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Developing a Novel Solvent System to Separate Polar and Nonpolar Leaf Pigments of Copperleaf (Acalypha wilkesiana) Using Thin Layer Chromatography

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Abstract

Plant species contain several pigments that are responsible for different functions. Depending on the structures of these pigments, some of these pigments are nonpolar (chlorophyll-a, chlorophyll-b, β-carotene, xanthophyll, etc.), whereas some are polar (anthocyanins), thus making them hydrophobic or hydrophilic, respectively. To understand more about the structure and properties of these pigments, it is essential to isolate them in pure forms. So far, planar chromatographic techniques have been mostly employed to separate nonpolar pigments from one another, but not from the polar ones. Here we are reporting a novel solvent composition (60% hexane, 10% ethyl acetate, 10% acetone, 10% isopropyl alcohol, 10% water), that can be used to separate the nonpolar pigments from the polar ones using thin-layer chromatography (TLC). This solvent composition enabled us to develop a chromatogram, where spots were distinctly separated, concentrated, and could easily be isolated. The pigments were identified from their colors and R_f values, followed by characterization using UV-Vis spectra.

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Introduction

Plants contain several pigments that are responsible for different physiological processes [1][2]. Some are responsible for photosynthetic processes (chlorophylls, pheophytins, xanthophylls, carotenes, etc.), whereas the others directly or indirectly help in several processes such as growth and development, protection, and pollination (phytochromes, anthocyanins, etc.) [1][2][3][4]. Several of these pigments are also medicinally important due to their anticancer, antioxidant, antidiabetic, and antimalarial properties [5][6]. Based on the structures of these pigments, some are nonpolar in nature (such as chlorophyll-a, chlorophyll-b, β-carotene, xanthophyll, etc.), whereas the rest are polar (for example, anthocyanins). To get insight into these pigments, it is critical to isolate and purify these pigments and conduct further studies on them. But the differences in structure and polarity of these pigments, along with their relative abundances make the isolation process challenging. To this end, different methodologies or protocols have been reported in the literature [7][8][9][10][11][12]. These methodologies have employed a wide variety of techniques, ranging from simple chromatographic techniques (paper chromatography (PC) and thin-layer chromatography (TLC)) to relatively complex ones (such as differential solvent extraction methods, high-pressure liquid chromatography (HPLC), and other biophysical processes of separation). The latter studies usually involve multiple steps and expensive instruments. Moreover, these techniques often consume more solvents (sometimes hazardous), corrosive chemicals, and are not very cost-effective. One of the most commonly used solvents is methanol, and some pigments have been found to be unstable in it [9]. Methanol is also known to be toxic in nature [13]. Sometimes these processes involve harmful or hazardous chemicals as well, such as acids, halogenated solvents, etc [14][15]. Hence, often planar chromatographic techniques are preferred due to their simplistic and convenient nature [7][8][16][17]. These methods are inexpensive and can be performed in basic lab settings, which is also ideal for educational purposes.

Even though there are a few reports in the literature on separating plant pigments using planar chromatography, most of these studies were focused on separating the nonpolar pigments such as chlorophyll-a, chlorophyll-b, pheophytin, β-carotene, and xanthophylls [7][16][17]. Hence, different mobile phases comprising of a relatively polar component (acetone, ethyl acetate, ethanol, methanol, chloroform, etc.) mixed with a nonpolar component (hexane, petroleum ether, isooctane, carbon tetrachloride, etc.) were sufficient to separate these pigments, On the other hand, several plants contain highly polar pigments such as anthocyanins in addition to the nonpolar ones [3][18]. Mobile phases mentioned above mostly

contain the nonpolar solvent as the major component and are not suitable to elude polar anthocyanin. Different techniques have been employed over the years to isolate anthocyanins as well, but usually not from a mixture containing highly nonpolar pigments using planar chromatography [19][20]. Even these studies used harmful chemicals that could be health hazards and were not environmentally friendly. A method to separate anthocyanins from the common nonpolar pigments has been reported earlier, but the expected separation pattern was found to be non-reproducible [21]. When repeated, the more nonpolar xanthophylls eluded before the highly polar stagnant anthocyanins, contradicting the reported data. Moreover, the solvent system included chloroform, which is nowadays avoided due to its carcinogenic nature [22]. Chloroform also has been found to be responsible for other severe diseases[23]. To the best of our knowledge, except for this report, separation and isolation of the nonpolar (chlorophylls, pheophytin, β-carotene, xanthophylls, etc.) and the polar anthocyanin pigments effectively using the same chromatogram have not been reported elsewhere. To achieve that, optimization of the commonly known mobile phases was required by introducing a more polar character. Yet, the effective polarity should be such that it does not compromise the separation of the nonpolar pigments.

Keeping this in mind, our aim was to develop a novel solvent system that can be used to separate the above-mentioned pigments using a simple experimental setup such as PC or TLC. We also wanted to ensure that the chemicals being used were not health hazards. To this end, we strategically designed a wide range of solvent systems with varying compositions and tested their efficacies for both paper and thin-layer chromatography in eluding the polar and nonpolar pigments in such a way so that they are well separated and could easily be isolated. In this communication, we report the screening of several solvent systems for both PC and TLC, optimizing the parameters for best results, followed by isolation and characterization of individual pigments.

Materials and methods

Materials

The stationary phase for the paper chromatography (PC) was obtained by cutting filter papers (Whatman, #1001-125) into rectangular shapes (7-8 cm x 1.5-2.5cm). For the thin-layer chromatography (TLC), silica-coated aluminium was chosen as the stationary phase (TLC Silica gel 60 F_{254}, Merck, Germany), with dimensions of 6-7 cm x 1.5-2 cm. The solvents used in this study were n-hexane (extrapure AR, 99%, SRL Chemical, India), ethyl acetate (extrapure AR, 99.5%, SRL Chemical, India), acetone (pure, 99%, SRL Chemical, India), and isopropyl alcohol (99%, Nice Chemicals, India). All the experimental procedures were carried out at room temperature (26-28 °C) and normal atmospheric pressure.

Plant pigment extract preparation

Two plant species were selected for this study – American basswood (*Tila americana*) as the control and copperleaf (*Acalypha wilkesiana*) as the sample of interest[24]. American basswood had green leaves, indicating the presence of chlorophylls. On the other hand, copperleaf had predominantly red leaves, but with a greenish tint. Studies have confirmed that the red color is due to the presence of anthocyanins in copperleaf. First, we collected 3-4 pieces of fresh

leaf samples from each plant, tore them into smaller pieces, and removed the veins and petioles. To 1 g of these leaf pieces, 3 mL of isopropyl alcohol was added in a mortar and these were ground to even smaller fragments using the pestle. At this stage, 2 more mL of isopropyl alcohol was added. The pigments started to get dissolved in isopropyl alcohol and this extraction process was facilitated by stirring the pestle for 2 minutes. The color of the solution changed depending on the color of the leaves. The supernatants from both these preparations were carefully decanted into 15 mL conical tubes and these sample solutions were used immediately for chromatographic purposes.

Chromatographic Separation

Chromatographic separation of the photopigments was done using two different chromatographic methods – paper chromatography (PC) and thin layer chromatography (TLC). 5 µL of these extracts were then spotted on the stationary phases (paper or silica-coated aluminium) using micropipette tips, dried and run inside the glass chamber containing the mobile phases for 7-8 minutes. The run was allowed to continue till the solvent fronts were 0.5 cm away from the top edge of the filter papers or TLC plates. We tried several mobile phases by trying out different combinations of solvents. Irrespective of the relative amount of the components, the total volume of the mobile phase used to run the PC or TLC experiments was fixed (10 mL). Throughout this paper, the relative compositions of the solvents have been expressed as volume percentages (v/v %). To compare, identify and assign different photopigments after separation, the retention factor (R_f) was calculated for each separated photo-pigment by dividing the distance traveled by the photopigments by the distance traveled by the solvent front [25].

UV-Vis spectroscopy

For each TLC, individual spots along with the silica were scratched off the plate using a small pipette tip and were transferred to 1.5 mL Eppendorf tubes. Then 1.5 mL of isopropyl alcohol was added to the Eppendorf tubes to dissolve and extract the photopigments. Extracted pigment fractions were filtered to remove the silica and the filtrates were transferred to a quartz cuvette for UV-Vis spectroscopy. Measurement was done on a Shimadzu UV-1780 UV-Vis spectrophotometer using UV Probe software.

Results and discussion

Paper chromatography (PC)

First, we chose paper chromatography (PC) as the separation method as it is simple, convenient, and easy to perform even in a basic lab setting. From the green leaves (control), we prepared the extract primarily containing the green pigments (chlorophylls) as described in the method section. We first started with a 100% polar (ethyl acetate) and another 100% non-polar solvent (n-hexane) to gauge the movement and separation of the pigments on paper. For n-hexane, the orange spot moved easily, almost with the solvent front. The rest of the spots were not well separated. On the other hand,

with pure ethyl acetate as the mobile phase, all the colored spots were overlapping with each other including the orange one. Interestingly, the red spot from copperleaf extract did not move at all either in hexane or in ethyl acetate. Other polar solvents (acetone, isopropyl alcohol, and water) in their pure form were not effective either (Table 1, Figure 1).

As the next step, we repeated the experiments using a combination of solvents and gradually changed the composition (constituents and their relative amounts). Details about different solvent compositions and the resulting chromatograms have been described in Table 1. A representative series of paper chromatography results are shown in Figure 1. As can be seen from the images, the green, yellow, or orange pigments traveled in a 10% ethyl acetate-hexane or 10% acetone-hexane mixture but did not move at all in water. On the other hand, the red spot did not move in any of these three solvents (Figure 1-A and 1-B).

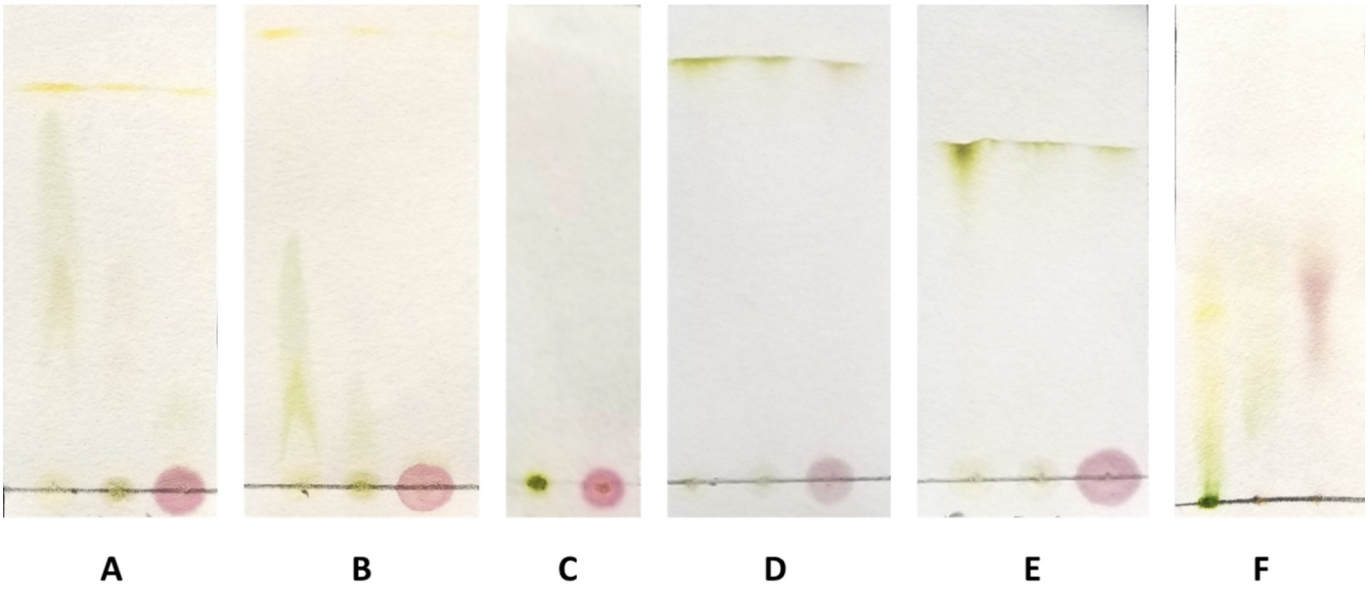

Figure 1. Representative images of some of the paper chromatography experiments done using different solvent systems as mobile phase. The samples were collected from green (left side spot of each chromatogram) and red leaves (right side spot of each chromatogram). The middle spots (where present) in the chromatograms were the diluted extracts from the green leaves. The solvent systems were: A) 10% ethyl acetate in hexane, B) 10% acetone in hexane, C) pure water, D) pure isopropyl alcohol, E) 50% isopropyl alcohol in n-hexane, F) 50% isopropyl alcohol in water

Table 1. Brief details about the paper chromatographic experiments conducted in this study using different solvent systems and interferences based on the observation

Solvent composition (Total volume 10 mL)	Observation	Inferences
Pure n-hexane	Only the orange spot moved with the solvent front, rest of the yellow/green pigments overlapped. The red spot did not move.	Only the orange spot moved as it was highly nonpolar. T of the spots were clubbed together and could not be separated. The highly polar red spot was insoluble in highly nonpolar n-hexane and did not move.
Pure ethyl acetate	All nonpolar pigments moved together. The red spot did not move at all.	Separation was not possible for any color (pigment).
Pure acetone	All nonpolar pigments moved together. The red spot did not move at all.	Separation was not possible for any color (pigment).
Pure water	None of the pigments moved from the baseline	Separation was not possible for any color (pigment) (Figure 1-C).
Pure isopropyl alcohol	All nonpolar pigments moved together, almost with the solvent front. Red colored spot did not move at all.	Separation was not possible for any color (pigment) (Figure 1-D).
10% ethyl acetate -90% hexane	Orange and greyish green moved separately, but the other green spots appeared to overlap with yellow. The red spot did not move at all.	Separation was possible only for the orange and greyish-green pigments, not for any other pigments (Figure 1-A).
10% acetone-90% hexane	Orange and greyish green moved separately, but the other green spots appeared to overlap with yellow. The red spot did not move at all.	Separation was possible only for the orange and greyish-green pigments, not for any other pigments (Figure 1-B).
50% isopropyl alcohol-50% hexane	All nonpolar pigments moved together, almost with the solvent front. The red spot did not move at all.	Separation was not possible for any pigment (Figure 1-E).
50% isopropyl alcohol-50% water	Both polar and nonpolar pigments moved but were tailing and overlapping	No separation was possible in this case also. But the encouraging fact was the significant movement of the red spot to the middle of the chromatogram. This was the first solvent system in our study that eluted the red spot (Figure 1-F).
80% isopropyl alcohol-20% water	All nonpolar pigments moved together, almost with the solvent front. The red spot did not move at all.	Even though all the spots moved (including the red one), separation was not possible due to extreme overlapping.
20% isopropyl alcohol-80% water	The red spot moved resulting in a faint long tail, however, the yellow and green spots did not move at all	No separation was possible as the orange, green, and yellow spots did not move at all. Perhaps the highly polar solvent composition was not suited to elude the nonpolar pigments,

It was clear that the mixture of solvents is a more suitable option for separating the plant pigments of varying polarity. Except for the red pigment, the other pigments were separated partially when a 10% ethyl acetate-hexane mixture or a 10% acetone-hexane mixture was used. Keeping the highly polar nature of anthocyanin in mind, next we explored more polar compositions. When a mixture of 50% isopropyl alcohol-water mixture was used, the red pigment moved along the stationary phase to the middle of the chromatogram (R_f = 0.51) (Figure 1-F). But in this case, the other nonpolar photopigments eluded in such a way that they were not well separated and were mixed with the red spot. 50% isopropyl alcohol-water also seemed to be a better option than the 20% or 80% isopropyl alcohol-water system (Table 1). From these results, we inferred that to have an ideal separation among the nonpolar and polar pigments, we must optimize the solvent composition in the following way:

i. It should contain isopropyl alcohol-water mixture so that it can facilitate the movement of anthocyanin (red spot).
ii. It should contain n-hexane, ethyl acetate, and acetone to facilitate the movement and separation of the nonpolar

pigments.

iii. 1:1 ratio of isopropyl alcohol to water would be a good starting point.

We then formulated several other solvent systems containing all five above-mentioned solvents (n-hexanes, ethyl acetate, acetone, isopropyl alcohol, and water). We varied the relative amounts of the individual solvents, but kept two conditions constant – hexane was the major constituent (accounting for 50-80 % of the mixture), and the ratio of isopropyl alcohol to water was 1:1 (even though their actual amount varied). Using these solvent systems as the mobile phase, we were able to elude the red spot (R_f = ~0.1) for the first time in our study, without it being mixed with the yellow/orange/green spots. The nonpolar spots traveled to different distances, often clubbed together, based on the nature of the mobile phase. Encouraged by this result, we aimed to fine-tune the experimental conditions (both mobile and stationary phases) next to produce a chromatogram with distinctly separated polar and nonpolar spots.

A persisting issue with the paper chromatography technique in our case was the tailing of the spots making the separation process even more difficult. Thin-layer chromatography is usually faster, more sensitive, and exhibits better resolution than paper chromatography [26]. TLC also enables recovery of the samples, allowing for characterization and studying the samples at a later stage. As a result, TLC has gained immense popularity over PC in the past few decades. Hence, we wanted to examine TLC-based separation next.

Thin-layer chromatography (TLC)

Just like the paper chromatography, we started with pure solvents such as hexane or ethyl acetate as the mobile phase, and then gradually changed the. Even though the trends of elution were similar for both the stationary phases (paper and silica on aluminium), the extents of elution and the separations were different between PC and TLC. But in general, separation seemed to be better for TLC as spots were concentrated, not tailing, and better separated. Details about the multi-solvent mobile phases and the resulting thin-layer chromatograms have been summarized in Table 2 (Figure 2). The issue of eluding the red spot along with the nonpolar ones still remained for the initial sets of solvent systems (compositions 1-3).

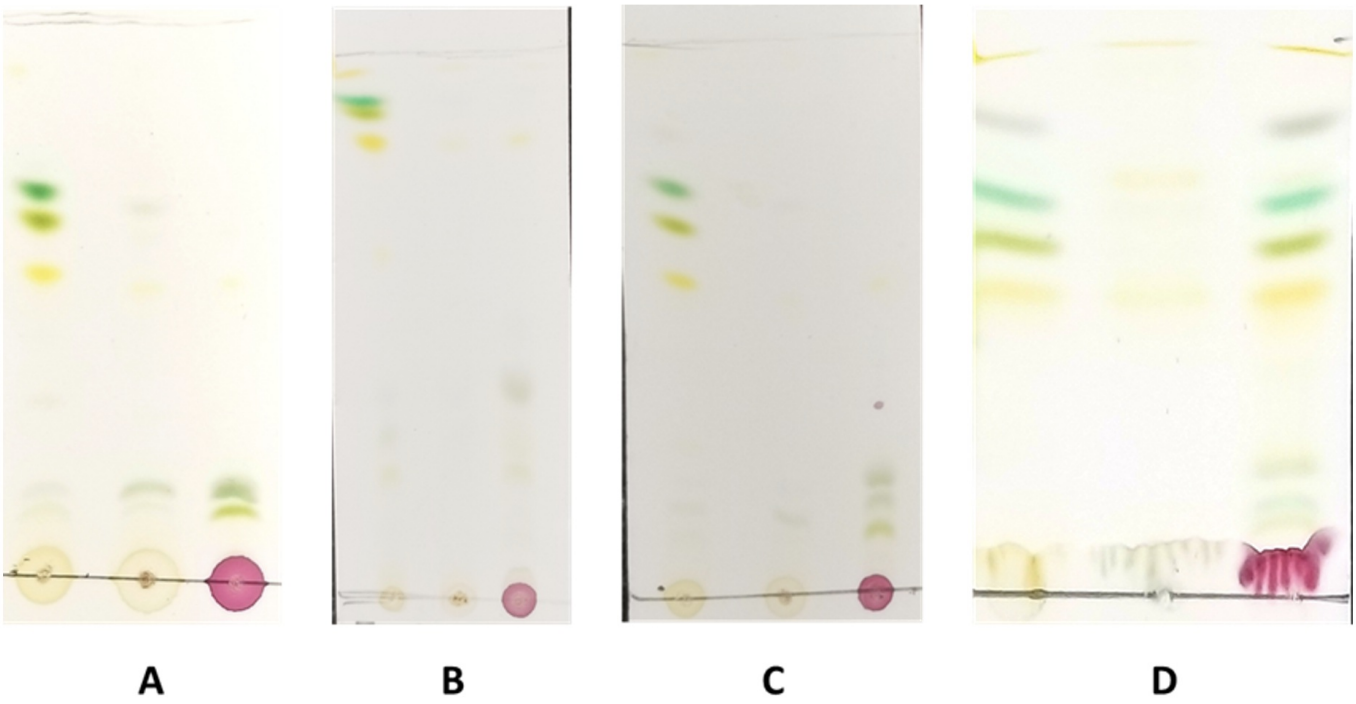

Figure 2. Representative images of a few TLC experiments done using different solvent systems, as mentioned in Table 2. The samples were collected from green (left spot) and red leaves (right spot). Panel A, B, C, and D represent compositions 2, 3, 5, and 6, respectively. Composition 6 containing 60% n-hexane, 10% ethyl acetate, 10% acetone, 10% isopropyl alcohol, and 10% water was found to be best suited to elude all the photopigments, facilitating their separation.

Table 2. Brief details about the thin-layer chromatographic experiments conducted in this study using different solvent systems and interferences based on the observation

Solvent composition (Total volume 10 mL)	Observation	Inferences
Composition 1: 10% acetone-10% isopropyl alcohol, 80% hexane	The orange, green, and yellow pigments moved up the chromatogram. Only the top part of the red spot showed a hint of partial movement	Unlike the paper chromatography for the same solvent composition, the spots were not tailing. It was possible to separate orange and dark green spots. But not the light green and yellow spots (as they overlapped). The red spot moved slightly.
Composition 2: 10% ethyl acetate, 10% isopropyl alcohol, 80% hexane	The orange, green, and yellow pigments moved up the chromatogram. The red spot did not move at all.	It was possible to separate all 5 nonpolar spots (orange, greyish green, dark green, light green, and yellow). It appeared that ethyl acetate was better at resolving the nonpolar spots, whereas the presence of acetone was key to moving the polar red spot. (Figure 2-A)
Composition 3: 10% ethyl acetate-10% acetone, 10% isopropyl alcohol, 70% hexane	The orange, green, and yellow pigments moved up the chromatogram. The red spot did not move at all.	Even though acetone and isopropyl alcohol were present, strangely the red spot did not move in this case. The absence of water seemed to be an influential factor in this case. As seen from the paper chromatography as well, a mixture of 1:1 isopropyl alcohol and water could be key in eluding the red spot. (Figure 2-B)
Composition 4: 10% acetone-10% isopropyl alcohol, 10% water, 70% hexane	The orange, green, and yellow pigments moved up the chromatogram but were very close. The red spot moved slightly.	In this case, the red spot moved a little, further supporting the importance of isopropyl alcohol-water mixture (1:1). However, separation of the nonpolar spots was compromised in this case.
Composition 5: 10% ethyl acetate-10% isopropyl alcohol, 10% water, 70% hexane	The orange, green, and yellow pigments moved up the chromatogram, but w better resolved. The red spot did not move at all.	The nonpolar spots moved and could be separated. However, the red spot did not move perhaps due to the absence of acetone. (Figure 2-C)
Composition 6: 10% ethyl acetate, 10% acetone-10% isopropyl alcohol, 10% water, 60% hexane	Movement and separation of all the colored pigments occurred. The orange one moved the farthest, the red one moved the least.	Based on the results of all the different compositions, this one seemed to work best in our study. The ethyl acetate-hexane component resolved the nonpolar spots, whereas, the acetone-isopropyl alcohol-water component made it possible to elude the red spot (Figure 2-D).

Based on our knowledge from the PC experiments discussed in the previous section, we included the polar solvent mixture of 50% isopropyl alcohol-water to the hexane and ethyl acetate/acetone mixture (compositions 4 and 5). In line with the data from the paper chromatography, an isopropyl alcohol-water mixture (1:1) appeared to be essential to move the red spot for TLC as well. The presence of acetone also seemed to be a key factor as the red spot did not move in its

absence (composition 5). However, in the presence of ethyl acetate, the separation of the nonpolar spots was better (composition 4). Putting together all these data, next, we prepared a solvent system with 60% hexane, 10% ethyl acetate, 10% acetone, 10% isopropyl alcohol, and 10% water. We found that this composition was indeed highly efficient in eluding all the pigments through the stationary phase and the separation was ideal for isolating them (Figure 2-D). To characterize different plant pigments separated using the chromatographic technique, we calculated their retention factor (R_f) values from the TLC (Table 3).

Table 3. Different bands observed during the separation of photopigments using TLC

Order from top of TLC	Color	Retention factor (R_f)	Probable photopigment
1	Orange	0.97	β-carotene
2	Greyish green	0.89	Pheophytin
3	Dark green	0.79	Chlorophyll-a
4	Light green	0.71	Chlorophyll-b
5	Yellow	0.66	Xanthophyll
6	Red	0.10	Anthocyanin

Isolation and characterization of the separated photopigments

Once the photopigments were separated, we wanted to isolate them and then characterize them. To achieve that, we loaded the leaf pigment extracts on the TLC plates with excess amounts and then let the TLC run using composition 6 (Figure 3-A and 3-B) [27]. The well-resolved and concentrated spots along with the silica were scratched off the plate using a small pipette tip and individual pigment solutions were prepared, as described in the ‘materials and methods’ section. Then UV-Vis absorption spectra were recorded for the isolated photopigments dissolved in isopropyl alcohol.

The UV-Vis spectroscopic data of all the photopigments were collected and matched with the reported ones[28][29][30]. Chlorophyll-a, chlorophyll-b, and pheophytin showed characteristic dual peaks (Figure 3-C) [28]. The peaks from both chlorophyll-a and pheophytin were around 400 and 680 nm, whereas the peaks from chlorophyll-b were around 450 and 650 nm. Due to low concentration, the absorbance values of xanthophylls and β-carotene were low, but characteristic peaks for both were obtained from the UV-Vis spectroscopy spreading from 350-500 nm (Fig. 3-D) [29]. Anthocyanin exhibited typical absorbance peaks around 200-400 nm (Fig. 3-E) [30].

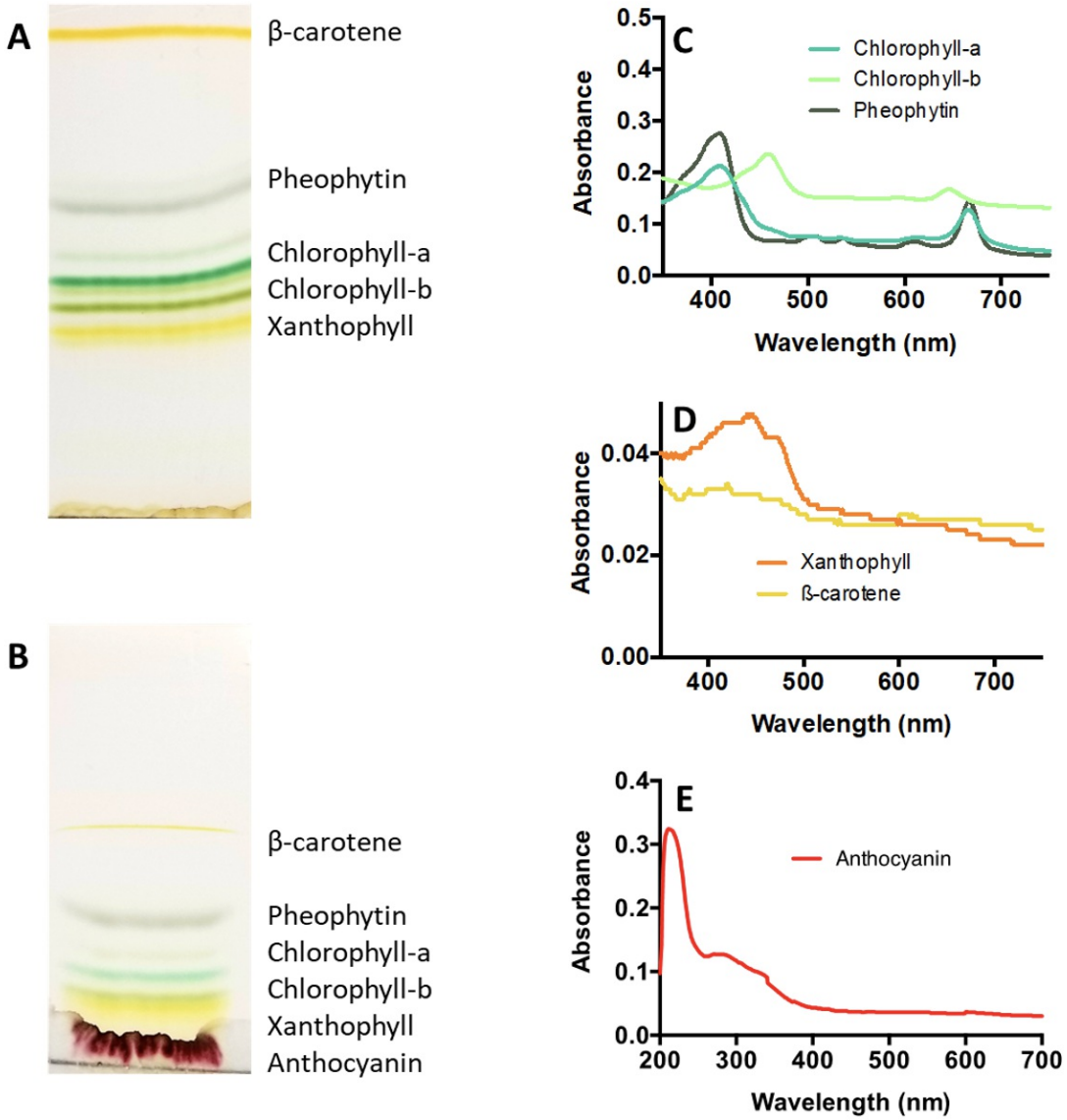

Figure 3. TLC images of pigments extracted from A) green leaves, and B) red leaves. These TLC plates were used for the isolation of individual photopigments that were used for UV-Vis spectroscopy. The UV-Vis absorption spectra for the isolated photopigments can be seen in panel C (chlorophyll-a and b, pheophytin), panel D (xanthophyll, β-carotene), and panel E (anthocyanin).

Discussion

In this study, we wanted to develop a solvent system that can separate the polar plant pigments from the nonpolar ones using planar chromatographic methods. Even though both paper and thin-layer chromatography techniques have been employed over the years, to this date, most of these techniques focused mostly on separating the nonpolar plant pigment mixture, and not a mixture of polar and nonpolar pigments (Table 4) [7][16][17][31][32][33][34]. A separate study claimed that anthocyanin moved after β-carotene and chlorophylls, but before xanthophylls when a mixture of hexane, acetone, and chloroform (3:1:1) was used [21]. Unfortunately, we could not reproduce this result in our lab as anthocyanins did not move at all, but xanthophylls did. This discrepancy could be probably due to the nature of anthocyanins and xanthophylls

explored in the original study, or some variances in the experimental conditions. The separation was not great either, and the spots were tailing.

Table 4. A synopsis of literature data available for the separation of nonpolar plant pigments using planar chromatography.

Samples	Pigments separated	Types of planar chromatography	Mobile phase (v/v % composition)	References
Spinach	Chlorophylls, Carotenes, Pheophytins, xanthophylls	Thin layer chromatography	60% isooctane 20% acetone 20% petroleum ether (or, carbon tetrachloride)	Reference [7]
Marine algae	Chlorophylls, Carotenes, Pheophytins, xanthophylls	Paper chromatography	4% propane-1-ol 96% light petroleum and 30% chloroform 70% light petroleum	Reference [16]
cocklebur, oats, wheat, spinach, and Elodea	Chlorophylls, Carotenes, xanthophylls	Paper chromatography	99% light petroleum ether-1% n-propanol	Reference [17]
Spinach	Chlorophylls, Carotenes, Pheophytins, xanthophylls	Thin layer chromatography	60% petroleum ether 16% cyclohexane 10% ethyl acetate 10% acetone 4% methanol.	Reference [31]
Alga	Chlorophylls, Carotenes, Pheophytins, xanthophylls	High performance thin layer chromatography	75% petroleum ether 25% acetone and medical petrol:isopropyl alcohol:ultrapure water (100:10:0.25)	Reference [32]
Ruccola	Chlorophylls, Carotenes, Pheophytins, xanthophylls	Normal and Reversed-Phase Thin Layer Chromatography	70% n-hexane 30% acetone and 15% n-hexane 35% acetonitrile 50% ethanol	Reference [33]
Spinach	Chlorophylls, Carotenes, xanthophylls	Thin layer chromatography	60% iso-octane 20% acetone 20% diethyl ether	Reference [34]

In our study, we found that no pure solvents (mobile phase) were able to elude the pigments separately on their own. Either they did not travel at all or some of them traveled together on paper making it impossible for us to distinguish between different spots. The only exception to this was when β-carotene moved separately with n-hexane as the mobile phase. The trickiest factor leading to this problem was the nature of the plant pigments. Out of these pigments, chlorophyll a and b, xanthophylls, and β -carotene are highly nonpolar in nature, whereas anthocyanin is highly polar [35]. Except for anthocyanin, which is hydrophilic in nature, all the other plant pigments mentioned here were hydrophobic. These nonpolar pigments are highly soluble in nonpolar solvents like hexane and some other polar organic solvents (ethanol, acetone, etc.). On the other hand, anthocyanin is highly soluble in water, methanol, ethanol, etc. but insoluble in nonpolar solvents like hexane. So, it was clear we needed to find a proper blend of polar and nonpolar solvents to elude all the pigments sequentially. To avoid the tailing and overlapping in PC, we shifted to TLC next. For TLC, the pigments were usually well resolved as distinct and concentrated spots, and not tailing, making it ideal for separation and detection. This is perhaps due to the differences in the nature of the materials, as cellulose and silica are the primary constituents for PC and TLC, respectively. The differences in properties of cellulose and silica, as well as the texture of the mobile phase, might have played a key role here.

Next, we decided to try out a wide range of solvent systems (consisting of hexane, ethyl acetate, acetone, isopropyl alcohol, and water in different proportions) to fine-tune the elution process using TLC. Consistent with the PC data, a 50% isopropyl alcohol-water mixture was found to be key to moving the polar anthocyanin for TLC as well. Interestingly, unlike PC, the presence of acetone was also necessary for TLC to facilitate the movement of anthocyanin along with the isopropyl alcohol-water system. The strong affinity between polar anthocyanin molecules and highly polar silica could be a possible reason behind this observation. The presence of ethyl acetate improved the separation between the nonpolar spots, making them well-resolved. In light of inferences drawn from these observations, we formulated a solvent system consisting of 60% n-hexane, 10% ethyl acetate, 10% acetone, 10% isopropyl alcohol, and 10% water. This formulation was able to dissolve and elude both the nonpolar and polar pigments in a sequential manner so that they were all well separated. The nonpolar ones traveled first, and polar anthocyanin started moving after the nonpolar ones.

Our data showed that we were able to isolate and purify all six major photopigments present in the red leaves of copperleaf. As we only used green and red leaves for this study, the major photopigments were chlorophylls and anthocyanins, respectively. This is also reflected in the absorption spectra (Figure 3C-3E). The absorbance values of β-carotene and xanthophylls were relatively low, perhaps due to low abundance leading to lower concentrations. The detection of pigments such as pheophytin, β-carotene, and xanthophylls in the red leaves of copperleaf further emphasized the high sensitivity of the TLC technique.

Conclusion

To explore the structures and properties of the plant photopigments, it is essential to isolate them in their pure forms. Some of the isolation techniques are expensive and complicated in nature, while others are simple, convenient yet

effective. Planar chromatography techniques belong to the latter category. Reports available in the literature successfully separated the nonpolar plant pigments using the planar chromatographic techniques, but not from a mixture with the polar ones. In this study, we conducted a series of experiments using both paper and thin-layer chromatography and reported a novel solvent composition (60% n-hexane, 10% ethyl acetate, 10% acetone, 10% isopropyl alcohol, and 10% water) that can be used to separate the nonpolar component from the polar ones using TLC. In addition to their colors and R_f values, we characterized the pigments through UV-Vis absorption spectra as well.

This method reported here has the potential to be beneficial for certain research activities where qualitative isolation of the plant pigments is sufficient. This could also be useful for educational purposes. Moreover, the solvent composition reported here can also lead to identifying the suitable solvents for HPLC for the same purpose in case of further quantification with more precision or handling a large quantity of samples. In future, a significant focus will be on isolating the other photopigments present in leaf extracts in addition to the major ones mentioned here.

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References

1. [a, b]*Sudhakar, P., Latha, P. and Reddy, P.V. Chapter 15 - Plant pigments. (2016); 121-127. doi.org/10.1016/B978-0-12-804073-7.00015-6.*
2. [a, b]*Niyogi, K.K., Bjorkman, O. and Grossman, A.R. The roles of specific xanthophylls in photoprotection. Proceedings of the National Academy of Sciences, (1997); 94(25): 14162–14167. https://doi:10.1073/pnas.94.25.14162.*
3. [a, b]*Swapnil, P., Meena, M., Singh, S.K., Dhuldhaj, U.P., Harish and Marwal, A. Vital roles of carotenoids in plants and humans to deteriorate stress with its structure, biosynthesis, metabolic engineering and functional aspects. Current Plant Biology, (2021); 26: 100203. doi:10.1016/j.cpb.2021.100203.*
4. [^]*Zheng, X.-T., Yu, Z.-C., Tang, J.-W., Cai, M.-L., Chen, Y.-L., Yang, C.-W., Chow, W.S. and Peng, C.-L. The major photoprotective role of anthocyanins in leaves of Arabidopsis thaliana under long-term high light treatment: antioxidant or light attenuator? Photosynthesis Research. (2021); 149: 25-40. doi:10.1007/s11120-020-00761-8.*
5. [^]*Gahlawat, I. N. Emerging New Insights into Significance and Applications of Plant Pigments. Journal of Integrated Science and Technology, (2019); 7 (2): 29–34.*
6. [^]*Naqvi, S.F.H. and Husnain, M. Betalains: Potential Drugs with Versatile Phytochemistry. Critical Reviews in Eukaryotic Gene Expression, (2020); 30(2): 169–189. doi.org/10.1615/critreveukaryotgeneexpr.2020030231.*
7. [a, b, c, d, e]*Anwar, M.H. Separation of plant pigments by thin layer chromatography. Journal of Chemical Education, (1963); 40(1): 29-31. doi:10.1021/ed040p29.*

8. [a, b]*Benson, E.E. and Cobb, A.H. The Separation, Identification and Quantitative Determination Of Photopigments From The Siphonaceous Marine Alga Codium Fragile. New Phytologist, (1981); 88(4): 627–632. doi:10.1111/j.1469-8137.1981.tb01738.x.*
9. [a, b]*Indriatmoko, Shioi, Y., Brotosudarmo, T.H.P. and Limantara, L. Separation of Photosynthetic Pigments by High-performance Liquid Chromatography: Comparison of Column Performance, Mobile Phase, and Temperature. Procedia Chemistry, (2015); 14: 202–210. doi:10.1016/j.proche.2015.03.029.*
10. [^]*Hosikian, A., Lim, S., Halim, R. and Danquah, M.K. Chlorophyll Extraction from Microalgae: A Review on the Process Engineering Aspects. International Journal of Chemical Engineering. (2010); Article 391632: 1-11. doi:10.1155/2010/391632*
11. [^]*Hu, X., Tanaka, A. and Tanaka, R. Simple extraction methods that prevent the artifactual conversion of chlorophyll to chlorophyllide during pigment isolation from leaf samples. Plant Methods, (2013); 9(1): 19, 1-13. doi:10.1186/1746-4811-9-19.*
12. [^]*Dunn, J.L., Turnbull, J.D. and Robinson, S.A. Comparison of solvent regimes for the extraction of photosynthetic pigments from leaves of higher plants. Functional Plant Biology, (2004); 31(2): 195-202. doi:10.1071/fp03162.*
13. [^]*Pressman, P., Clemens, R., Sahu, S. and Hayes, A.W. A review of methanol poisoning: a crisis beyond ocular toxicology. Cutaneous and Ocular Toxicology, (2020); 39(3): 173–179. doi:10.1080/15569527.2020.1768402.*
14. [^]*Forgacs, E. and Cserhati, T. Thin-layer chromatography of natural pigments: New advances. Journal of Liquid Chromatography & Related Technologies, (2002); 25(10-11): 1521–1541. doi:10.1081/jlc-120005702.*
15. [^]*Wolf, F.T. The Separation of Chloroplast Pigments by Unidimensional Ascending Paper Chromatography. Bulletin of the Torrey Botanical Club, (1958); 85(2): 128-133. doi:10.2307/2483026.*
16. [a, b, c, d]*Jeffrey, S.W. Paper-chromatographic separation of chlorophylls and carotenoids from marine algae. Biochemical Journal, (1961); 80(2): 336–342. doi: 10.1042/bj0800336*
17. [a, b, c, d]*Strain, H.H., Sherma, J., Benton, F.L. and Katz, J.J. One-way paper chromatography of the chloroplast pigments of leaves. Biochimica et Biophysica Acta (BBA) - Biophysics including Photosynthesis, (1965); 109(1): 1–15. doi:10.1016/0926-6585(65)90084-1.*
18. [^]*Santos-Buelga, C., Mateus, N. and De Freitas, V. Anthocyanins. Plant Pigments and Beyond. Journal of Agricultural and Food Chemistry, (2014); 62(29): 6879–6884. doi:10.1021/jf501950s.*
19. [^]*Welch, C., Wu, Q. and Simon, J. Recent Advances in Anthocyanin Analysis and Characterization. Current Analytical Chemistry, (2008); 4(2): 75–101. doi:10.2174/157341108784587795.*
20. [^]*Cretu, G.C. and Morlock, G.E. Analysis of anthocyanins in powdered berry extracts by planar chromatography linked with bioassay and mass spectrometry. Food Chemistry, (2014); 146: 104–112. doi:10.1016/j.foodchem.2013.09.038.*
21. [a, b]*Reiss C. Experiments in Plant Physiology. Englewood Cliffs, NJ, USA: Prentice Hall. ISBN: 0137012853. (1994).*
22. [^]*Reuber, M.D. Carcinogenicity of chloroform. Environmental Health Perspectives, (1979); 31: 171–182. doi:10.1289/ehp.7931171.*
23. [^]*Sridhar, N., Krishnakishore, C., Sandeep, Y., Sriramnaveen, P., Manjusha, Y. and Sivakumar, V. Chloroform Poisoning – A Case Report. Renal Failure, (2011); 33(10): 1037–1039. doi:10.3109/0886022x.2011.618920.*
24. [^]*Chandra, S.P., Adiga, V., Chinmaya, P., Kikkeri, V.V. and Senapati, S. Modulation of stomatal conductance in*

response to changes in external factors for plants grown in the tropical climate. Journal of High School Science, (2023); 7(1): 1-17. Available at: https://jhss.scholasticahq.com/article/66172

25. ^*Silver, J. Let Us Teach Proper Thin Layer Chromatography Technique! Journal of Chemical Education, (2020); 97(12): 4217–4219. doi:10.1021/acs.jchemed.0c00437.*
26. ^*Hosikian, A., Lim, S., Halim, R. and Danquah, M.K. Chlorophyll Extraction from Microalgae: A Review on the Process Engineering Aspects. International Journal of Chemical Engineering, (2010); Article 391632, 1-11. doi.org/10.1155/2010/391632.*
27. ^*Sherma, J. and Fried, B. Chapter 2 Preparative Thin Layer Chromatography. (1987); 38: 105-127. doi.org/10.1016/S0301-4770(08)60365-6*
28. a, b*Borello, E., Roncucci, D. and Domenici, V. Study of the Evolution of Pigments from Freshly Pressed to 'On-the-Shelf' Extra-Virgin Olive Oils by Means of Near-UV Visible Spectroscopy. Foods, (2021); 10(8): 1891, 1-25. doi:10.3390/foods10081891.*
29. a, b*Domenici, V., Ancora, D., Cifelli, M., Serani, A., Veracini, C.A. and Zandomeneghi, M. Extraction of Pigment Information from Near-UV Vis Absorption Spectra of Extra Virgin Olive Oils. Journal of Agricultural and Food Chemistry, (2014); 62(38): 9317–9325. doi:10.1021/jf503818k.*
30. a, b*Wahyuningsih, S., Wulandari, L., Wartono, M.W., Munawaroh, H. and Ramelan, A.H. The Effect of pH and Color Stability of Anthocyanin on Food Colorant. IOP Conference Series: Materials Science and Engineering, (2017); 193, 012047. doi.org/10.1088/1757-899x/193/1/012047.*
31. a, b*Quach, H. T., Steeper, R. L., & Griffin, G. W. An Improved Method for the Extraction and Thin-Layer Chromatography of Chlorophyll a and b from Spinach. Journal of Chemical Education, (2004); 81(3), 385-387. doi.org/10.1021/ed081p385*
32. a, b*Hynstova, V., Sterbova, D., Klejdus, B., Hedbavny, J., Huska, D. and Adam, V. Separation, identification and quantification of carotenoids and chlorophylls in dietary supplements containing Chlorella vulgaris and Spirulina platensis using High Performance Thin Layer Chromatography. Journal of Pharmaceutical and Biomedical Analysis, (2018); 148, 108–118. doi.org/10.1016/j.jpba.2017.09.018.*
33. a, b*Sjursnes, B.J., Kvittingen, L. and Schmid, R. Normal and Reversed-Phase Thin Layer Chromatography of Green Leaf Extracts. Journal of Chemical Education, (2014).; 92(1), 193–196. doi.org/10.1021/ed400519v.*
34. a, b*Sherma, J. and Fried, B. Separation and determination of chloroplast pigments from spinach by thin-layer chromatography: A student laboratory experiment. Journal of Planar Chromatography – Modern TLC, (2004); 17(4), 309–313. doi.org/10.1556/jpc.17.2004.4.13.*
35. ^*Sousa, C. Anthocyanins, Carotenoids and Chlorophylls in Edible Plant Leaves Unveiled by Tandem Mass Spectrometry. Foods, (2022); 11(13): 1924, 1-31. doi:10.3390/foods11131924.*