provided by this mode was that it allowed cleanup of the sample to occur on the silicagel side using organic solvents, which were easierto dry at room temperature than water. This plate was usedfor resolving the components antineoplastic bryostatin 1 and bryostatin 2 from *Bugulu neritina* [9] as shown in figure 4.

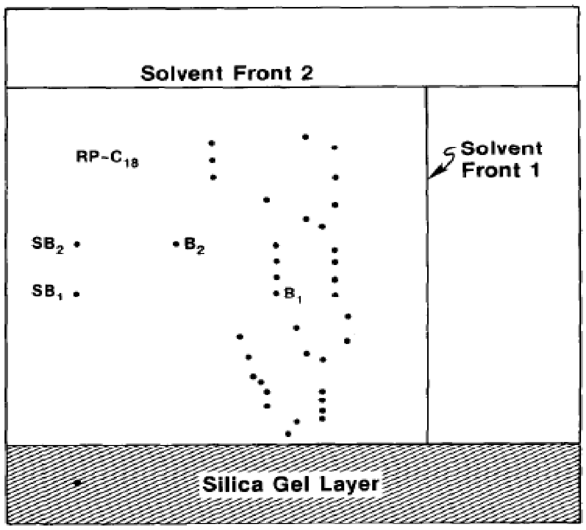


Figure 4 Multimodal side by side separation of Bryostatin 1 and Bryostatin 2 [figure from ref 9]

Thin layer chromatography is considered a useful tool in the characterisation of plant extracts. Typically, the chromatogram is used as fingerprint for comparison and identification of material as a way of quality control without having to identify each component. HPLC can be used for separation, however, only marker compounds are generally identified. In that sense, fingerprinting by TLC or HPTLC is more convenient tool that can be used on routine bases for quality control purposes.

With this view point we made an attempt to develop mixed stationary phase thin layer chromatography so that polar nature of the highly polar silica gel can reduced. As phytochemicals are a complex mixture of compounds of varying polarities and chiral compounds which tend to adhere very strongly on only silica gel.

Stationary phases containing two or more kinds of adsorbents (with different functional group or donor sets) are mixed in a controlled proportion [13] to enhance the separation of phytochemical constituents which tend to adhere strongly on silica gel. The success of separation of a complex mixture greatly depends on the choice of the stationary phase. The separation (chemical and physical) properties of two different types of adsorbents are combined into a mixed stationary phase that can provide better resolution. It is capable of providing good resolution for target analytes which are retained most strongly on any of the parent individual adsorbents. Need is felt for modifying the selectivity of stationary phase, since Phytochemicals are a complex mixture of different organic compounds. Mixing of small proportions of other material in silica gel as stationary phase is expected to give improvement in selectivity and resolution. In literature, analyses of food components on unconventional stationary phases such as starch and talc layers having specific characteristics compared with silica gel have been described [14]. Penicillium fungal extract has been resolved on cyano derivatized silica gel TLC plate using 2D development [15], first dimension in methylene chloride: hexane: acetic acid in 9:10:1 ratio respectively and then developed in the second dimension in CH_3CN: CH_3OH: H_2O in the ratio of 40:37:32. Literature shows use of ternary solvents for the separation of phytochemicals with toxic solvent such as benzene [16]. This can be avoided if selectivity of the stationary phase is improved. Therefore, addition of different materials like lignin, fuller's earth and microcrystalline cellulose to silica gel has been studied for the improvement in resolution of separation of various components in extracts prepared from roots of *Rubia cordifolia* in various solvents.

Other materials screened for mixed stationary phase in this work were lignin and fuller's earth. The word Lignin is derived from the Latin word *lignum* which means wood. Lignins are multifunctional phenolic polymers containing hydroxyl, carboxyl and carbonyl groups. These natural polymers have a 3D (three dimensional) structure when isolated from wood by acid hydrolysis [17]. Fuller's earth is essentially a hydrousaluminium silicate containing small proportions of lime, magnesia, alkalies and iron oxide [18]. These structural properties of the material can attribute to the separation of chemical constituents from phytochemicals as they can bind to the stationary phase through hydrogen bonding, chirality, intermolecular or metal-ligand bonding and thus can efficiently help in resolving the compounds better which otherwise may not resolve on the pure silica stationary phase.

We chose fuller's earth because it is known to contain traces of metals which can affect separation process in TLC. Similarly, choice of lignin and microcrystalline cellulose was based on the fact that both the materials contain chiral centres and it is expected that this should influence separation of phytochemicals, many of which are chiral.

2a.2.1. Materials and instrumentation

All the chemicals and solvents used for the synthesis, characterization and extraction were of analytical grade and used as purchased. TLC grade silica (Acme's Laboratory Chemicals Silica Gel G (100 – 200 mesh size), particle size: max.76μ) was used for Thin layer chromatography. Analytical grade solvents methanol, ethyl acetate, chloroform, Petroleum ether (60-80°C), diethyl ether, toluene, glacial acetic acid, THF, hydrochloric acid (1M to 6M), ethanol, dichloromethane, acetonitrile were used as such. Lignin (analytical grade) was purchased from TCI chemicals and Fuller's earth stone was purchased from local shop, which was ground and sieved before use. For cleaning glassware technical grade acetone was used. Commercial grade cotton and roots of Manjistha were procured from Manikarnika Aushadhalaya, Pune. Micro crystalline cellulose (MCC) from Sigma Aldrich was used for comparison. In laboratory TLC plates were prepared using pour and dry method, glass slides of dimensions 7.5cm × 2.5 cm were used for preparing TLC plates.

The plant material was identified and duly authenticated from Botany department, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, which has been preserved as herbarium with the reference no. NK 03.

The roots of *Rubia cordifolia* L. were washed extensively with tap water and then with distilled water to ensure the removal of soil and dust particles. They were first sun dried and then dried at 60 °C under vacuum for 24 hours and then ground to fine powder in domestic mixer grinder, sieved through 0.425 mm mesh for the preparation of extracts.

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 and spectra in the range of 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 found to be satisfactory. Thermo gravimetric analysis of MCC was done with the SII TG/DTA6300 EXSTAR. The analysis was carried out in N_2 atmosphere and the amount of MCC taken was 2.299 mg which was heated from 30º to 550º C at a heating rate of 10º $Cmin^{-1}$. 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.

2a.2.2. Method

MCC was synthesized in laboratory from commercially available cotton. By modifying the method described by Chauhan et al, we have performed the hydrolysis of cotton using 2.5 M HCl. This results into partial hydrolysis of the cotton fibre to result in microcrystalline cellulose.

The synthesis method was optimized by changing various conditions of reaction temperature and acid strength so as to get better yield and quality of the final material.

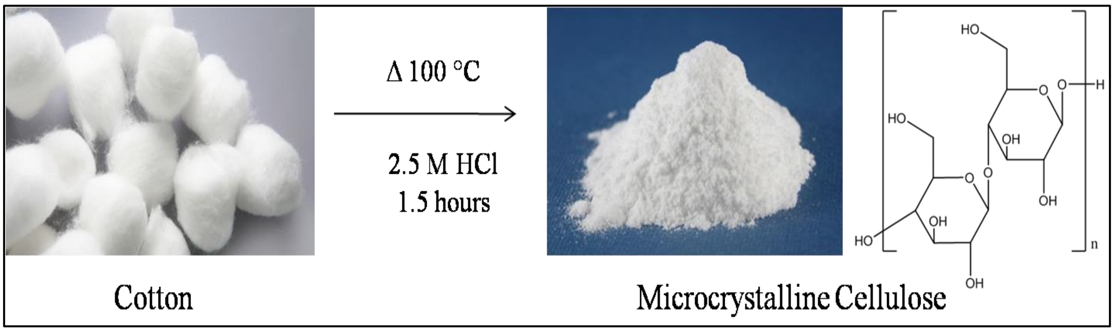


Figure 5 Synthesis scheme for microcrystalline cellulose (MCC)

The MCC prepared was characterized using FT-IR, TGA analysis, XRD analysis, and SEM analysis. Since we wanted to have control over the properties of MCC as we were not using pre-coated TLC plates, we have synthesized and optimized the parameters (as shown in table 1, results and discussion) for synthesis of MCC. Further, the morphology of prepared MCC was studied by means of SEM analysis. To study the particle size of prepared MCC, Dynamic light scattering (DLS) study was carried out.

To extract the components of *Rubia cordifolia* L. 1.0 g root powder was weighed in a stopper conical flask to which 10 mL solvent (CH_3OH and $CHCl_3$used respectively) was added and kept for extraction for 24 hours with occasional stirring. After 24 hours, it was filtered with Whatmann filter paper® 1 and used for analysis. Secondary metabolites were determined in all these extracts.

In order to prepare TLC plates MCC was mixed with silica gel in different weight proportions, which were used to resolve the constituents of *Rubia cordifolia* L. The extracts (CH_3OH, $CHCl_3$) prepared were administered as a spot on the prepared mixed stationary phase TLC plates. Development was carried out in different solvent systems which showed different R_F values.

2a.2.3. Results and discussion

To determine the presence of secondary metabolites in Manjistha, the root powder was treated with solvents to prepare their extract and then performed the identification tests for determining the classes of secondary metabolites in all the extracts, which are tabulated in the table below.

Class	***H_2O extract***	***CH_3OH extract***	***$CHCl_3$ extract***
Alkaloids	-	-	-
Carbohydrates	+	+	-
Anthraquinone	+	+	+
Flavanoids	-	-	-
Phenols	-	+	-
Saponins	+	+	+
Coumarins	-	-	-
Terpenoids	-	+	-
Tannins	-	-	-

Table 1 Class tests for Manjistha extracts (+ = positive; - = negative)

Microcrystalline cellulose, an important cellulose derivative is a white, fine, odourless and bio-degradable material. It is manufactured industrially by acid hydrolysis of wood cellulose. It has a unique advantage of being relatively mild stationary phase compared to silica gel and also shows good separation of chiral compounds. The adsorption of chemical constituents occurs through hydrogen bonding with the abundant aliphatic hydroxyls and some carboxyl groups present in MCC. It is mainly used for separation of compounds such as amino acids, carboxylic acids, carbohydrates, nucleic acid derivatives, tetracyclines, phosphates, lactones [20].

Our aim was to use solvents which are not toxic like benzene and instead make modifications to the stationary phase to achieve better resolution of mixture of phytochemicals. The properties that MCC offers are as follows:

- Binder not required.
- Needs no activation
- Slurry spreads evenly

- Plates don't crumble
- Adheres firmly, that plates can be stacked one upon another
- Cellulose and derivatives are natural chiral sorbents for enantiomeric separation.

Owing to these properties exhibited by MCC, it was chosen and mixed with silica gel to prepare mixed stationary phase. The prepared MCC was fully characterized by spectral and analytical techniques. The parameters were optimized for the synthesis of MCC and it was found that method M4 gave us the best results and it was chosen for the preparation of MCC and its characterization.

Method	Cotton (g)	100 mL Acid Strength (M)	Δ time (Hrs)	T (°C)	% yield
M1	5	3.0	1.5	100	72.04
M2	5	2.5	3.0	85	71.45
M3	5	3.0	2.5	85	75.30
M4	5	2.5	1.5	100	79.00

Table 2 Optimization of parameters for synthesis of MCC

The FT-IR spectrum of prepared microcrystalline cellulose has been shown in figure 6. The characteristic peak at 1113 cm^{-1} (1120 cm^{-1}) is attributed to C-O-C stretch of β-1, 4-glycosidic linkage in cellulose. The wide absorption band at 3341 cm^{-1} is due to -OH stretching and 2900 cm^{-1} is due to $-CH_2$ groups [21]. The absorbance peak observed at 1,164 cm^{-1} (1,160 cm^{-1} reported in literature) is assigned to –C–O– stretching vibration within the anhydroglucose ring. The absorption peaks at 1635 cm^{-1} and around 1640 cm^{-1} correspond to water absorption as a consequence of presence of strong cellulose-water interaction or bending mode of absorbed water in cellulose.A characteristic that all samples have cellulose I crystals is proved by the $-CH_2$ scissors mode observed at 1428 cm^{-1} (1,429 cm^{-1}). Absence of vibration at 750 cm^{-1} (characteristic of cellulose I_β), along with the shoulder at 710 cm^{-1} in all spectra helped us determine and establish that the crystalline structure of I_α, which consists of a parallel alignment of the chains in a monoclinic arrangement). A peak at 3,270 cm^{-1} which is overlapped by the strong stretching vibration of –OH is another indication (fingerprint) of I_α [22]. The removal of hemicelluloses was indicated by the absence of absorption band in the region 1700–1740 cm^{-1} which corresponds to either the acetyl or uronic ester groups of hemicelluloses. FT-IR absorbance peaks are tabulated in table 3 for the synthesis method of MCC. The peak at 2900 cm^{-1} was due to C-H stretching.

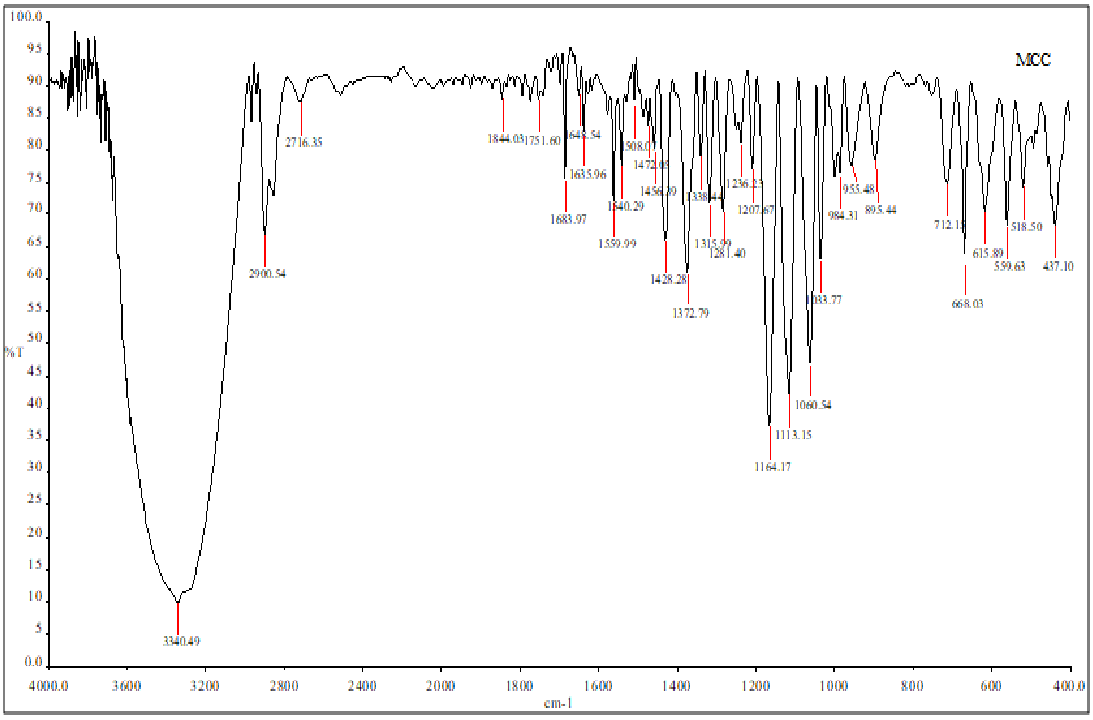


Figure 6 FT-IR spectrum of microcrystalline cellulose (MCC)

Method	*– OH stretch (cm^{-1})*	*v –C-O-C cm^{-1}str.*	*-CH_2 bending*	*–C-O cm^{-1} stretch*	*v C-H cm^{-1}str.*	*v C-Hcm^{-1}rocking vib*
M1	3341.19	1113.77	1429.41	1163.97	2901.31	895.83
M2	3344.17	1113.07	1430.66	1164.96	2899.68	894.87
M3	3347.92	1112.59	1431.05	1163.82	2901.14	895.74
M4	3340.49	1113.15	1428.28	1164.17	2900.54	895.44

Table 3 FT-IR peaks for synthesis method of MCC

Thermogravimetric analysis of prepared MCC was performed to investigate the thermal stability and compare it with that of literature. The minor loss observed at 100 °C was attributed to the loss (or desorption) of H_2O from polysaccharide. Also, the maximum degradation of the cellulose fibre was observed between 300 °C to 400 °C. The temperature at the maximum degradation was 332.5 °C which was comparable with the literature value 328.5 °C for MCC. This maximum degradation was due to the decomposition of cellulose. The thermal stability of the acid treated MCC was maintained [23] in all the cases (M1 to M4).

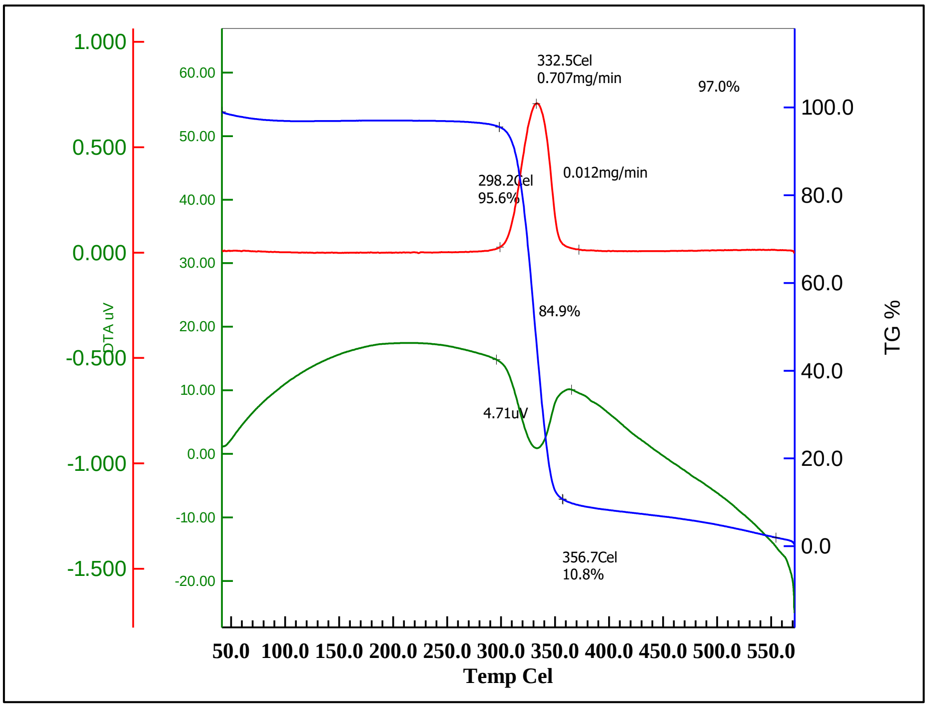


Figure 7 TGA of MCC (by M4)

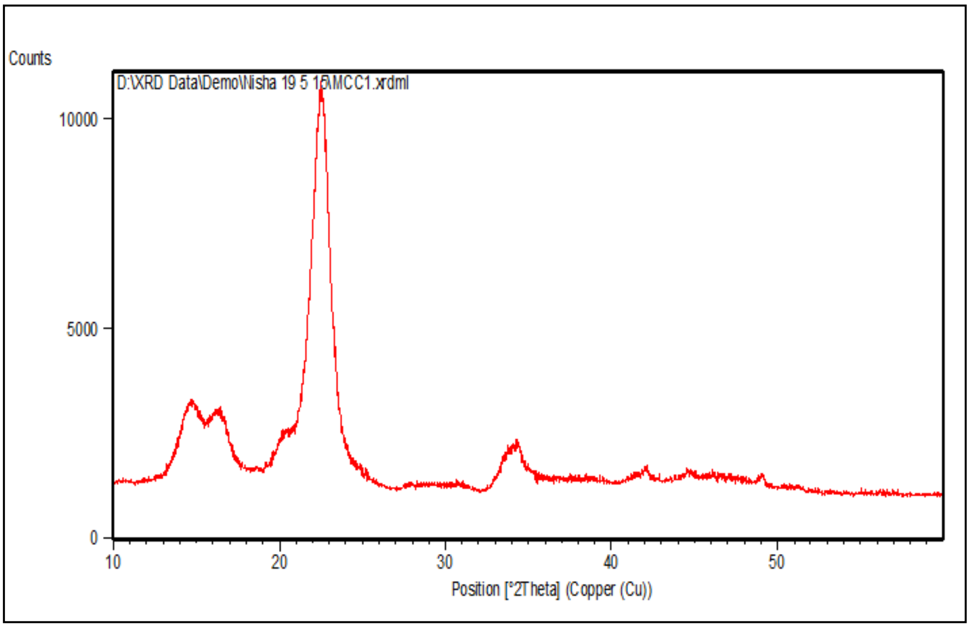
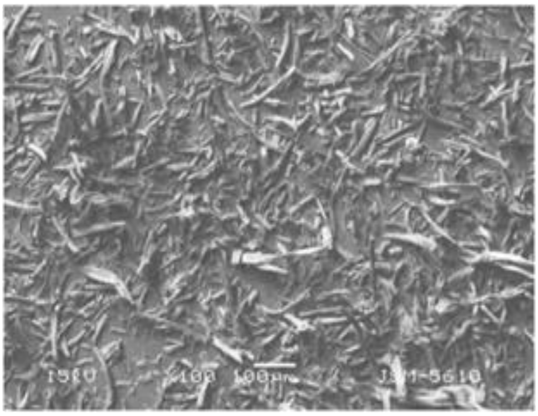


Figure 8 XRD pattern of MCC

X-ray diffraction study of the prepared MCC was carried out and it was observed that there were four main peaks present at 2θ = 14.54°, 16.38°, 22.51° and 34.5° corresponding to the cellulose I crystals where the parallel chains are strongly intermolecularly hydrogen bonded [22, 24]. These are the main characteristic peaks localized at 14.9°, 16.8°, 22.8° and 34.5° for the reference, which are assigned to the diffraction planes of 101, 101, 002 and 040 respectively [25]. Crystallinity index was calculated to be 90.06% which was comparable with literature value of 87.1%.

SEM analysis of the prepared MCC was carried out to determine and confirm if the prepared MCC had similar morphology as that of commercial or literature reported MCC.



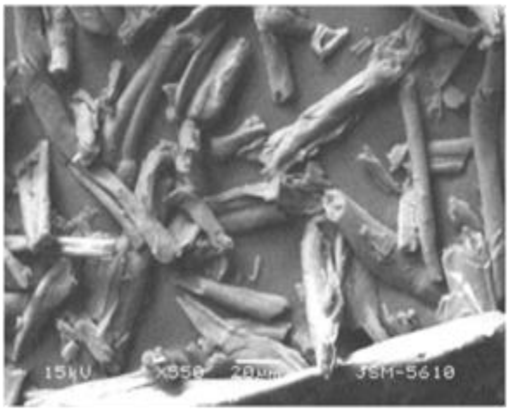


Figure 9 SEM micrographs for MCC

The prepared MCC displayed irregularly shaped fibrils which were aggregated and each fibril had a rough surface morphology, with presumably lower aspect ratios. From the literature reports it was suggested that these aggregated MCC are composed of strong hydrogen bonding between hundreds of individual cellulose whiskers [26].

Method for MCC preparation	Mean particle diameter from DLS
M3	1.131 µm
M2	0.796 µm
M1	0.823 µm
M4	0.715 µm

Table 4 Particle size of MCC determined by DLS

The particle size determined by Dynamic light scattering studies exhibited that MCC prepared by M4 was 0.715 µm. The particle size was smaller than the MCC prepared by other three methods. Particle size has strong bearing on the separation efficiency. Thus, the particle size for MCC achieved by M4 can prove to be efficient in achieving better separation. Therefore, these data and studies exhibit the formation of MCC and found to be in agreement with the literature reports.

TLC plates were prepared by mixing MCC to silica in different ratios (w/w) to resolve the constituents of Manjistha which was compared with that of TLC plate prepared using only silica. Manjistha extracts prepared in solvents such as CH_3OH and $CHCl_3$ were subjected to separation on silica and mixed stationary phase TLC plates. The different stationary phase that were studied and application of these plates on separation of methanol extract of Manjistha was achieved and compared in terms of their retention factor R_F, tabulated in the table 5.

SEM analysis of the mixed stationary phase from TLC plate (taken from TLC plate) was carried out to observe if the prepared stationary phase exhibited distribution of MCC in silica and if there was any change in the morphology of silica.

Figure 10 SEM analysis of MCC

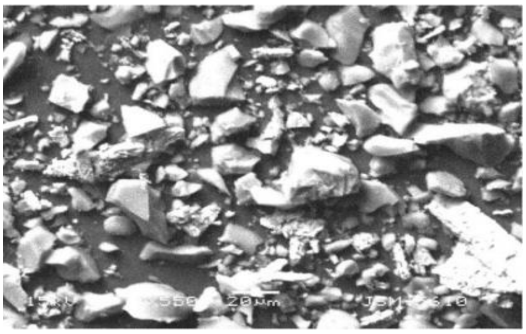


Figure 11 SEM analysis of Silica

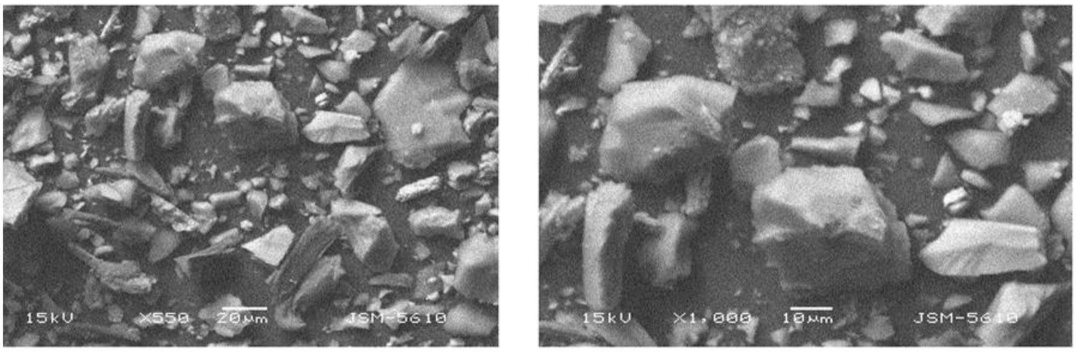


Figure 12 SEM image of MCC + Silica

Scanning electron microscopic image for the mixed stationary phase exhibited that there was no change in the morphological properties of both silica and MCC. Micro fibrils of MCC were found to be distributed within the silica matrix (figure 12).

The modifications done by changing the composition of mobile phase and developing the components on silica TLC plate were studied. Polarity of mobile phase was varied by combination of polar and non-polar solvents to resolve the chemical constituents of Manjistha. But in most cases, spots with tail were observed.

Solvent system	R_F values
100 % EA	0.10, 0.74, 0.84
100 % $CHCl_3$	0.34 (tail), 0.41, 0.47
90 : 10 = $CHCl_3$: EA	0.86
100 % PE	0.09, 0.20, 0.64
96 : 4 = PE: EA	0.208, 0.354 (both with tail)
92 : 8 PE:EA	0.36 (tail), 0.40, 0.56, 0.70
90 : 10 = PE : EA	0.204 (tail), 0.306, 0.408, 0.571
85 : 15 = PE : EA	0.26, 0.34, 0.50 (all in tail), 0.82
95 : 5 = $CHCl_3$: EA	0.583 (trial), 0.75
95 :5 = PE: CH_3OH	0.18 (tail), 0.34 (tail), 0.50, 0.70

Table 5 Effect of solvent polarity on separation. (EA: ethyl acetate, PE: Petroleum ether)

We then attempted to separate components of methanol extract by changing the stationary phase. We wanted to observe the effect of change in stationary phase by keeping the mobile phase same in all the cases of Petroleum ether and ethyl acetate in the ratio of 90: 10 respectively.

Stationary phase (SP) (w/w)	R_F values		
100% MCC plate	0.775 (tail)	-	-
Whatmann filter paper 1	No separation		
100 % Silica	0.90 (tail)	-	-
1:1= MCC : Silica	0.30 (tail)	0.88	
1:9 =MCC : Silica	0.30 (tail)	0.78	-
1.5:8.5 =MCC : Starch	0.30 (tail)	0.46	0.58

Table 6 Comparison of stationary phase for methanol extract

These results led us to modify the stationary phase to mixed stationary phase to separate the component. Comparisons of R_F values observed for the methanolic extract of *Rubia cordifolia* L. on MCC, silica gel and mixed TLC plates have been demonstrated. Mobile phase of Petroleum ether and ethyl acetate in the ratio of 90:10 (v/v) respectively was used to separate and develop the components on TLC plate of methanol extract (table 7) and chloroform extract of Manjistha (table 8).

R_F value on 100% silica plate	R_F value on 10% MCC in silica plate
0.204 (tail)	0.30
0.306	0.38
0.408	0.50
0.571	0.62
-	0.76

Table 7 Methanol extract of Manjistha on MCC –silica TLC plate

R_F value on 100% silica plate	R_F value on 5% MCC in silica plate
0.982	0.983
0.446	0.525
0.357	0.372
0.267	0.271
-	0.254
0.178	0.169
0.107	0.084
0.071	0.033

Table 8 Chloroform extract of Manjistha on MCC – silica TLC plate

We then investigated another material, Fuller's earth, to observe any enhancement in the separation efficiency. Powdered fuller's earth was added to silica gel, mixed well and its slurry was applied to glass slide. The effect of particle size of fuller's earth added to silica gel was studied. For this in one case the fuller's earth was sonicated using an ultrasonication system and in the other we used it directly keeping the weight ratio of fuller's earth to silica gel constant in both the cases. The particle size of the fuller's earth dispersed in ethyl acetate was measured by Dynamic light scattering (DLS).

Fuller's earth	**Mean particle diameter (µm)**
With sonication	0.799
Without sonication	1.325

Table 9 Particle size of fuller's earth

Sonication decreases the particle size of fuller's earth (FE), which was used as adsorbent as it provides more surface area, which in turn helps resolve the compounds efficiently. Thus, we could achieve separation of constituents of chloroform extract of *Rubia cordifolia* L. on mixed stationary phase TLC plates. In this case, the mobile phase for developing the components was Petroleum ether and ethyl acetate in the ratio of 92: 8 respectively; R_F values are tabulated in table 10.

R_F value on 100% silica plate	R_F value on 0.5% FE (w/o sonication) in silica plate	R_F value on 0.5% FE (with sonication) in silica plate
0.462	0.454	0.448
0.570	0.563	0.603
0.660	0.672	0.689
0.761	0.836	0.758
-	-	0.896
0.973	0.963	0.982

Table 10 Chloroform extract of Manjistha on FE – silica TLC plate

R_F value on 100 % silica plate	R_F value on 0.5 % lignin in silica plate
0.942	0.977
0.854	0.889
-	0.834
0.683	0.756
0.362	0.400

Table 11Methanol extract of Manjistha on lignin – silica TLC plate

Thus, we were able to achieve better resolution of components of Manjistha on these mixed stationary phase TLC plates. The tailing of spots also reduced and additional spots were resolved.

Conclusion

The prepared MCC was fully characterized using spectroscopic and analytical techniques. We have achieved good separation of phytochemical constituents on mixed stationary TLC plates of Manjistha plant extracts.

References

1. D Priya, E A Siril. Traditional and modern use of Indian Madder (*Rubia cordifolia* L.): An overview. *International Journal of Pharmaceutical Sciences Review and Research* **2014**. 25 (1), 154 – 164.
2. P S Vankar, R Shanker, D Mahanta and S C Tiwari. Ecofriendly sonicator dyeing of cotton with *Rubia cordifolia* Linn. using biomordant. *Dyes and Pigments* **2008**. 76, 207 – 212.
3. Y Chen, P Chen, B H Bao, M Shan, K Zhang, F Cheng, Y Cao, L Zhang, A Ding. Anti-thrombotic and pro-angiogenic effects of *Rubia cordifolia* extract in zebrafish. *Journal of Ethnopharmacology* **2017**, 1 – 34. doi: 10.1016/j.jep.2017.11.005.
4. K M Nadkarni. 3rd edition. New Delhi, Popular Prakashan. *Indian Materia Medica* **1982**, 1075–1077.
5. YB Tripathi, S Pandey, SD Shukla. Anti-platelet activating factor property of *Rubia cordifolia* Linn. *Ind J Exp Biol* **1993,** 31: 533–535.
6. YB Tripathi, M Sharma, S Shukla, P Tripathi, P Redanna. Rubia cordifolia inhibits potato lipoxygenases. *Ind J Exp Biol* **1995**, 33: 109–112.
7. R Baskar, L M Bhakshu, G V Bharathi, S S Reddy, R Karuna, G K Reddy and D Saralakumari. Antihyperglycemic activity of aqueous root extract of *rubia cordifolia*. in streptozotocin-induced diabetic rats. *Pharmaceutical Biology* **2006**, 44 (6), 475–479. doi: 10.1080/13880200600798593.
8. D E Jaenchen and H J Issaq. Modern thin-layer chromatography: advances and perspectives. *Journal of Liquid Chromatography* **1998**, 11, 9 – 10, 1941 – 1965. doi: 10.1080/01483918808069035.
9. H J Isaaq. Recent advances in multimodal thin-layer chromatography. *Trends in Analytical Chemistry* **1990**, 9, no. I.
10. B Spangenberg. Quantitative Thin-Layer Chromatography. Chapter 3. The stationary phase in thin layer chromatography, **2011.** doi: 10.1007/978-3-642-10729-0_3.
11. S Kirchert, M Schulz, M Oberle, G E Morlock. Development of a new particulate 4-micron;m adsorbentlayer for ultrathin-layer chromatography (miniaturized chromatogram). *Journal of Chromatography A* **2018.** doi:10.1016/j.chroma.2018.11.035.

12. E Stahl. Gradient and low temperature thin layer chromatography. *Angew. Chem. Internat*. **1964**, Edit. I, 3, 12.

13. I V Pletnev, E I Morosanova, Y A Zolotov. Use of Mixed Stationary Phases for the separation of metal ions. *Analytical sciences* **1990**, 6, 863 - 866. doi: 10.2116/analsci.6.863.

14. S Gocan. Stationary phases for thin layer chromatography. *Journal of Chromatographic Science* **2002**, 40.

15. H Xu, S H Issaq, T G McCloud and H J Issaq. The separation of a penicillium fungal extract by thin layer chromatography and HPLC: a comparative study. *Journal of Liquid Chromatography & Related Technologies* **2001**, 24 (5), 625 – 633. doi: 10.1081/JLC-100103399

16. S Pathania, R Daman, S Bhandari, B singh and B Lal. Comparative studies of *Rubia cordifolia* L. and its commercial samples. *Ethanobotanical Leaflets* **2006**, 11, 179 -188.

17. S Lin, C W Dence. Book on Methods in Lignin chemistry. *Springer* **1992**. Doi: 10.1007/978-3-642-74065-7.

18. M L Wolfrom, D L Patin, R M D Lederkremer. *Journal of Chromatography A* **1965**, 17, 488 – 494. doi: 10.1016/S0021-9673(00)99899-6.

19. Y P Chauhan, R S Sapkala, V S Sapkala and G S Zamre. Microcrystalline cellulose from cotton rags (waste from garment and hosiery industries). *Int. J. Chem. Sci*. **2009**: 7(2), 681-688.

20. Adel et al, T Kowalska, J Sherma. Book on TLC in chiral separations and analysis. *Carbohydrate Polymer* **2011,** Vol: 98.

21. M K M Haafiz, S J Eichhornc, A Hassan, M Jawaid. Isolation and characterization of microcrystalline cellulose from oil palm biomass residue. *Carbohydrate Polymers* **2013,** 93, 628– 634. doi: 10.1016/j.carbpol.2013.01.035.

22. R Xiong, X Zhang, D Tian, Z Zhou, C Lu. Comparing microcrystalline with spherical nanocrystalline cellulose from waste cotton fabrics. *Cellulose* **2012,** 19:1189–1198. Doi: 10.1007/s10570-012-9730-4.

23. S Ren, X Sun, T Lei and Q Wu. The Effect of Chemical and High-Pressure Homogenization Treatment Conditions on the Morphology of Cellulose Nanoparticles. *Journal of Nanomaterials* **2014**, 1 – 11. doi: 10.1155/2014/582913.

24. D Trache, A Donnot, K Khimeche, R Benelmir, N Brosse. Physico-chemical properties and thermal stability of microcrystalline cellulose isolated from Alfa fibres. *Carbohydrate Polymers* **2014,** 104, 223–230. doi: 10.1016/j.carbpol.2014.01.058

25. X Sun, C Lu, Y Liu, W Zhang, X Zhang. Melt-processed poly(vinyl alcohol) composites filled with microcrystalline cellulose from waste cotton fabrics. *Carbohydrate Polymers* **2014**,101, 642– 649. doi: 10.1016/j.carbpol.2013.09.088

26. M K Haafiza, A Hassan, Z Zakaria, I M Inuwaa. Isolation and characterization of cellulose nano whiskers from oil palm biomass microcrystalline cellulose. *Carbohydrate Polymers* **2014**, 103,119– 125. doi: 10.1016/j.carbpol.2013.11.055.

•