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RESEARCH ARTICLE

Identification of Natural Madder and Indigo Dyes by Novel HPTLC Method

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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 analog. Madder (Rubia cordifolia n Rubia tinctorum L.) and Indigo (Indigofera tinctoria) dyes were used for this study. Both the dyes are of pharmacological importance as they are used in TCM (Traditional Chinese Medicine) and Ayurveda (Traditional Indian Medicine). HPTLC with imaging system and densitometer (scanner) were used to identify the purity content. The technique has shown to provide a high degree of chemical information making dye identification highly specific. Taking photos through imaging system of the dye eluted plate and developing chromatogram of the same under the densitometer with a light beam of UV ranges 254and 366 nm yielded very distinctive features. Fluorescence was also measured and recorded.

Abbreviations: ND: Natural Dyes; NI: Natural Indigo; SI: Synthetic Indigo; NPRA: Natural Product Reagent A; Rc: Rubia Cordifolia; Rt: Rubia Tinctoria; Al: Alizarin; Rf: Retention Factor; hRf: 100 fold value of Retention Factor.

Introduction

There is great demand for natural dyes in the present times due to resurgence of natural dyes (ND) in dyeing industry. Market is full of samples from different sources; however identification and standardization of natural dye are two major issues. This is primarily because of purity content, method of extraction and of course also due to different sources of the dyes. There are no testing protocols available for natural dyes in the literature among DIN, ASTM, BIS or others. Therefore there is a need to develop 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 is reliability.

Some methods are known in the literature but they are not very specific. A simple method of identification of Indigo with the help of color change by the addition of sodium hydrogen sulfite has been discussed [1]. The solvent system used is Chloroform–hexane-methanol system for the separation of blue and red pigments of indigo dye have also been used [2]. Use of only chloroform for separation of indigo was mentioned [3]. TLC method was also used to identify Indigo dye [4]. A study on components of Indigo from different

sources was carried out using TLC method among others [5]. Similarly for Madder dye too very few references can be found in the literature. TLC method of identification of Madder dye was mentioned [6,7]. Rubia tinctorum L was also analyzed by TLC method [8].

Although it is easy to explain scientifically how and why the colors produced by natural indigo and synthetic indigo can differ, the natural indigo and synthetic indigo were reduced by zinc powder which is considered as a relatively weak reducing agent and sodium hydrosulfite which is commonly used as reducing agent [9-11]. Color can be compared using a spectrophotometer and a colorimeter and thus correlated with dye diffusion and dye penetration. However, this sort of comparison of natural indigo and synthetic indigo in terms of four key color characteristics: brightness of color, running of color color unevenness and color fading as well as of coloring materials in dye and dyed cloth, dye diffusion, dye aggregation, and dye penetration are very difficult to assess. Even brightness of color includes properties such as clarity, and color related to croma. These specific nuances are not easily identified. All this is post dyeing information which are obtained only when the indigo dye is used. How to ascertain that the dye used in the first place is natural and not synthetic?

Similar problems are encountered with Madder. Synthetic alizarin and Madder outwardly give same shades on the dyed fabric, however it is essential to find out the authenticity of the dye used in the dyed fabrics. The non-uniformity in dyeing is a very characteristic of natural dyes. But all this is known after dyeing the fabric.

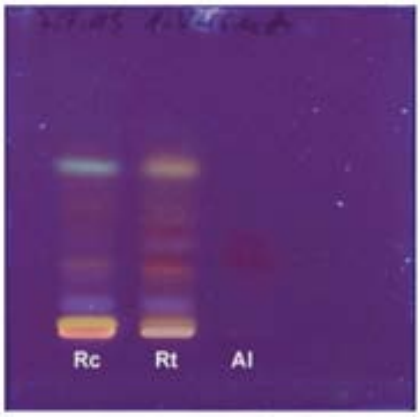

Figure 1: 1st run of Rc (20µl), Rt (9µl) and Al (3µl), solvent system II.

Therefore it was necessary to develop methods for identification of Natural dyes Madder and Indigo for the ease of buyers as well as customers. Three dyes from the anthraquinoid series i.e. Madder (Rubia cordifolia and Rubia tinctorum L.) from the natural sources and alizarin, the synthetic analog were selected. Similarly two dyes were selected from indigoid series- Indigofera tinctoria from the natural source and synthetic indigo.

Materials and Methods

1. The natural dyes sources

Rubia cordifolia and Indigofera tinctoria dye extracts were from AMA herbals, Lucknow India; Rubia tinctorum L. dye extract was from NIG GmbH, Magdeburg, Germany. Alizarin and synthetic indigo are from Carl Roth, Karlsruhe, Germany. Application of dyes was done by HPTLC Applicator AS 30. All the solvents used for these experiments and natural product reagent A (NPA) were purchased from Carl Roth GmbH, Karlsruhe, Germany. Precoated silica gel plates HPTLC Silica Gel 60 F254Multiformat, pre-scored to 5.0 x 5.0 cm were purchased from Merck, KGaA, Germany. Model name of the imaging system used was ProViDocDD70. Model name of the densitometer used was HPTLC Densitometer CD 60, Germany.

2. Sample preparation

Rubia cordifolia (Rc) and Rubia tinctorum L. (Rt) were prepared as 0.2% solution in ethanol, sonicated for 2 minutes for best dissolution of the dye extracts and then filtered through filter paper and used for analysis. Alizarin (Al) was taken0.1% solution in ethanol, sonicated for 2 minutes and filtered through filter paper and used for analysis. Indigofera tinctoria and synthetic indigo were prepared in 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.

3. Method for HPTLC

Samples were dissolved in appropriate solvents and applied on pre-coated silica gel plates through applicator AS 30.The plates were then developed in a solvent system which would provide very efficient

separation of the chemical components and finally the plates would be used for taking photos through imaging system ProViDoc DD70 and developing chromatogram of the same under the densitometer CD 60 with a light beam of visible and UV ranges 254 and 366 nm yielding very distinctive features. As an additional measure to highlight the bandstand their intensity derivatization with a reagent- natural product reagent A (NPRA) was also used in each case.

4. Spotting of the plates

Applicator AS30 was used for the loading of 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.

5. Solvent system used

In all the 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: 120µl. One dimensional study with single run, double run and then two dimensional studies of the eluted plates were carried out.

6. Derivatization of the eluted plates by Natural Product Reagent

A (0.1% in EtOH) applied with sprayer SGe1.Detection under ProViDoc DD70 was carried out with a light beam of visible and UV ranges 254 and 366 NMAND photos were collected for each run and identification of the bands were done by Rf value. Detection under Densitometer CD 60 was carried out in the case of indigo dyes for additional information using the mercury arc lamp as source light for detection. The device was used for Fluorescence measurements at366 nm excitation with a cut-off filter of 420 nm. Derivatization on the TLC plate helps in identification of the spots more easily.

7. HPTLC Analysis for Natural and Synthetic Dyes

Stationary phase used was Silica gel 60 F254 and two mobile phases were used which were specially found to be very appropriate for the separation of the components: 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 sizes loaded were: 9 up to 20µl each for Rubia cordifolia and Rubia tinctorum L. 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.

Stationary phase used was silica gel and the mobile phase used was light petroleum ether: ethyl acetate: formic acid (7.5: 2.5: 1.0) in first run. The 2 D 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 was: 40µl for Indigofera tinctoria (NI) sample in single dimension run; 40µl was used in2-dimensional experiment, while for synthetic indigo (SI) 40µl was used.

8. Parameters of Densitometer

The parameters of densitometer are as Start coordinate X: 20.0 mm, Start co-ordinate Y: 10.0 mm, End coordinate Y: 50 .0 mm, Slit width: 4.00mm, Slit height: 0.10 mm, Wavelength: 366 nm, Distance of the lanes: 15.0mm, Filter position: 420nm, Evaluation mode: Fluorescence, Lamp: Mercury

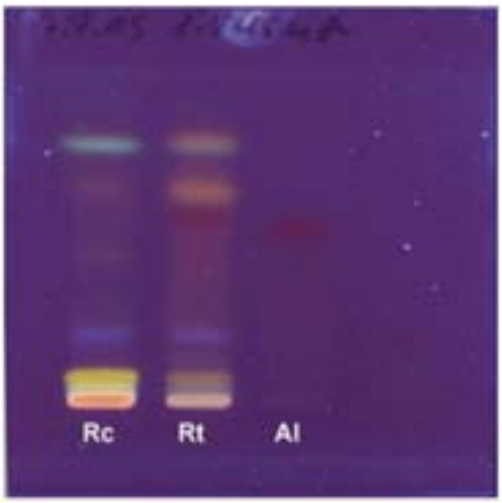

Figure 2: 2nd run of the plate of Fig. 1 in solvent system II.

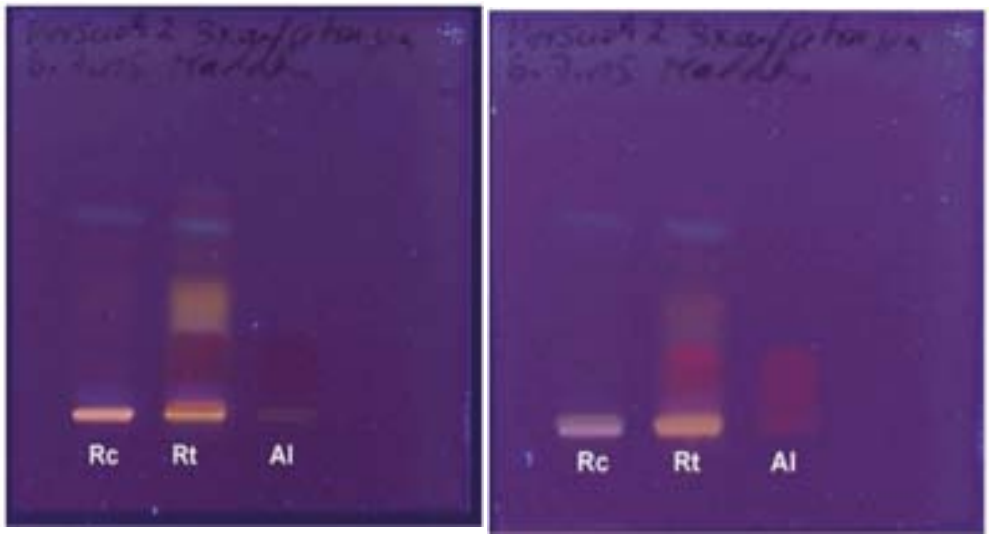

Figure 3: left: Application of each 9µl Rc and Rt and 3µl Al and run in solvent system I, Right: the same plate after spraying with Nat. Prod. Reg A.

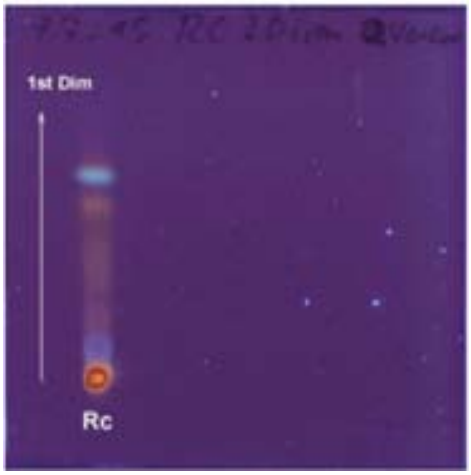

Figure 4: 1st dimension run of 20µl Rc in solvent system I: 9 spots visible

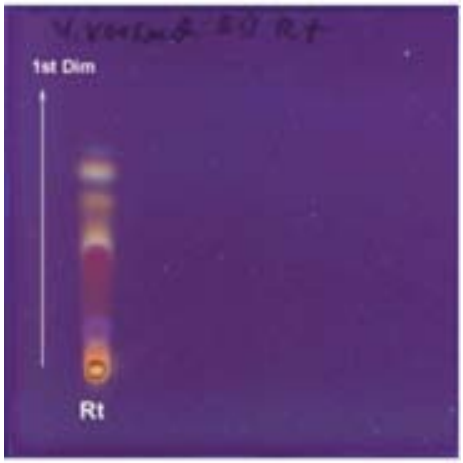

Figure 5: 1st dimension run of 12µl Rt in solvent system I: 10 spots visible

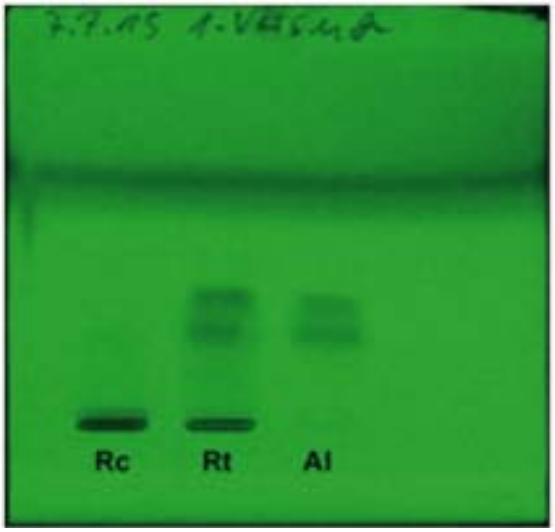

Figure 6: Plate as in Figure 1 under UV 254nm. Rf differences for Alizarin are visible. Figure 5, while Al shows only 1 broad band (Figure 3).

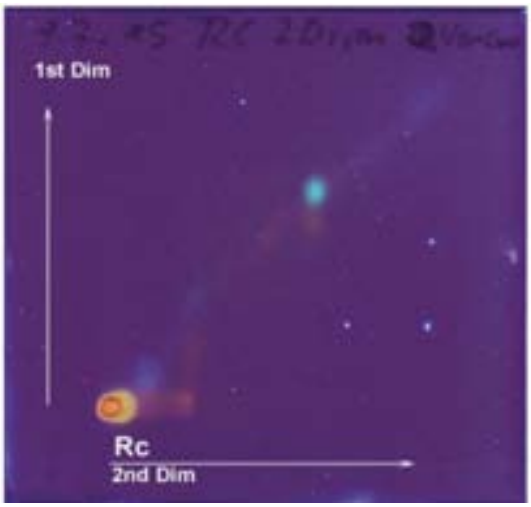

Figure 7: 2-dimensional separation of 20µl Rc. Plate of Fig 4 after 2nd run in solvent system II

Results

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

1. Madder analysis

In one dimensional study following observations were made:

All the 3 samples (Rc, Rt, Al) showed distinct bands as shown in (Figure 1-3). Fluorescent bands common to Rc and Rt are observed at the spotting area as well as a blue band at Rf0.5, seems to be very characteristic of Madder dyes (Figure 3). Rc shows 9 apparent bands while Rt shows10 apparent bands of different intensities as shown in (Figure 4). Disappearance of bright orange band in Rt after derivatization with Natural product reagent A as shown in (Figure 3). Another blue band appeared in second run of the plate in solvent I with formic acid system as shown in (Figure 2). Double run of the plates in the solvent system light petroleum ether: ethyl acetate: formic acid brings about very good separation of the components. Rf of Reddish spot in Rt under 366nm illuminations corresponding with Alizarin's reddish spot is slightly different as shown in (Figure 3). The difference is also observed under 254nm illuminations as shown in (Figure 5). Rt shows many spots of different colors and intensity. The intensity of the reddish spot corresponding to ALIS also different in Rc and Rt in Rf as well. The orange band in Rc seen under 366nm illuminations corresponds to the dark band seen under 254nm illuminations (Figure 6).

In 2-dimensional study following observations were made:

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

Take in Figure 1: 1st run of Rc (20µl), Rt (9µl) and Al(3µl), solvent system II

Take in Figure 2: 2nd run of the plate of (Figure 1) in solvent system II

Take in Figure 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 Nat. Prod. Reg A

Take in Figure 4: 1st dimension run of 20µl Rc in solvent system I: 9 spots visible

Take in Figure 5: 1st dimension run of 12µl Rt in solvent system I: 10 spots visible

Take in Figure 6: Plate as in (Figure 1) under UV 254nm. Rf differences for Alizarin are visible. (Figure 5), while Al shows only 1 broad band (Figure 3).

Take in Figure7: 2-dimensional separation of 20µl Rc. Plate of (Figure 4) after 2nd run in solvent system II

Take in Figure 8: 2-dimensional separation of 12µl Rt insolvent system I used in both dimensions

Take in Figure 9: 2 dimensional separation of 20µl Rt. Solvent systems used: 1st dimension: I, 2nddimension II.

2. Indigo analysis

In one dimensional study following observations were made:

A very prominent pink band can be seen in Natural Indigo (NI) at Rf 0.45 under visible illumination as shown in (Figure 10), which is missing in SI.A weak blue band with a lower pink band at Rf 0.48in NI, while SI has a very intense blue band at that Rf under 366 nm illumination as shown in (Figure 11). 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 (Figure 12) left. An intense fluorescent blue band after treatment with NPRA can be seen in NI at Rf 0.03 while in SI a similar band

appears a bit higher at Rf 0.10 as showing on right side of (Figure 12). Use of natural product reagent A used as derivatization makes band more prominent and fluorescent. Very brilliant bands appear in SI after derivatization near Rf 0.1 and 0.15.In Natural Indigo new fluorescent band appears just above the point of spotting as shown in (Figure 12) right. The top pink band of NI disappeared after derivatization as seen in (Figure 12).

In 2-dimensional study following observations were made:

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

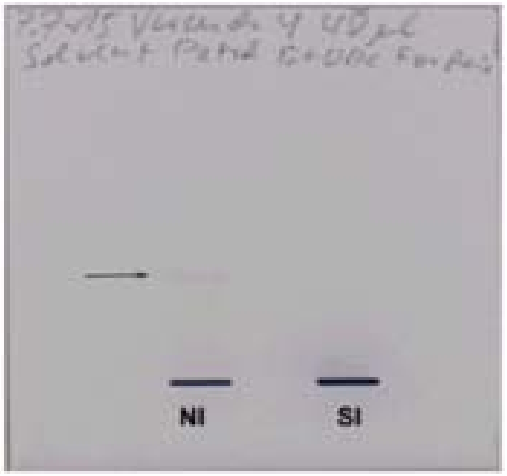

Figure 10: One-dimensional separation of NI and SI under visible light. NI shows a pink band missing in SI.

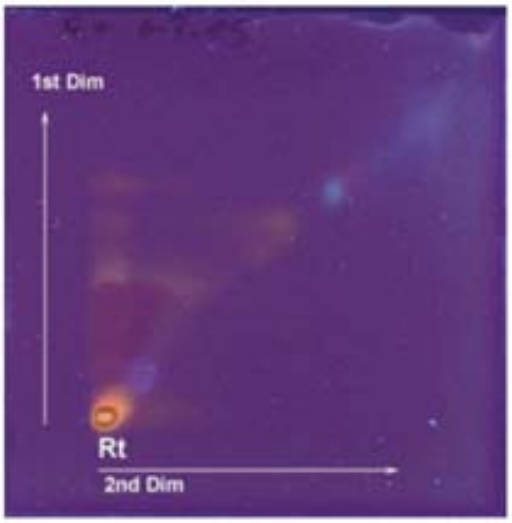

Figure 8: 2-dimensional separation of 12µl Rt insolvent system I used in both dimensions

Figure 9: 2 dimensional separation of 20µl Rt. Solvent systems used: 1st dimension: I, 2nddimension II.

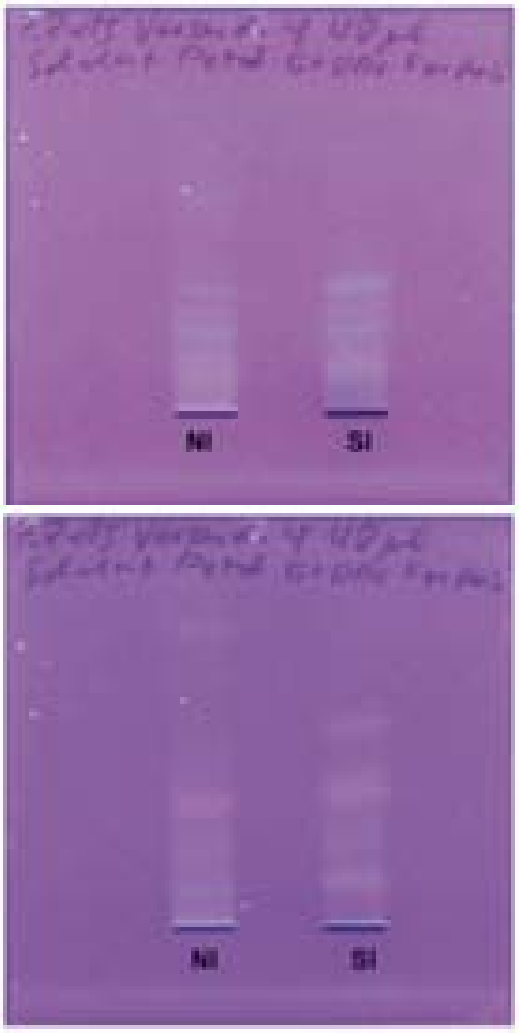

Figure 11: One-dimensional separation of NI and SI. Left: 1st run, right: 2nd run. Illumination with UV 366nm.

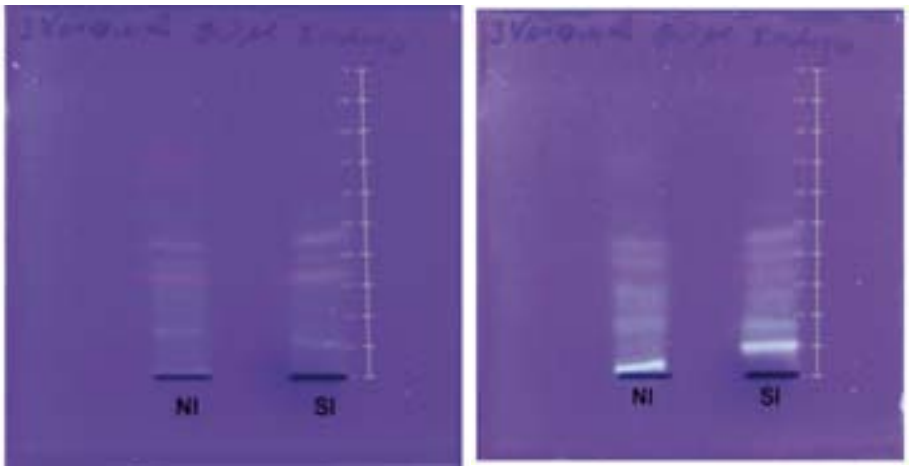

Figure 12: Left: one-dimensional separation of 60µl NI and SI, right: the same plate after spraying with NPRA. Illumination with UV 366nm. Rf-scale was placed with ProViDoc.

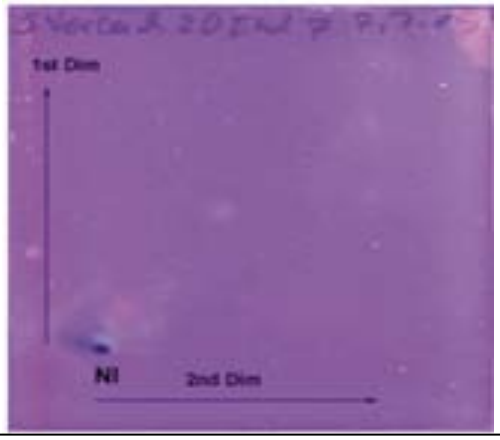

Figure 13: 2D-Separation of 40µl NI

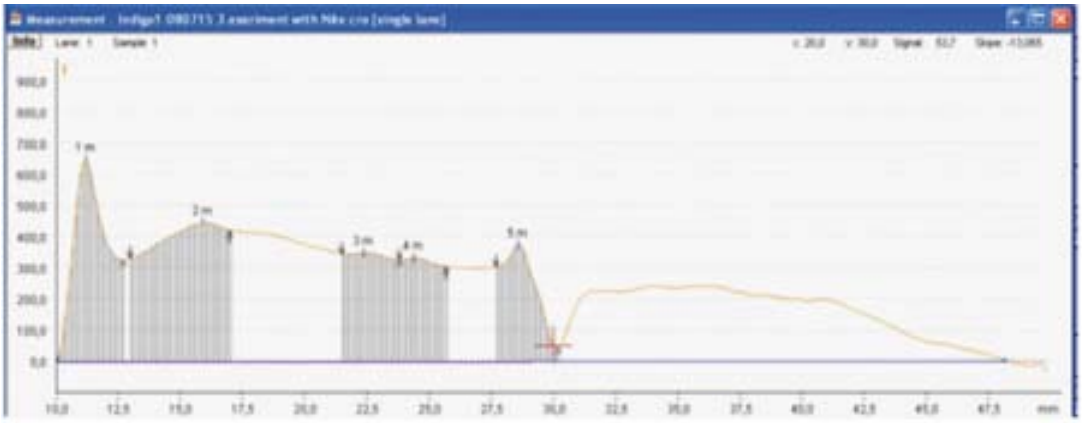

Figure 14: Chromatogram of Natural Indigo after derivatization with NPRA

2.1. Densitometer analysis of indigo samples: The chromatogram of NI (Figure 14) showed initially three main peaks at hRf 13, 35 and74, 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 NPRA 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 synthetic indigo is shown. (Figure 15) showed initially three main peaks at hRf 8, 13and 32 however after modified 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 NPRA 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.

Take in Figure 12: Left: one-dimensional separation of 60µl NI and SI, right: the same plate after spraying with NPRA. I llumination with UV 366nm. Rf-scale was placed with ProViDoc.

Take in Figure 13: 2D-Separation of 40µl NI

Take in Figure 14: Chromatogram of Natural Indigo after derivatization with NPRA

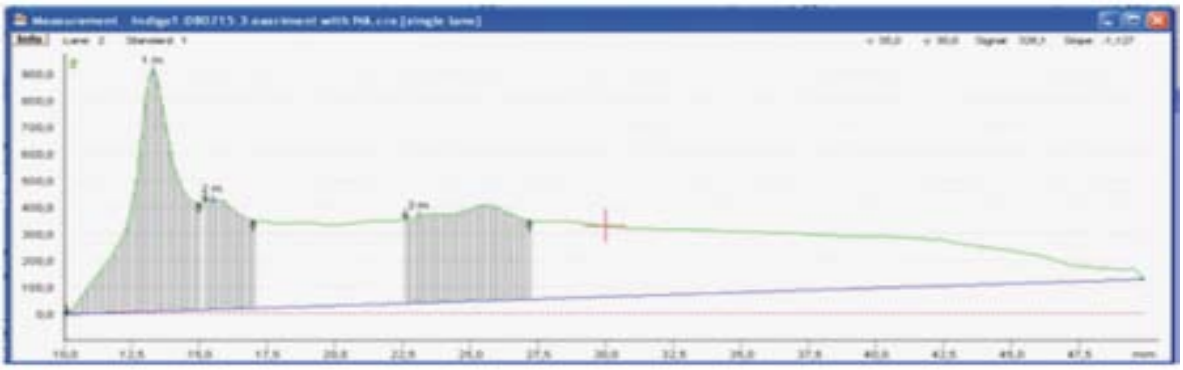

Figure 15: Chromatogram of Synthetic Indigo after derivatization with NPRA.

The Madder samples Rubia cordifolia Rc and Rubia tinctorum L. Rt can be easily distinguished from synthetic alizarin and identified by HPTLC with this new and very special solvent system petroleum ether: ethyl acetate: formic acid in the ratio of 7.5ml: 2.5ml: 120µl. One dimensional study with single run subsequently with double run and further on with two dimensional study of the eluted plates were carried out. Derivatization of the eluted plates by Natural Product Reagent A (0.1% in EtOH). Detection with a light beam of visible and UV ranges 254 and 366 nm and photos thus collected for each run showed identification of the bands by Rf value. Similarly in the case of Indigo samples NI and SI the same methodology along with densitometry analysis showed the distinction very clearly.

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

Take in Figure 11: One-dimensional separation of NI and SI. Left: 1st run, right: 2nd run. I llumination with UV 366nm.

Take in Figure 15: Chromatogram of Synthetic Indigo after derivatization with NPRA

Conclusion

Natural Rubia and Indigo dyes have been studied. With the help of imaging system, it could be seen that the color of the spots in Rc and Rt were very distinctively different, their Rf values with synthetic alizarin was also very clearly different, thereby making this test method as appropriate for the identification of the different sources of madder dye such as Rubia cordifolia and Rubia tinctorum L. 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 nine components in Rc and 10 components in Rt. Double run of the silica gel plates is recommended. Derivation with Natural Product Reagent A makes further more distinctive differences among the three anthraquinoid samples. Similarly, in indigo samples with the help of the imaging system very distinctive features for Indigo feratinctoria and synthetic indigo were observed. Appearance of a pink spot in natural indigo at 0.45 Rf under visible illumination is a

diagnostic feature of this analysis assynthetic indigo does not show that spot. The solvent system consisting of light petroleum ether: ethyl acetate: formic acid (7.5ml: 2.5ml: 120µl) developed for the method is very appropriate for very efficient separation of the chemical components. Further on Densitometer analysis substantiated the results of the imaging system. The Rf values matched with the bands. Derivatization with natural product reagent A makes further more distinctive differences among the two indigoid samples. Thus it is a very efficient test method for the identification of the different sources of indigo dye.

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