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Identification and Characterization of Natural dyes Maddar and Indigo by HPTLC Imaging System ProViDoc DD70 and Densitometer CD60

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Abstract: In this communication, an attempt has been made to compare the two major classes of natural dyes Anthraquinoid and Indigoid with their synthetic analogs. Maddar (*Rubia cordifolia* and *Rubia tinctoria*) and Indigo (*Indigofera tinctoria*) dyes were used for this study. HPTLC imaging system ProViDoc DD 70 and densitometer CD 60 (scanner) were used to identify the purity content. The techniques are shown for providing a high degree of chemical information making dye identification highly specific. Taking images of the dye eluted plate by imaging system ProViDoc DD70 and developing chromatogram of the same by the densitometer CD 60 with a light beam of visible light and UV ranges of 254 and 366nm yielded very distinctive features. Fluorescence was measured and recorded.

Keywords: Maddar dye, Indigo dye, HPTLC, Imaging system ProViDoc DD70, Densitometer CD 60, Scanner, Chromatogram

Introduction:

In present times, there is a great demand of natural dyes times due to resurgence of natural dyes (ND) in dyeing industry. Market is full of samples from different sources, however, standardization of ND is a major issue. This is primarily because of purity content, method of extraction and of course due to different sources of dyes. In literature, there are no testing protocols available for NDs or testing methods such as DIN, ASTM, BIS or others. Therefore, developing easy methods for identification of natural dyes based on thin layer chromatography which would also reveal the purity content and provide chemical information having good repeatability and reliability method was important for us.

Three dyes from the anthraquinoid series were selected: Maddar (*Rubia cordifolia* and *Rubia tinctoria*) from the natural sources and Alizarin the synthetic analog. Similarly, two dyes were selected from indigoid series: *Indigofera tinctoria* from the natural source and synthetic indigo. Samples were dissolved in appropriate solvents and applied on precoated silica gel plates by the HPTLC applicator AS 30. Afterwards, the plates were developed in a solvent system which provided very efficient separation of the chemical components. Finally, the plates were documented by the imaging system ProViDoc DD70; developped chromatogram of the same detected by the densitometer CD 60 with a light beam of visible light and UV ranges of 254 and 366nm resulted in very distinctive features. As an additional measure to highlight the bands and their intensity, derivatization with a reagent: Natural Product Reagent A (NPR A) was also used in each case.

Materials and methods:

The natural dyes sources:
Rubia cordifolia and *Indigofera tinctoria* from AMA herbals, Lucknow India;
Rubia tinctoria from NIG GmbH, Magdeburg, Germany.
Alizarin and synthetic indigo from Carl Roth, Karlsruhe, Germany.

Application of dyes was done by biostep® HPTLC Applicator AS 30 (Order No. BS130.500) from biostep® GmbH, Burkhardtsdorf, Germany.
All solvents used for these experiments and NPR A were bought from Carl Roth GmbH, Karlsruhe, Germany.

Precoated silica gel plates HPTLC Silica Gel 60 F254 multiformat, pre-scored to 5.0 x 5.0 cm were bought from Merck, KGaA, Darmstadt, Germany.
Imaging system used was biostep® ProViDoc DD 70, (Order No. BS140.061), biostep® GmbH, Burkhardtsdorf, Germany.
Densitometer used was biostep® HPTLC Densitometer CD 60 (Order No. BS131.800), biostep® GmbH, Burkhardtsdorf, Germany.

Sample preparation:
Rubia cordifolia (Rc) and *Rubia tinctoria* (Rt) were prepared as 0.2% solution in ethanol, sonicated for 2 minutes and then filtered through filter paper and used for analysis. Alizarin (Al) was taken 0.1% solution in ethanol, sonicated for 2 minutes and filtered through filter paper and used for analysis. *Indigofera tinctoria* and synthetic indigo were prepared as 0.2% solution in ethyl acetate with 3-5 drops of chloroform, sonicated for 2 minutes and filtered through filter paper and used for analysis.
Spotting of the plates:
HPTLC Applicator AS 30 was used for loading the dyes onto the silica gel plates. 40µl samples were applied in the case of Rc and Rt while 20µl was applied for synthetic Alizarin. Similarly, 40µl samples were applied for natural indigo and synthetic indigo.
Solvent system used:
In all cases, the following solvent system was used for elution of the plates:
light petroleum ether:ethyl acetate:formic acid in the ratio of 7.5ml:2.5ml:120 µl.
One dimensional study with single run, double run and then two dimensional study of the eluted plates were carried out.
Derivatization of the eluted plates by Natural Product Reagent A (0.1% in EtOH) applied with sprayer SGe1 by biostep® GmbH, Burkhardtsdorf, Germany.

Detection by ProViDoc DD70 was carried out with a light beam of visible light and UV ranges of 254 and 366nm. Images were collected for each run and identification of the bands was done by Rf value.
Detection by HPTLC Densitometer CD 60 was carried out in the case of indigo dyes for additional information using the mercury lamp as light source for detection. The device was used for fluorescence measurements at 366nm excitation with a cut-off filter of 420nm.

Results and discussion:
In the present study of High Performance Thin Layer Chromatography (HPTLC), natural dye samples were compared with their synthetic analogs. There are very apparent differences observed in the samples with the imaging system DD 70 and densitometer CD 60. Spraying the eluted plates with natural product reagent A also showed some marked changes in the imaging as well as densitometeric analysis. These results can thus be used as a mark of identification of the natural dye samples and labelled by its origin.

Maddar analysis:
Stationary phase used was Silica gel 60 F254 and two mobile phases were used:
i) light petroleum ether:ethyl acetate:MeOH (7.5ml:2.5ml:120µl) and
ii) light petroleum ether:ethyl acetate:formic acid (7.5ml:2.5ml:100µl)
Sample size loaded were: 9 up to 20µl each for *Rubia cordifolia* and *Rubia tinctoria* samples in one dimensional run; 20µl in 2-dimensional experiments for each one. While for Alizarin 3µl was used in one dimensional run and 6µl was used in 2-dimensional experiment.

In one dimensional study, following observations were made:

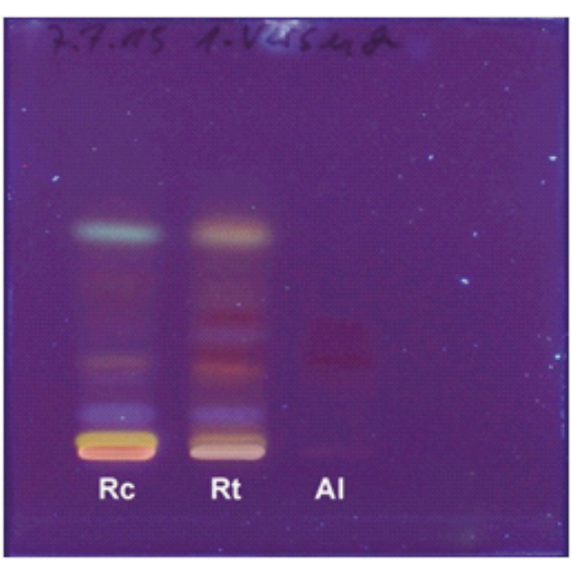


Fig.1: 1st run of Rc (20µl), Rt (9µl) and Al (3µl), solvent system ii.

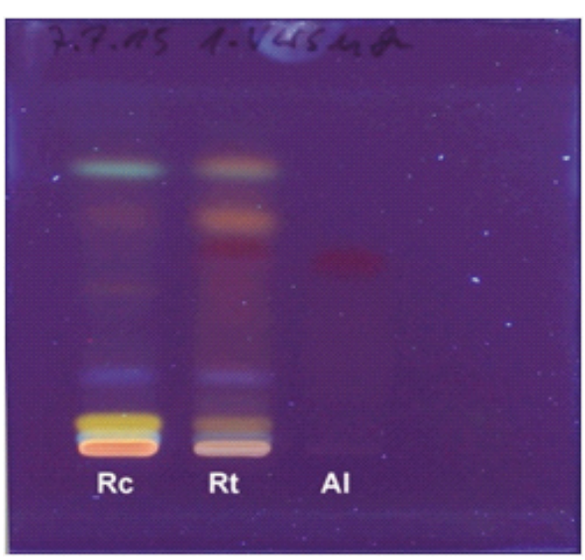
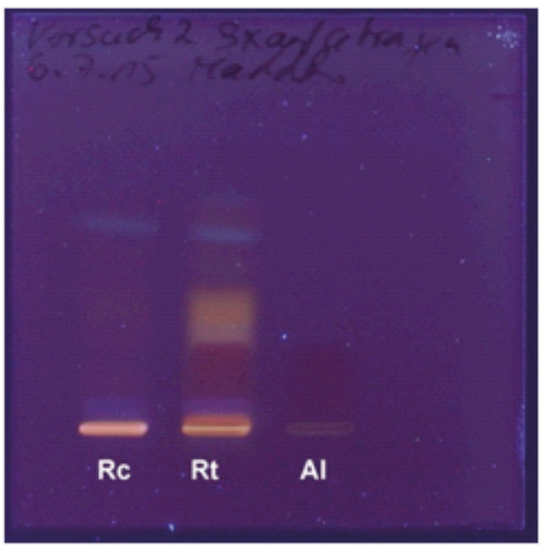


Fig. 2: 2nd run of the plate of Fig. 1 in solvent system ii.



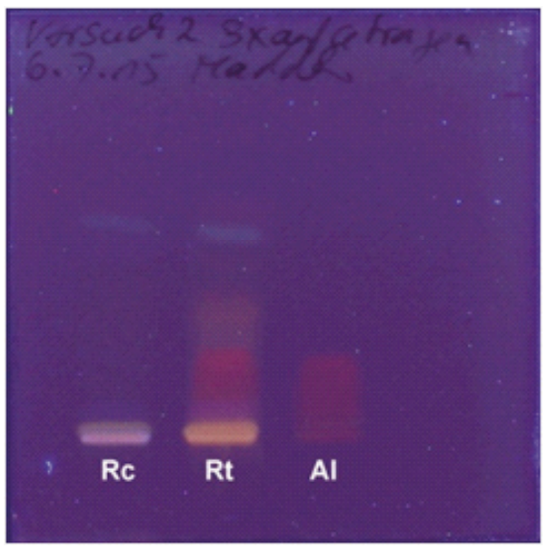


Fig. 3: Left: Application of each 9µl Rc and Rt and 3µl Al and run in solvent system i.
Right: The same plate after spraying with NPR A

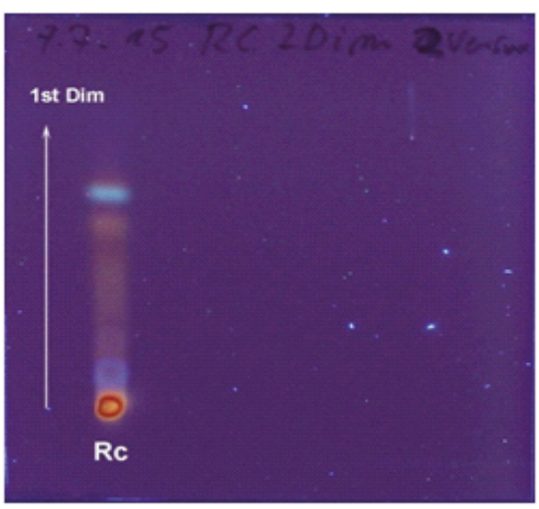


Fig. 4: 1st dimension run of 20µl Rc in solvent system i: 9 spots visible

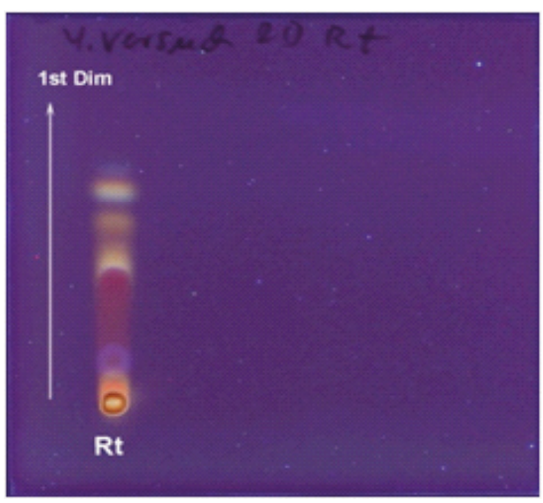


Fig. 5: 1st dimension run of 12µl Rt in solvent system i: 10 spots visible

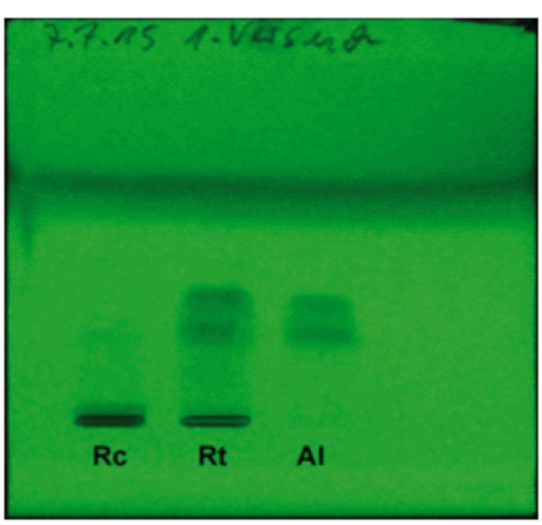


Fig. 6: Plate as in Fig. 1 under UV 254nm.
Rf differences for Alizarin are visible.

1. All 3 samples (Rc, Rt, Al) showed distinct bands as shown in Fig. 1, 2 and 3.
2. Fluorescent bands common to Rc and Rt are observed at the spotting area as well as a blue band at Rf 0.5, seems to be very characteristic of maddar dyes (Fig. 3).
3. Rc shows 9 apparent bands (Fig. 4) while Rt shows 10 apparent bands of different intensities as shown in Fig. 5, while Al shows only 1 broad band (Fig. 3).
4. Disappearance of bright orange band in Rt after derivatization with NPR A as shown in Fig. 3.
5. Another blue band appeared in second run of the plate in solvent i with formic acid system as shown in Fig. 2.
6. Double run of the plates in the solvent system light petroleum ether:ethyl acetate:formic acid causes

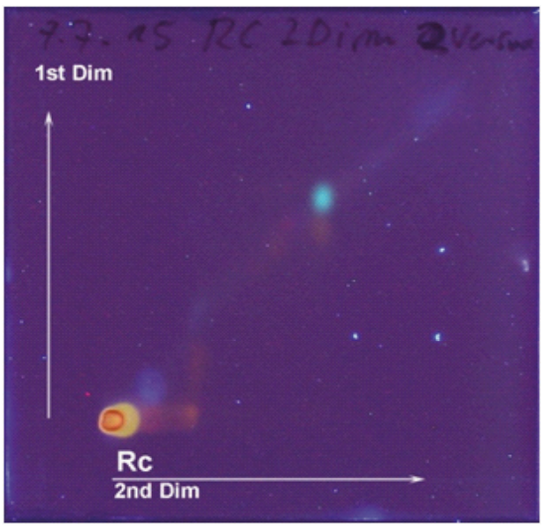


Fig.7: 2-dimensional separation of 20µl Rc. Plate of Fig 4 after 2nd run in solvent system ii.

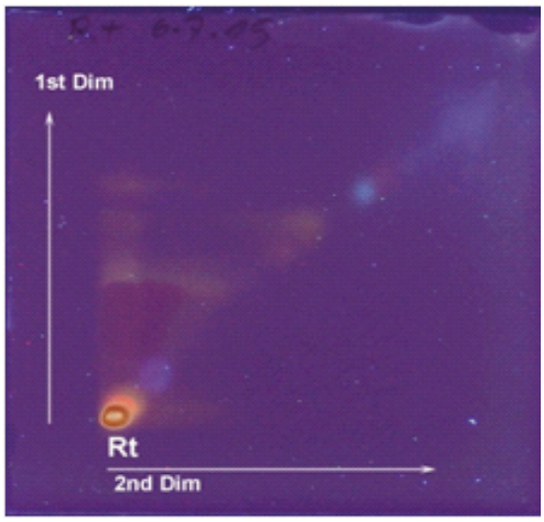


Fig. 8: 2-dimensional separation of 12µl Rt in solvent system i used in both dimensions.

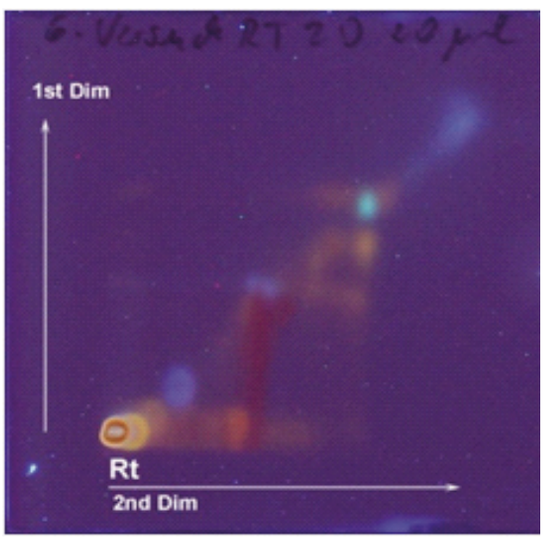


Fig. 9: 2-dimensional separation of 20µl Rt. Solvent systems used:
1st dimension: i, 2nd dimension ii.

very good separation of the components.

7. Rf of reddish spot in Rt under 366nm illuminations corresponding with Al's reddish spot is slightly different as shown in Fig. 3. The difference is also observed under 254nm illuminations as shown in Fig. 6.
8. Rt shows many spots of different colours and intensities.
9. The intensity of the reddish spot corresponding to Al is also different in Rc and Rt in Rf as well.
10. The orange band in Rc seen under 366nm illuminations corresponds to the dark band seen under 254nm illuminations (Fig. 6).

In 2-dimensional study, following observations were made:

11. In 2-D study of Rc and Rt diagonal movement of the spots make the image very clear and the differences are more apparent as shown in Fig. 4 and 5.
12. In 2-D run of Rt 4 differently coloured spots appear in a square showing a blue, orange, purple and a red band (Fig. 9).
13. In 2-D run of Rt in Fig. 9 shows not only a diagonal pattern under 366nm illumination but also a real areal distribution showing that the solvent systems used have different separation properties.
14. Fig. 7 and 9 show clear differences in the spot pattern of Rc and Rt, respectively.
15. 2-D separation pattern of Rt was almost diagonal when in both dimensions solvent system i was used (Fig. 8).

Indigo analysis:

Stationary phase used was silica gel 60 F254 and the mobile phase used was light petroleum ether:ethyl acetate:formic acid (7.5:2.5:1.0) in first run. The 2D experiment was done with light petroleum ether:ethyl acetate:formic acid (7.5:2.5:1.0) in first run and CHCl3:hexane:formic acid (8:2:1).

Sample size loaded were:

40µl for *Indigofera tinctoria (NI)* sample in single dimension run; 40µl was used in 2-dimensional experiment, while for synthetic indigo (SI) 40µl was used.

In one dimensional study, following observations were made:

16. A very prominent pink band can be seen in Natural Indigo (NI) at Rf 0.45 under visible illumination as shown in Fig. 10, which is missing in SI.
17. A weak blue band with a lower pink band at Rf 0.48

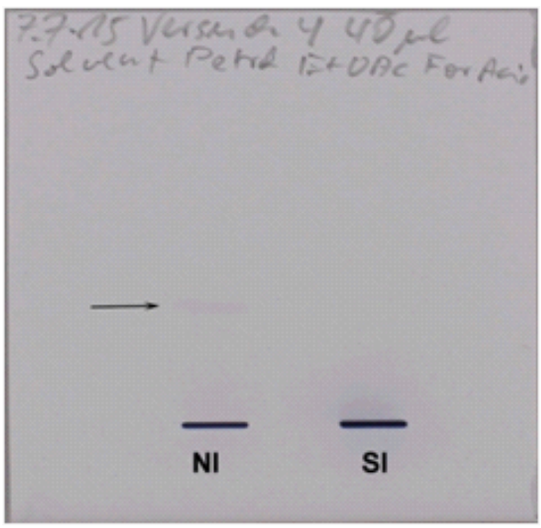


Fig. 10: One-dimensional separation of NI and SI under visible light. NI shows a pink band missing in SI.

in NI, while SI has a very intense blue band at that Rf under 366 nm illumination as shown in Fig. 11

18. An intense pink band at Rf for NI at 0.35, while in SI there is a weak bluish band at that Rf as shown in Fig. 12 left.
19. An intense fluorescent blue band after treatment with NPR A can be seen in NI at Rf 0.03 while in SI a similar band appears a bit higher at Rf 0.10 as shown in Fig. 12 right.
20. Use of natural product reagent A used as derivatization makes band more prominent and fluorenscent.
21. Very brilliant bands appear in SI after derivatization near Rf 0.1 and 0.15.
22. In NI new fluorescent band appears just above the point of spotting as shown in Fig. 12 right.
23. The top pink band of NI disappeared after derivatization as seen in Fig. 12.

Fig. 11: One-dimensional separation of NI and SI. Left: 1st run, Right: 2nd run. Illumination with UV 366nm.

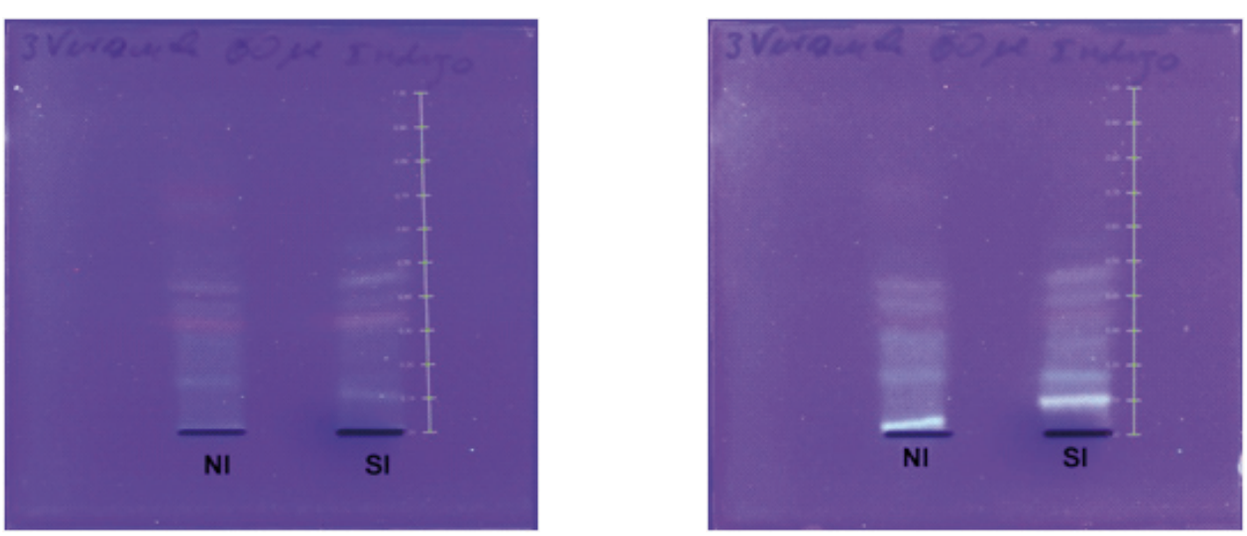


Fig. 12: Left: one-dimensional separation of 60µl NI and SI // Right: the same plate after spraying with NPR A. Illumination with UV 366nm. Rf-scale was placed with ProViDoc.

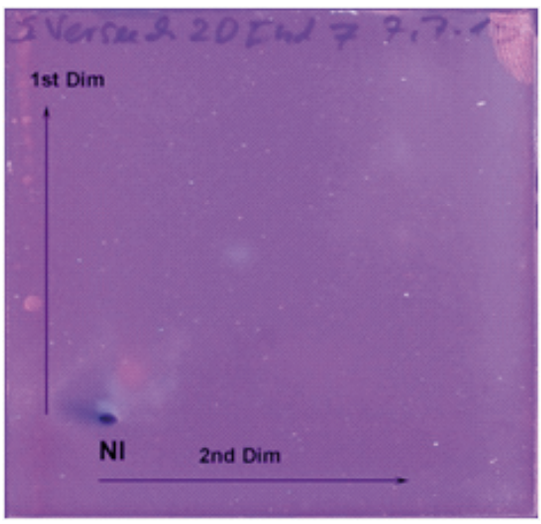


Fig. 13: 2D-Separation of 40µl NI.

In 2-dimensional study, following observations were made:

24. In 2-dimensional study of NI diagonal movement of the spots can be seen as shown in Fig. 13.
25. Pink and blue bands move in diagonal positions as shown in Fig. 13.

Densitometer analysis of indigo samples:
The parameters of densitometer were as follows:
Start coordinate X: 20.0mm
Start coordinate Y: 10.0mm
End coordinate Y: 50.0mm
Slit width: 4.00mm
Slit height: 0.10mm
Wavelength: 366nm
Distance of the lanes: 15.0mm
Filter position: 420nm
Evaluation mode: Fluorescence
Lamp: Mercury

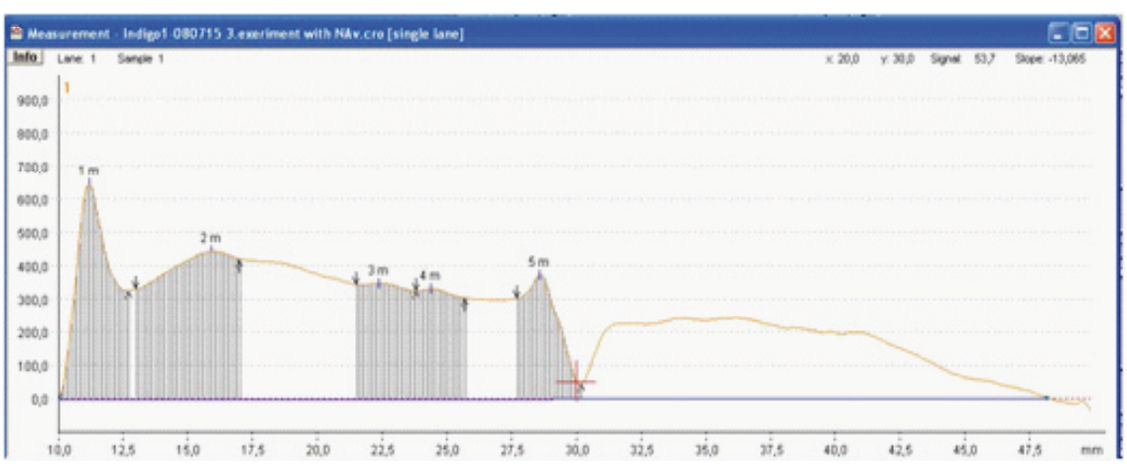


Fig. 14: Chromatogram of natural indigo after derivatization with NPR A

Fig. 15: Chromatogram of synthetic indigo after derivatization with NPR A

The chromatogram of NI is shown in Fig. 14. Initially, three main peaks can be seen at hRf 13, 35 and 74. However, after modification five peaks were obtained at hRf 13, 31, 35, 58 and 74 which correspond to the blue band, pink band, violet band, bluish red band and pink band. But after derivatization with NPR A, the hRf changed to 3, 14, 46 and 54 and thus it is proven that the pink band appearing at hRf 74 is completely quenched.

The chromatogram of SI is shown in Fig. 15. Initially, three main peaks can be seen at hRf 8, 13 and 32. However, after modification five peaks were obtained at hRf 8, 13, 31, 34 and 39 which correspond to the blue band, pink band, violet band, bluish red band and pink band. But after derivatization with NPR A, the hRf changed to 9, 20, 28, 33, 38 and 45 which correspond to blue band, faded blue band, faded blue band, overlayed of pink band and blue band, very thin blue band and blue band, respectively.

Conclusion: With the help of the imaging system Providoc DD70 alone, it could be seen that the colour of the spots in Rc and Rt were very distinctively different. Their Rf values with synthetic Alizarin were also very clearly different, thereby making this test method as appropriate for the identification of the different sources of madder dyes such as *Rubia cordifolia* and *Rubia tinctoria*.

The solvent system consisting of light petroleum ether:ethyl acetate:formic acid developed for the method seems very appropriate for very efficient separation of the 9 components in Rc and 10 components in Rt. Double run of the silica gel plates is recommended. Derivation with NPR A makes further more distinctive differences among the three anthraquinoid samples.

Similarly, in indigo samples with the help of the imaging system Providoc DD70, we observed very distinctive features for *Indigofera tinctoria* and synthetic indigo. Appearance of a pink spot in NI at 0.45 Rf under visible illumination is a diagnostic feature of this analysis as synthetic indigo does not show that spot. The solvent system consisting of light petroleum ether:ethyl acetate:formic acid developed for the method seems very appropriate for very efficient separation of the chemical components. Furthermore, densitometer CD 60 analysis substantiated the results of the imaging system. The Rf values matched with the bands. Derivatization with NPR A makes further more distinctive differences among the two indigoid samples. We conclude that we found a very efficient test method for the identification of the different sources of indigo dyes.