Developing A Novel Solvent System to Isolate Plant Pigments of Different Polarities 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 that can be used to separate the nonpolar pigments from the polar ones using thin-layer chromatography (TLC). Using a mixture of hexane:ethyl acetate:acetone:isopropyl alcohol:water = 6:1:1:1:1 as the mobile phase, we were able to obtain a TLC, where spots are distinctly separated, concentrated, and could easily be isolated. The pigments were identified from their colors and R_f values, and characterized 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, carotenoids, etc.), whereas some other pigments directly or indirectly help in other processes such as growth and development, protection, and pollination (phytochromes, anthocyanins to name a few) [1][2][3]. To get insights into these pigments, it is critical to isolate and purify these pigments and conduct further studies on them. To this end, different methodologies or protocols have been reported in the literature [4][5][6][7][8][9]. These methodologies have employed a wide variety of techniques, ranging from simple chromatographic techniques (paper chromatography, 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 involved multiple steps, expensive instruments, more solvents, and sometimes corrosive or hazardous chemicals such as acids, and were not very cost-effective. One of the most commonly used solvents is methanol, and some pigments have been found to be unstable in it [6]. Methanol is also known to be toxic in nature[10]. Sometimes these processes involve some harmful or hazardous chemicals as well, such as acetic acid, halogenated solvents, etc [11][12]. Hence, often planar chromatographic techniques (paper chromatography and TLC) are preferred due to their simplistic and convenient nature [4][5][13][14]. 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 chromatographic techniques, most of these studies were focused on separating the nonpolar pigments such as chlorophyll-a, chlorophyll-b, pheophytin, β-carotene, and xanthophylls [4][13][14]. On the other hand, several plants contain highly polar pigments such as anthocyanins in addition to the nonpolar ones [3][15]. Different techniques have been employed over the years to isolate anthocyanins as well, but usually not from a mixture containing highly nonpolar pigments [16][17]. 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 [18]. When repeated, the more nonpolar xanthophylls eluded before the highly polar anthocyanins that did not move, contradicting the reported data. Moreover, the solvent system included chloroform, which is nowadays avoided due to its carcinogenic nature [19]. Chloroform also has been found to be responsible for several other severe diseases [20]. 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.

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 planar chromatography (thin-layer chromatography (TLC) or paper chromatography). We also wanted to ensure that the chemicals being used were not threats to health under normal circumstances. 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. Our results indicated that a solvent composition of hexane:ethyl acetate: acetone:isopropyl alcohol:water = 6:1:1:1:1 as the mobile phase was ideal for this purpose using silica-coated aluminium as the stationary phase.

Experimental

Materials

The stationary phase for the paper chromatography was obtained by cutting filter papers (Whatman, #1001-125) into rectangular shapes. For the thin-layer chromatography (TLC), silica-coated aluminium was chosen as the stationary phase (TLC Silica gel 60 F_{254}, Merck, Germany). All the solvents used as the constituents of the mobile phase were purchased from SRL Chemical (India).

UV-Vis spectroscopy

UV-Vis absorption spectra were recorded for the isolated photopigments dissolved in isopropyl alcohol. 1-1.5 mL of the sample solutions were transferred to a quartz cuvette and measurement was done on a Shimadzu UV-1780 UV-Vis spectrophotometer using UV Probe software.

Methods

Plant pigment extract preparation

Two plant species were selected for this study – American basswood (*Tila americana*) and copperleaf (*Acalypha wilkesiana*). 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 comes due to the presence of anthocyanins in copperleaf. First, we collected 3-4 pieces of fresh leaf samples from each plant and tear them into smaller pieces. Then 2-3 mL of isopropyl alcohol was added to the smaller leaf fragments in a mortar and these were ground to even smaller fragments using the pestle. At this stage, the photopigments started to get dissolved in isopropyl alcohol and this extraction process was facilitated by stirring the pestle. The color of the solution changed

depending on the color of the photopigments. Acetone was also used instead of isopropyl alcohol giving the same result.

Chromatographic Separation

Chromatographic separation of the photopigments was done using two different chromatographic methods – paper chromatography and thin layer chromatography (TLC). The stationary phase for the paper chromatography was obtained by cutting the Whatman filter paper into rectangular shapes. The stationary phase for the TLC was silica gel on aluminium support (Silica gel 60 F254, Merck, Germany). These extracts were then spotted on the stationary phases (paper or TLC) and run inside the TLC chamber carrying the mobile phases. We tried several mobile phases by trying out different combinations of solvents such as water, hexane, ethyl acetate, acetone, and isopropyl alcohol. 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 travelled by the photopigments by the distance travelled by the solvent front [21].

Results

Paper chromatography

As our first method to isolate the photopigments, we chose paper chromatography as the separation method as it is simple, convenient, and easy to perform even in a basic lab setting. From the green leaves, we prepared the extract primarily containing the green pigments (chlorophylls) as described in the method section. We first started with a purely polar (ethyl acetate) and another purely non-polar solvent (n-hexane) to gauge the movement and separation of the photopigments on paper. For hexane, we observed multiple spots of different colors – green, yellow, orange, etc. Most of them moved easily with the solvent but were not well separated, making it difficult to isolate the photopigments. On the other hand, with pure ethyl acetate as the mobile phase, the spots did not move much. Before trying different solvent compositions based on their polarities, we used the extract from the red leaf as well, but the red spot from the red leaf extract did not move at all either in hexane or in ethyl acetate. As the next step, we tried different other solvents, first in pure form and then as solvent mixtures, gradually changing the composition (constituents and their relative amounts). 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 these solvents. In pure isopropyl alcohol, the green, yellow, and orange pigments moved, but, the red one did not move. After the initial round of experimenting with different compositions, 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 in a relatively better way 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 to elude anthocyanins. When a mixture of 50% isopropyl alcohol-water mixture was used, the red pigment moved along the stationary phase. But in this case, the other nonpolar photopigments eluded in such a way that they were not well separated and the separation was found to be difficult using paper

chromatography. Another issue with the paper chromatography technique was the tailing of the spots making the separation process even more difficult.

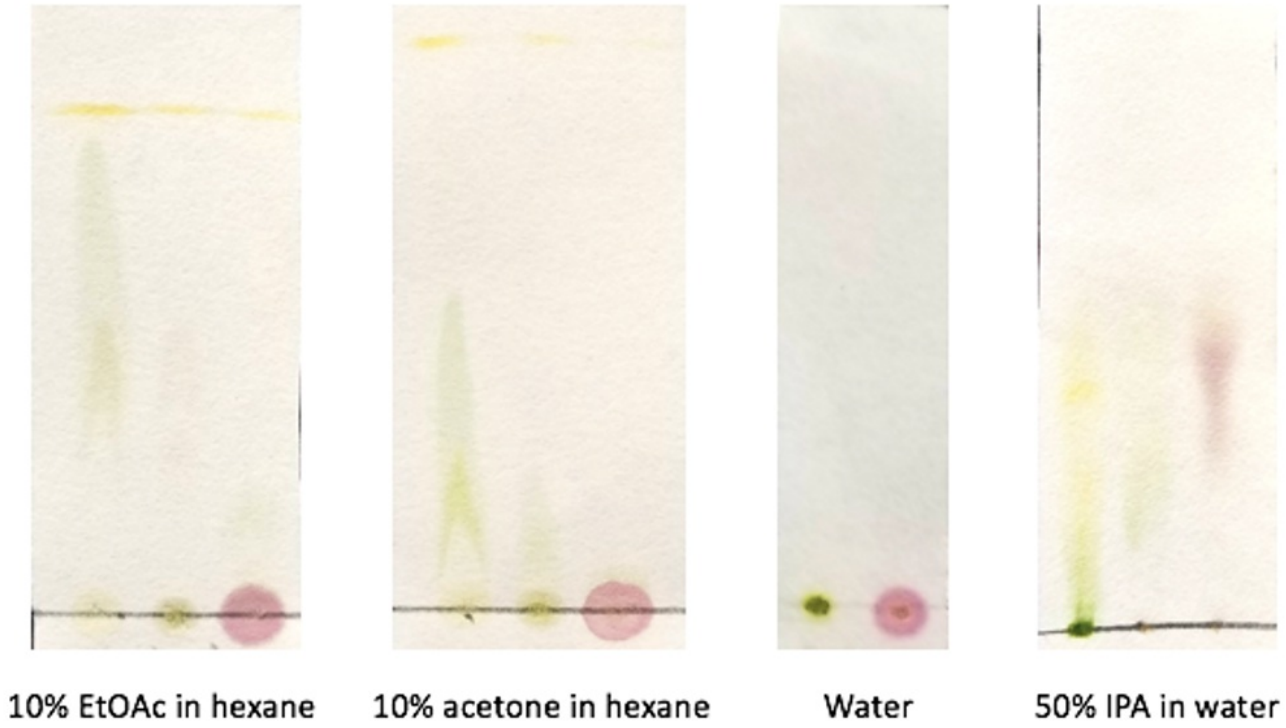

Figure 1. Representative images of some of the paper chromatography experiments done using different solvents as mobile phase. The samples were collected from green and red leaves.

Thin-layer chromatography (TLC)

We used thin-layer chromatography as our next stationary phase. Silica coated on aluminium support was used for this purpose. Just like the paper chromatography, we started with pure solvents such as hexane or ethyl acetate as the mobile phase, and then 10% ethyl acetate in hexane or 10% acetone in hexane. Even though the trend of elution was similar for both the stationary phases (paper and silica on aluminium), the extent of elution and the separation were different for different stationary phases. But in general, separation seemed to be better for TLC as spots were not tailing and were well separated. But the issue of eluding the red spot along with the other ones still remained for all the solvent compositions tried till this point.

Based on our knowledge from the paper chromatography experiments discussed in the previous section, we included the polar solvent mixture of 50% isopropyl alcohol-water to the hexane-ethyl acetate-acetone mixture. The composition of the solvent mixture was chosen in such a way that it can dissolve both the polar and nonpolar pigments and elude them sequentially. As seen from the paper chromatography, water was essential to move the red spot when combined with isopropyl alcohol. We wanted to test if the presence of water can move the red spot for TLC as well. Hence, we prepared a mixture with the composition of hexane:ethyl acetate:acetone:isopropyl alcohol:water = 6:1:1:1:1. We found that this

composition was indeed highly efficient in eluding all the photopigments through the stationary phase and the separation was found to be better in case of TLC compared to paper chromatography (Figure 2). To characterize different photopigments separated using the chromatographic technique, we calculated their retention factor (R_f) values from the TLC. R_f values of the photopigments from a typical TLC run are given in Table 1.

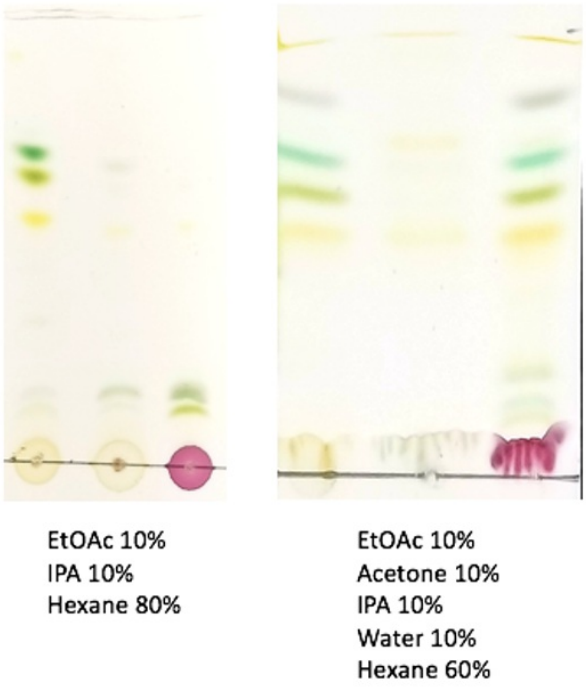

Figure 2. Representative images of some of the TLC experiments done using different solvents as mobile phase. The samples were collected from green and red leaves. The solvent system hexane:ethyl acetate:acetone:isopropyl alcohol:water = 6:1:1:1:1 was found to be best suited to elude all the photopigments separately (image on the right)

Table 1. 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	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 extracts on the TLC plates with excess amounts and then let the TLC run using the aforementioned solvent system (Figure 3-A and 3-B) [22]. Once the spots were well separated, the spots along with the silica were scratched off the plate using a small pipette tip and were transferred to small Eppendorf tubes. Then 1-1.5 mL of isopropyl alcohol was added to the Eppendorf tubes to dissolve and extract the photopigments. Extracted pigment fractions were used for UV-Vis spectroscopy.

The UV-Vis spectroscopic data of all the photopigments were collected and matched with the reported ones. Chlorophyll-a, chlorophyll-b, and pheophytin showed characteristic dual peaks (Figure 3-C) [23]. 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 ß-carotene were low, but characteristic peaks for both of them were obtained from the UV-Vis spectroscopy spreading from 350-500 nm (Fig. 3-D) [24]. Anthocyanin exhibited typical absorbance peaks around 200-400 nm (Fig. 3-E) [25].

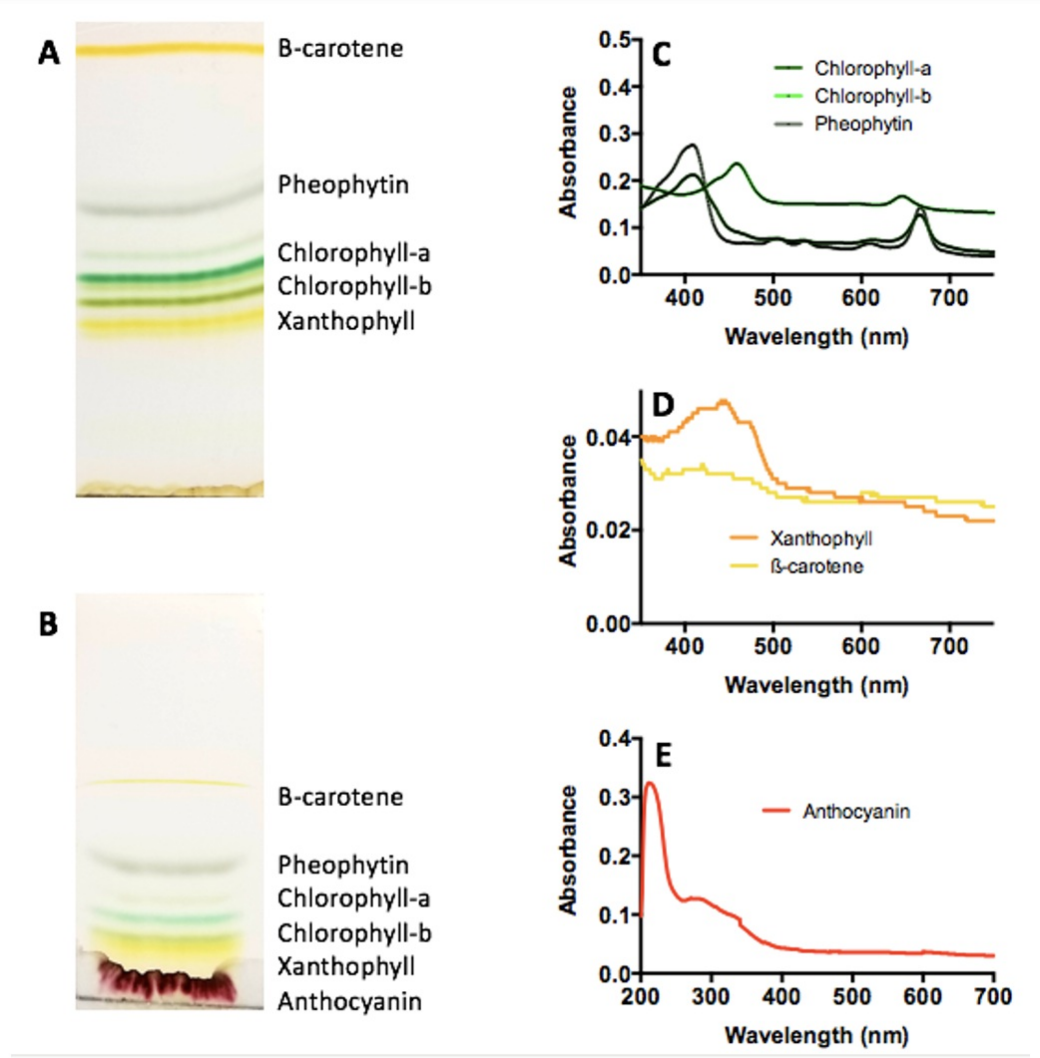

Figure 3. TLC images of pigments extracted from A) green leaves, and B) red leaves. These TLC plates were used for the isolation of individual photopigments that were used for UV-Vis spectroscopy. Panel C, D, and E exhibits the UV-Vis absorption spectra for the isolated photopigments.

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. Separation of these pigments can be a complex process, involving a few pieces of equipment/instruments, and is often expensive. Hence, planar chromatographic techniques such as paper or thin-layer chromatography are preferred under certain circumstances. Planar chromatographic techniques are easy to carry out, convenient, sensitive, and inexpensive and yet serve the main purpose of separating components from a mixture in a very basic lab setting [4][5][13][14]. One major advantage of planar chromatography is the ability to analyze multiple samples in the same run. It also requires less volume of samples and less run time to conduct the separation. 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 [4][13][14]. In most of the studies, anthocyanins did not move at all from the baseline due to their highly polar nature. In one study, it was claimed anthocyanins moved after β-carotene and chlorophylls, but before xanthophylls when a mixture of hexane, acetone, and chloroform (3:1:1) [18]. 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. Moreover, the separation was not great either, and the spots were tailing.

In our study, we found that no pure solvents (mobile phase) were able to differentiate between the photopigments. 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 trickiest factor leading to this problem was the nature of the photopigments. It is well established that the green, yellow/orange, and red colors of leaves are due to the presence of chlorophylls (a and b), xanthophylls, ß-carotene, and anthocyanin, respectively. Out of these pigments, chlorophyll a and b, xanthophylls, and ß-carotene are highly nonpolar in nature, whereas anthocyanin is highly polar [26]. Except for anthocyanin, which is hydrophilic in nature, all the other plant pigments mentioned here were hydrophobic. As a result of that, except for anthocyanin, all the other 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 highly nonpolar solvents such as hexane. So, it was clear we needed to find a proper blend of polar and nonpolar solvents to elude all the photopigments. Pure hexane (nonpolar) or mixtures of hexane with polar solvents such as ethyl acetate or acetone were able to elude the nonpolar photopigments, but not anthocyanin. Interestingly, pure water or pure IPA was not able to elude anthocyanin individually, but a 50% mixture of IPA in water did. But the 50% IPA-water system also eluded the nonpolar pigments clubbed together, making it impossible to separate them. We also noticed that the pigments were tailing in the paper chromatography, even if they are moving across the paper. Hence, we shifted to thin-layer chromatography using the same solvent systems. We found that for TLC, the pigments were now well isolated 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 paper chromatography

and TLC, respectively. The properties of these ingredients, as well as the texture, might have played a key role in this case.

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. We observed that a solvent composition of hexane:ethyl acetate: acetone:isopropyl alcohol:water = 6:1:1:1:1 was able to dissolve and elude both the polar and nonpolar pigments in a sequential manner so that they were all well separated. The nonpolar ones travelled first, and polar anthocyanin started moving only after the nonpolar ones. As anthocyanin, unlike the others, had both polar and nonpolar ends, both types of solvents were needed for the mobile phase.

As the first step of characterization, we assigned the types of photopigments based on the color of the photopigments (table 1). We then calculated and compared their R_f values to further characterize the pigments as reported in the literature. We focused on the major bands that we observed during the chromatographic separation. From the top, these bands are for ß-carotene (orange), pheophytin (greyish green), chlorophyll-a (dark green), chlorophyll-b (yellowish green), xanthophylls (yellow), and anthocyanin (red). To further characterize these bands using analytical techniques, we separately scratched the silica-containing pigments and dissolve them in IPA. We recorded the UV-Vis absorption spectra using each separated fraction and compared them with the literature. Our data showed that we were able to isolate and purify all six major photopigments present in the leaves chosen for this study. As we only collected green and red leaves for this analysis, the major photopigments were chlorophylls and anthocyanins, respectively. This is also reflected in the absorption spectra as absorbance values of ß-carotene and xanthophylls were relatively low, perhaps due to low abundance leading to lower concentrations. It should also be noted that even though the copperleaf was primarily red in color, still some of the other pigments were still present in it as evident from Figure 2-B, albeit in lesser amounts.

Conclusion

To explore the structures and properties of the plant photopigments, it is essential to isolate them in their pure forms. Different strategies have been adopted in the past for this, and some of them are expensive and complicated in nature, while others are simple, convenient yet effective. Planar chromatography techniques (i.e. paper and TLC) 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 that can be used to separate the nonpolar component from the polar ones using TLC. After trying out several solvent compositions in both parallel and sequential manners, we found that a mixture of hexane:ethyl acetate:acetone:isopropyl alcohol:water = 6:1:1:1:1 is suitable for this purpose. In addition to the colors and R_f values, we characterized the pigments through UV-Vis absorption spectra as well.

One major limitation of the planar chromatographic technique is its incapability to handle a large amount of sample. Also, the quantification of samples is much more suited for sophisticated techniques such as HPLC than paper or thin-layer

chromatography. Having said that, the ease and simplicity of planar chromatographic techniques give them a definite edge based on the nature of the application. Hence, this method reported here has the potential to be beneficial for certain research activities where qualitative isolation of the plant pigments is sufficient. This also could be impactful for educational purposes for teaching the students several concepts such as plant pigments, polar and nonpolar compounds, and different chromatographic techniques. 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 containing the pigments.

In the future, a significant focus will be on isolating the other photopigments present in these extracts in addition to the major ones mentioned here and further optimization might be needed to achieve that. Also, in addition to the green and red ones, yellow leaves will also be explored. Yellow leaves might give us more information about the different types of carotenoids and xanthophylls. Finally, characterization will also be done using a suitable mass spectroscopic method.

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Other References

- 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. (2020) doi:10.1007/s11120-020-00761-8.

References

1. [a, b] *Sudhakar, P., Latha, P. and Reddy, P.V. Chapter 15 - Plant pigments. (2016). https://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. [a, b, c, d, e] *Anwar, M.H. Separation of plant pigments by thin layer chromatography. Journal of Chemical Education, (1963); 40(1): 29. doi:10.1021/ed040p29.*
5. [a, b, c] *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.

6. [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.*
7. [^]*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*
8. [^]*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. doi:10.1186/1746-4811-9-19.*
9. [^]*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. doi:10.1071/fp03162.*
10. [^]*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.*
11. [^]*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.*
12. [^]*Wolf, F.T. The Separation of Chloroplast Pigments by Unidimensional Ascending Paper Chromatography. Bulletin of the Torrey Botanical Club, (1958); 85(2): 128. doi:10.2307/2483026.*
13. [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*
14. [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.*
15. [^]*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.*
16. [^]*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.*
17. [^]*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.*
18. [a, b]*Reiss C. Experiments in Plant Physiology. Englewood Cliffs, NJ, USA: Prentice Hall. ISBN: 0137012853. (1994).*
19. [^]*Reuber, M.D. Carcinogenicity of chloroform. Environmental Health Perspectives, (1979); 31: 171–182. doi:10.1289/ehp.7931171.*
20. [^]*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.*
21. [^]*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.*
22. [^]*Sherma, J. and Fried, B. Chapter 2 Preparative Thin Layer Chromatography. (1987). ScienceDirect.*

sciencedirect.com/science/article/abs/pii/S0301477008603656

23. ^*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. doi:10.3390/foods10081891.*
24. ^*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.*
25. ^*Ge, Q. and Ma, X. Composition and antioxidant activity of anthocyanins isolated from Yunnan edible rose (An ning). Food Science and Human Wellness, (2013); 2(2): 68–74. doi:10.1016/j.fshw.2013.04.001.*
26. ^*Sousa, C. Anthocyanins, Carotenoids and Chlorophylls in Edible Plant Leaves Unveiled by Tandem Mass Spectrometry. Foods, (2022); 11(13): 1924. doi:10.3390/foods11131924.*