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THIN LAYER CHROMATOGRAPHIC ANALYSIS OF FOOD COLORANTS FROM THREE MORPHOTYPES OF ANNATTO (*Bixa orellana* L.)

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Abstract

This article describes a simple solvent extraction method for the extraction of colorants from the three morphotypes such as, (Morphotype-1 (M_1)), Morphotype-2 (M_2), and Morphotype-3 (M_3) of Annatto (*Bixa orellana* L.) seeds, and their separation, vivid, and qualitative demonstration by thin-layer chromatography. Several solvent systems (hexane, chloroform, acetone, ethanol, and a mixed-solvent having composition of $\text{CHCl}_3/\text{C}_2\text{H}_5\text{OH}/\text{CH}_3\text{COOH}$ (80:2:1)) were applied for extraction of colored components. It was observed that a large portion of colorants was extracted by chloroform. Its effluent was deep brick red in color and transparent. Furthermore, various carrier solvent systems (Benzene-Ethyl acetate) were used to separate the components from the extracts. Carrier solvent system with the ratio of 7:3 was found as superior solvent for chloroform extracts. Three colored-spots were observed for all morphotypes. Among them, the first one was yellow colored having very low polarity and the second and third spots were both redbrick colored having medium and higher polarity respectively. In addition, for M_1 no colorless-spot was observed in low and medium polar systems, revealing that the amount of wax and gum were minimum in the extract and superior morphotype among the three.

Keywords: Solvent Extraction, Seed Coat, *Bixa orellana*, Natural Colour, R_f Value

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Introduction

Over the past few decades, it has been a growing concern in worldwide for food quality, safety, and security. Particular attention has been drawn to colorants used in food, drug, cosmetic, and textile industry, where the safety of colorants is one of the most burning issues. While, most of the synthetic colorants tend to be perceived as undesirable and harmful; some are considered to be responsible for allergenic and intolerance reactions (Wissgott and Bortlik, 1996). In contrary, nature-derived products are considered as health friendly and of good quality.

A growing number of natural food colorants are being isolated and excepted to use commercially, such as Caramel coloring (E150) made from caramelized sugar used in cola products and also in cosmetics; Annatto, a reddish-orange dye extracted from the seed of the *Bixa orellana* L.; Cochineal (E120), a red dye derived from the cochineal insect, *Dactylopius coccus*; Pandan a green food coloring extracted from *Pandanus*

amaryllifolius; Butterfly pea a blue food dye from *Clitoria ternatea*. Among them, annatto (E160b) appears as an important one for food and drug industries owing to its potential uses as a substitute of the synthetic colorant Tartrazine which is prohibited in several countries (JECFA, 1982). Annatto was classified by the Food and Drug Administration in the U.S.A. as a "color additive exempt of certification" (Hallagan *et al.*, 1995). In addition, this red-orange colorant is extracted from the seeds of Annatto (*Bixa orellana* L.), can be a potential candidate for replacing the costly "Saffron" which is widely used in medicine, food and cosmetics industry. Annatto tree is frequently seen as an ornamental plant in various parts of the world. It is a tropical tree native to the Central and South American rain forest, and is vastly cultivated in warm regions of the world, such as India, Bangladesh, Sri Lanka, and Java (Wolf, 1997). The dye extracted from the seed coat of the Annatto seeds can be used in coloring cheese, soft drinks, oil, butter, sondesh, vanilla ice-cream, and soap.

Further, it can be used in some regions to color textiles (Lata *et al.*, 1990) and color fabrics and weapons (Rao *et al.*, 2002). Recently, Ali (2006) reported three morphotypes of Annatto namely, morphotype-1 (M_1), morphotype-2 (M_2), and morphotype-3 (M_3) were found in Bangladesh.

Here we reported qualitative assessment of three morphotypes of Annatto in order to develop a simple inexpensive process for extracting the colorants.

Materials and Methods

Extraction of colorants

The matured-fruit-capsule of Annatto (*Bixa orellana* L.) of three morphotypes: morphotype-1 (M_1), morphotype-2 (M_2), and morphotype-3 (M_3) were collected from Botanical Garden of Bangladesh Agricultural University (BAU). The fruit-capsules were dried in sunlight for 30 hours and seeds were separated out from the capsules. After that, the seeds were dried in sunlight for another 30 hours and preserved in a refrigerator at 20°C for further analysis. To extract the colorants, 100 g of seeds were soaked in 200 ml particular organic solvent for 30 minutes and stirred abrasively by a glass rod for one hour (Islam, 2008). Extracting solvents were selected based on their polarity, viz. hexane (Polarity: 0), chloroform (Polarity: 4.1), acetone (Polarity: 5.1), and ethanol (Polarity: 5.2) (Finar, 2000). In addition, a mixed-solvent system of $\text{CHCl}_3/\text{C}_2\text{H}_5\text{OH}/\text{CH}_3\text{COOH}$ (80:2:1) also was employed. Finally, it was filtered by Whatman Grade-114 filter paper to prepare the stock solution for Thin Layer Chromatographic (TLC) analysis.

Thin layer chromatography analysis of colorants

The chromoplate were prepared by dispersing a suspension of finely powdered silica gel adsorbent (50 g silica gel thoroughly mixed and swelled in 125 ml of water) on a glass strip (EMerck India limited, Mumbai, 400018) of suitable size (5 cm \times 1 cm \times 0.3 cm). A very thin uniform layer of silica was formed on glass strip with the aid of a spreader. It was then dried in an oven at 105 ± 5 °C for 12 hours. For obtaining a chromatogram, the chromoplate was spotted with a minute drop of annatto extract with the help of a capillary tube, and then placed vertically in a solvent tank. Development of the chromatogram occurred by capillary movement of the solvent up the silica-adsorbent layer. When solvent front reached at the upper end of the TLC plate, it was marked and the plate was taken out and allowed to dry. If the components were colored, then the spots were readily located. To ascertain the presence of colorless spots, the TLC plate was transferred to iodine chamber. Both colored and colourless spots

on the TLC plate were recorded and R_f values were calculated. The R_f value depends on the relative polarities of the carrier solvent and spot-components. It is defined as the ratio of the distance travelled by a component to the distance travelled by the solvent front (Finar, 2000). R_f value indicates the polarity of the spot-components in the TLC plate (Saraswathy *et al.*, 2004). Similar polarities of carrier solvent and spot-components leading higher R_f value and vice-verse.

Results and Discussion

Extraction of colorant

Colorants were extracted from the three morphotypes of Annatto seeds by employing variety of solvents (hexane, chloroform, acetone, ethanol, and a mixed solvent system), shown in Table 1. Solvents were selected based on their polarity to find out the suitable solvent for extracting the colorants. The hexane extracted effluent was transparent; however, the intensity of color was very light, indicating that only components of low polarities were extracted by hexane. When chloroform was employed, the effluent solution was very transparent and deep red brick color, shown in Fig. 1 and Table 1, suggesting that chloroform is one of the suitable solvents to extract the colorants. Furthermore, the colorants extracted by more polar solvents such as, acetone and ethanol were dark and cloudy, like emulsion. It might be due to some undesired highly polar components were also extracted along with the colorants. In addition, mixed solvents extracted effluent was transparent and brick red color, indicating that it is only selective to brick red colored pigments (Saraswathy *et al.*, 2004).



Fig. 1. Colorants extracts by solvents

Thin layer chromatographic analysis of colorant

To separate and evaluate the coloring pigments from the solvent extracted effluents, TLC was employed. Both colored and colorless spots and their R_f values were recorded (Finar, 2000; Saraswathy *et al.*, 2004).

Table 1. Physical properties of colorant extracts

Extracting solvent	Polarity	Color/Intensity	Transparency	Inferences
Hexane	0.0	Yellow/Light	Transparent	Suitable for non-polar dyes
Chloroform	4.1	Brick red/Deep	Transparent	Suitable
Acetone	5.1	Amber/Dark	Cloudy	Not suitable
Ethanol	5.2	Orange/ Dark	Cloudy	Not suitable
Mixed solvent	-	Amber/Light	Transparent	Suitable for selected colorants

Hexane extracts

Table 2 shows the R_f values of both colored and colorless spots of n-hexane extracts. The starting carrier solvent was benzene (100%), in this case, colored-spots were not developed regardless of three morphotypes. However, after exposing in I_2 vapor, M_1 and M_2 showed few spots, attributing for the colorless compounds. When mixed solvent systems of benzene/ethyl acetate (Bz/EtOAc) with the ratio of 3:2, 7:3, and 4:1 were employed, respectively, M_1 as well as M_3 showed one colored spot respectively and no colorless spot was observed, indicating that they were exclusion of

colorless compounds (gum, wax, etc.). In case of M_2 , two colored spots were seen: first one was yellow in color with low R_f value, and second one was brick red in color with higher R_f value respectively. It suggested that both low and high polar pigments (i.e. yellow and brick red colored constituents respectively) were present in the hexane extracted annatto. After exposing to I_2 vapor, few spots were also observed. On the basis of colored and colorless spots, and their R_f values, it can be concluded that Bz/EtOAc (3:2) and (7:3) solvent mixture were the superior carrier solvents for M_1 and M_3 morphotypes.

Table 2. TLC analysis of hexane extracted colorants

Morphotype	Eluting solvent	Value of R_f	
		Colored spot	Colorless spot
M_1		-	0.416
			0.602
M_2	Bz = 100 %	-	0.504
			0.416
M_3		-	-
M_1		0.113	-
M_2	Bz/EtOAc = 3:2	0.939	0.965
		0.173	0.913
M_3		0.156	-
M_1		0.084	-
M_2	Bz/EtOAc = 7:3	0.940	0.983
		0.110	0.840
M_3		0.067	-
M_1		0.940	0.983
		0.060	
M_2	Bz/EtOAc = 4:1	0.949	0.967
		0.093	
M_3		0.084	-

Chloroform extracts

Solvent systems of Bz/EtOAc with the ratio of 1:0, 9:1, 7:3, 1:1, and 0:1 were exploited separately as carrier solvent, presented in Table 3. Bz (100%) provided single color spot for all the morphotypes. After transferring to I_2 chamber, only M_2 showed two spots. In case of solvent systems 9:1 and 7:3, three colored spots were observed for all morphotypes. Among them, the first one was yellow colored spot with very low polarity and the second spot was red color with moderate polarity and the third one was brick red color. Most of the cases for M_1 and M_3 colorless

spots were not appeared, suggesting that colorless compounds were absent in these morphotypes. These two carrier solvent systems seemed to be suitable solvent for separating the dyes from the chloroform extract. Further increasing in portion of EtOAc, as in case of 1:1 and EtOAc (100%), both color and colorless spots were observed.

Acetone extracts

Table 4 shows the R_f values for chromatogram of acetone extracts. Similar trend was observed in using Bz (100%); only colorless components were present.

Table 3. TLC analysis of chloroform extracted colorants

Morphotype	Eluting solvent	Value of R_f	
		Colored spot	Colorless spot
M ₁	Bz (100 %)	0.235	-
		0.227	0.504
		0.219	0.390
M ₁	Bz/EtOAc = 9:1	0.983	-
		0.845	-
		0.128	-
M ₂	Bz/EtOAc = 9:1	0.990	0.983
		0.846	0.957
		0.213	0.897
M ₃	Bz/EtOAc = 9:1	0.957	-
		0.803	-
		0.128	-
M ₁	Bz/EtOAc = 7:3	0.929	-
		0.835	-
		0.144	-
M ₂	Bz/EtOAc = 7:3	0.945	-
		0.850	0.976
		0.236	0.912
M ₃	Bz/EtOAc = 7:3	0.958	-
		0.883	0.983
		0.166	-
M ₁	Bz/EtOAc = 1:1	0.140	-
		0.932	0.907
		0.210	0.974
M ₂	Bz/EtOAc = 1:1	0.924	-
		0.180	-
		-	-
M ₁	EtOAc (100%)	0.122	-
		0.967	-
		0.131	0.934
M ₂	EtOAc (100%)	0.959	-
		0.114	0.943
		-	-

Table 4. TLC analysis of acetone extracted colorants

Morphotype	Eluting solvent	Value of R_f	
		Colored spot	Colorless spot
M ₁	Bz (100 %)	-	-
		-	0.983
		-	0.459
M ₂	Bz (100 %)	-	0.344
		-	-
		0.949	0.983
M ₃	Bz (100 %)	0.168	0.967
		0.957	0.967
		0.176	0.918
M ₁	Bz/EtOAc = 9:1	0.210	-
		0.084	-
		0.924	0.983
M ₂	Bz/EtOAc = 9:1	0.101	0.840
		0.067	-
		0.14	-
M ₃	Bz/EtOAc = 9:1	0.932	0.974
		0.210	0.907
		0.924	-
M ₁	Bz/EtOAc = 7:3	0.180	-
		-	-
		-	-
M ₂	Bz/EtOAc = 7:3	-	-
		-	-
		-	-
M ₃	Bz/EtOAc = 7:3	-	-
		-	-
		-	-
M ₁	Bz/EtOAc = 1:1	-	-
		-	-
		-	-
M ₂	Bz/EtOAc = 1:1	-	-
		-	-
		-	-
M ₃	Bz/EtOAc = 1:1	-	-
		-	-
		-	-

While mixed solvents were employed, one/two colored spots were developed. Between them one was very low polarity with yellow colored spot and other was brick red with moderate polarity. M_2 provided two colorless spots; however, M_1 and M_3 did not show any spot under iodine vapor. On depending of the colored and colorless spots, and their R_f values, it seemed that Bz/EtOAc (9:1) solvent system was the superior carrier solvent for separating dyes from acetone extracts. In this case, TLC chromatogram of M_1 and M_2 showed two colored spots, and its R_f value was 0.949 and 0.168 for M_1 ; and 0.957 and 0.176 for M_2 , respectively. For the M_3 , one colored spot was appeared with R_f of 0.210 and did not show any spots under iodine vapor.

Table 5. TLC analysis of ethanol extracted colorants

Morphotype	Eluting solvent	Value of R_f	
		Colored spot	Colorless spots
M_1		-	0.50
M_2	Bz (100 %)	-	0.528
M_3		-	0.50
M_1		0.983 0.122	0.959
M_2	Bz/EtOAc = 7:3	0.983 0.114	0.959
M_3		0.983 0.098	0.959
M_1		0.975 0.173	0.967
M_2	Bz/EtOAc = 6.5:3.5	0.983 0.190	0.967
M_3		0.983 0.247	0.967
M_1		0.459	-
M_2	Bz/EtOAc = 6:4	0.475	-
M_3		0.508	-

Considering these chromatograms, it seemed that Bz/EtOAc with the ratio of 6:4 solvent system can be used for separating selective dye. Its chromatogram indicated that M_1 , M_2 , and M_3 contained one colored spots with R_f value of 0.459, 0.475, and 0.508, respectively.

Mixed-solvent of chloroform/ethanol/acetic acid (80:2:1) extracts

Table 6 shows the R_f values of colored and colorless spots of mixed solvents extract; and Bz/EtOAc solvent systems were attempted to separate the components. In every case, only Table 6. TLC analysis of mixed-solvents extracted colorants

Ethanol extracts

For developing the chromatogram of ethanol extract, Bz (100%) and mixture of Bz/EtOAc were used as carrier solvent, shown in Table 5. As expected, Bz did not carry any color components. With the inclusion of EtOAc, in 7:3 and 6.5:3.5 ratios two colored spots were observed for all the morphotypes. Between them one was yellow color with very low polarity and the second spot was brick red color. After exposing to iodine vapor, one spot was appeared for all morphotypes. Further increasing the portion of EtOAc, i.e., in 6:4, only single color spot was seen for all the morphotypes.

colored spots were observed in chromatogram and it was brick red color having high polarity, suggesting that extracts of mixed solvent comprised of mostly colored constituents. On the basis of separation of colored and colorless spots, and their R_f values; it may be concluded that Bz/EtOAc (1:1) solvent mixture was most suitable carrier solvent for separating the dyes from the extract of mixed solvent systems. In all case, M_1 , M_2 , and M_3 developed only one colored spot respectively and, their R_f value were 0.809 for M_1 , 0.826 for M_2 and 0.843 for M_3 .

Table 6. TLC analysis of mixed-solvents extracted colorants

Morphotype	Eluting solvent	Value of R_f	
		Colored spot	Colorless spots
M_1		0.104	-
M_2	Bz (100 %)	0.104	-
M_3		0.104	-
M_1		0.295	-
M_2	Bz/EtOAc = 9:1	0.305	-
M_3		0.315	-
M_1		0.184	-
M_2	Bz/EtOAc = 7:3	0.193	-
M_3		0.202	-
M_1		0.809	-
M_2	Bz/EtOAc = 1:1	0.826	-
M_3		0.843	-

Conclusion

Three coloring constituents in each morphotypes (M_1 , M_2 , and M_3) of Annatto were observed. Among the three pigments, one was associated with high R_f value, i.e., very low polarity and yellow in color. The rest of the two pigments were brick red and orange colored associated with medium and low R_f value, respectively. Moreover, after exposing in iodine vapor, most of the chromatogram indicated the presence of colorless components. Studies from R_f values of colorless components revealing that one was low polar colourless components and other two colorless compounds were of medium polarity. Furthermore, chloroform extract effluent was very deep brick red in color and transparent. On depending of the chromatogram, mixed solvent systems of Bz/EtOAc of 7:3 was superior carrier solvent for separating the dyes from the chloroform extracts. Thus, dyes separated from the extract can be used in coloring the food, medicine, cosmetic and other purpose.

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