Separation of photosynthetic pigments: paper chromatography

Plant pigments have the ability to absorb visible light, which can be used in order to harvest energy for photochemical reactions. There are a variety of pigments present in plants, and for this experiment, these pigments were separated using paper chromatography. Absorbance spectrophotometry was also used in order to obtain the different absorbances of the pigments. The data was then used to compare relative chlorophyll content in both old and young leaves, and to identify the pigments present in the leaves. It was seen that chlorophyll a and b were present, and young leaves yielded more chlorophyll than old leaves as reflected by the high absorbance of the young leaf extract as compared to the old leaf extract. The performance of this experiment can be deemed successful in separating pigments, however identification of the pigment concentration could also be done to better quantify the amount of pigment in the leaves.

INTRODUCTION

Chromatography is a technique used to separate complex mixtures between a stationary phase and a mobile phase. (Craig) There are several types of chromatography, namely: gas chromatography, liquid chromatography, ion exchange chromatography, affinity chromatography, adsorption chromatography, partition chromatography, and molecular exclusion chromatography. (Carrier, Bordonaro and Yip) The concept behind this process is that the smaller the affinity a molecule has for the stationary phase, the faster it migrates. The stationary phase varies depending on the type of chromatography used (Carrier, Bordonaro and Yip). Most of the time, chromatography is used for purification techniques. The process is also used in separation of substances, an example of which is plant pigments.

The result of chromatography is a chromatogram. From the chromatogram, different information about the test sample can be attained. By counting the number of peaks in the chromatogram, one can deduce the complexity of the mixture. The more peaks there are, the more complex the mixture is. Also, qualitative information about the composition of the mixture can be deduced by comparing the peak positions with a standard. Quantitative assessment of the relative concentration of the components can also be attained by comparing the peak areas (Carrier, Bordonaro and Yip).

In this experiment, chromatography was used to separate pigments extracted from old and young leaves. The pigments were further identified using absorbance spectrophotometry.

MATERIALS AND METHODS

For this experiment, photosynthetic pigments were separated through paper chromatography and the absorption spectra of these were measured through absorbance spectrophotometry.

Pigment Extraction

Old and young leaf samples were obtained around the UP campus. Each set of samples was shredded into smaller pieces and weighed to twenty grams. Using mortar and pestle, each sample was immersed in 50 ml acetone and ground thoroughly to fully extract the chloroplast pigments. After this, the extracts were filtered by using filter paper and Buchner funnel. The eluted extracts were collected in separate beakers and transferred into separate test tubes, and labelled accordingly as OLD and YOUNG.

Paper Chromatography

The extract from young leaves was subjected to paper chromatography. Three pieces of 3cm x 11cm Whatman no. 42 filter paper were cut out. The sheets of paper were marked 3 cm from one end, and 2 cm from the other. The 3cm-point served as the point where the extract would be loaded, while the 2cm-point dictates the point of termination of the chromatogram. This end was tied with a string so that it could easily be suspended on a Gatorade bottle cap.

With a Pasteur pipette, one drop of extract was loaded on each sheet of the filter paper. To further saturate the loading, the filter paper sheets were loaded ten times. However, for each loading, the previous spot was allowed to dry before an additional drop of extract was added.

Developing solvent of petroleum ether was prepared. The solvent was then poured into three Gatorade bottles, filling only up to two centimetres of the bottle. The paper strips were then secured on the bottle caps with tape and hung, with its end touching the developing solvent, but not the loaded extract.

The development of the chromatogram was terminated after the pigments or the developing solvent has reached the 2cm-line. Of the three chromatograms, only one was presented for the results because it has the clearest separation.

Absorbance Spectrophotometry

Absorbance spectrophotometry was also done to obtain absorbance spectra of the different pigments present in leaves. Both the old and young leaf

extracts were used and the absorbances of extracts were obtained for the following wavelengths (nm): 400, 450, 470, 500, 550, 600, 647, 650, 663, 700. Two trials were done per leaf sample. The absorbances obtained were then plotted against wavelength.

RESULTS AND DISCUSSION

Pigments are defined as substances in plants that are able to absorb visible light. These can be classified into three basic groups. (University of California – Berkeley, 2006)

The first are the chlorophylls which are greenish pigments containing a porphyrin ring. The ring contains several double bonds which makes it stable, and at the same time allows free migration of electrons. In this way, when sunlight strikes the plant surface, electrons in the pigment molecules of the chloroplast thylakoid become excited which in turn pass on this excitation to the photosystems that begins the series of cellular events that generate oxygen and sugar.

Four species of chlorophyll – a, b, c, and d – are known. Chlorophyll a is the primary photosynthetic pigment in all higher plants, algae, and the cyanobacteria. Chlorophyll b is found virtually in all higher plants and green algae, differing from the former only in that a formyl group substitutes for the methyl group in ring II. Chlorophyll c is meanwhile found in the diatoms, dinoflagellates, and brown algae and lacks the phytol tail of chlorophyll a. Lastly, chlorophyll d is found only in the red algae and has an (-O-CHO) group in place of the (-CH= CH2) group on ring I of chlorophyll a. (Taiz and Zeiger, 2008)

The second class of pigments are the carotenoids. They are usually red, orange, or yellow pigments composed of two small six-carbon rings connected by a chain of carbon atoms. Their high carbon content prevents them from dissolving in water and as such they must be attached to membranes within the cell. They have several functions, including the broadening of the spectrum of colors able to drive photosynthesis (especially in seasons with shortened days such as fall and winter), and in photoprotection; they are able to absorb and dissipate excessive light energy that can otherwise damage chlorophyll or interact with oxygen to produce reactive oxidative molecules that can damage the cell. (Cain, et. Al, 2011)

The third class of pigments are the phycobilins. They are water-soluble pigments found in the chloroplast stroma or the cell cytoplasm. Occurring only in the Cyanobacteria and Rhodophyta, they are efficient in absorbing light wavelengths that are not well absorbed by chlorophyll a. These pigments are bound to phycobiliproteins which pass on the absorbed light energy to chloroplasts for photosynthesis.

Knowledge of which types of pigments are present in a plant is useful in the field of agriculture. Using this information, lights that promoting the optimal growth of plants having certain pigments can be developed, increasing their yield. Additionally, pigments extracted from plants can be used as dyes in scientific research.

One way of determining the pigments present in a plant sample is through paper chromatography. Paper chromatography separates pigments present in the plant sample based on their solubilities in the solvent; compounds

which are very soluble move along with the advancing solvent front, while less soluble compounds travel slowly through the paper, well behind the solvent front. Chlorophyll a is slightly soluble in a 3: 1: 1 mixture of petroleum ether, acetone, and water (which was the resulting mixture used in the study), while carotenoids are very soluble in this system. This difference in solubility should allow the separation of chlorophyll a from the carotenoids and chlorophyll b, which is less soluble than chlorophyll a. (Bowen and Baxter, 1980)

The following chromatogram was obtained from the chromatography done in the study.

chlorophyll B

chlorophyll A

carotenoid

Figure 1. Obtained chromatogram from the paper chromatography of (young or old?) leaf extracts. Only one trial was used in the identification of pigments, with the topmost band determined to be a carotenoid, followed by chlorophyll A and chlorophyll B.

The first pigment band was identified as a carotenoid due to its yellow-orange color, the second band identified as chlorophyll A due to its darker green color, and the third band identified as chlorophyll B due to its yellow-green color. The study is said to be a success in this respect as the results obtained matched the theoretical results.

Additionally, pigments can also be identified based on the value of their retention factor; the retention factor (Rf) is calculated as the distance the pigment travels (in centimeters) divided by the distance the solvent travels (in centimeters). Standard values of the Rf are compared to the calculated values and the closest standard value that matches the calculated value is used to identify the pigment. This step however, was no longer done in the study.

Figure 2. A graph showing the absorbance obtained from the old and young leaf extracts through absorptive spectrophotometry.

Spectrophotometry can be utilized for measuring the chlorophyll content of a leaf by measuring the absorbances of the plant extract at red and far red regions of the visible light spectrum. The absorbance of the extract is directly proportional to its chlorophyll content. The experiment measured the absorbances of old and young leaves coming from the same plant in order to compare their chlorophyll content. In the experiment, the pigments from old and young plants were subjected to spectrophotometry to identify which wavelength would yield the highest absorption therefore identifying the pigments present, and also compare at which level of maturity plants would yield more chlorophyll. The former is possible because plant pigments participate in photosynthesis by absorbing light, and there is the optimal wavelength wherein they can absorb the most amount of light and can therefore enhance the process of photosynthesis.

Pigments in seed plants may be present as chlorophyll a, b, and carotenoids, all with varying abundance. For the old and leaf samples, it can be seen that

the measured absorbances peaked at two wavelengths (Figure 2). The first peak is around 450-470 nm while the second peak is around 663 nm. This data implies that most of the pigment extracted must be from chlorophyll a and b, since theoretically, these pigments peak at 430-450 nm and 640-660 nm. It should also be remembered that peaks in an absorbance vs. wavelength pigment spectra means that these pigments absorb and utilize light best in these wavelengths. In Figure 3 below, the other pigments and corresponding peak wavelengths can be seen.

Figure 3. Absorption spectra of pigments found in seed plants

Aside from knowing the pigments present in the leaves, the graph could also show the relative amount of chlorophyll present in the leaves. Theoretically, older leaves contain much more chlorophyll than younger leaves; this is contrary to the results as depicted by Fig. 2. These results can only make sense if the plant from which the extract was taken from has a magnesium deficiency, assuming that no methodological error was committed. Plant with Mg deficiency tend to sequester Mg from old leaves by degrading chlorophyll and then transporting the retrieved Mg to the younger leaves which have higher photosynthetic needs.

Other methods of measuring the pigment content of leaves include using chlorophyll content meters, which do not require an extract to be prepared, and the more superior technique known as chlorophyll fluorescence where the ratio of chlorophyll fluorescence at certain wavelengths give a linearly proportional estimation of the chlorophyll content.

Literature Cited

Hopkins, W. G. & Hüner, N. P. A. (2009). Introduction to plant physiology (4th ed). Ontario, Canada: John Wiley & Sons.

Cain, M. L, Jackson, R. B., Minorsky, P. V., Reece, J. B., Urry, L. A., & Wasserman, S. A. (2011). Campbell biology (9th ed). San Francisco, USA: Benjamin/Cummings.

University of California – Berkeley. (2006). Photosynthetic pigments. Retrieved from http://www. ucmp. berkeley. edu/g lossary/gloss3/pigments. html on August 23, 2012.

Bowen, W. R., & Baxter, W. D. (1980). Experimental cell biology: an integrated laboratory guide and text. New York, USA: Macmillan Publishing Co.

Taiz L and Zeiger E. (2008). Plant Physiology. 3rd ed. Sinauer Associates, Inc.

Hermans C.; Vuylsteke F.; Coppens F.. 2009. Systems Analysis of the responses to long-term magnesium deficiency and restoration in Arabidopsis thaliana. New Phytologist 187: 132-144.

Carrier, Rebecca, Julie Bordonaro and Kevin Yip. Chromatography. 1997. 23 August 2012 Retrieved from: http://www. rpi. edu/dept/ chem-eng/Biotech Environ/CHROMO/chromintro. html.

Craig, Paul A. Chromatography. 2007. 23 August 2012 Retrieved from: http://people. rit. edu/pac8612/ webionex/website/html/ione8h o9. html.