Using a Microscale Approach To Rapidly Separate and Characterize Three Photosynthetic Pigment Species from Fern

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ABSTRACT: A rapid separation of three photosynthetic pigments (chlorophyll a and b and xanthophyll) from fern (Polystichum acrostichoides) is described using microscale solvent extraction and traditional thin layer chromatography that minimizes use of harmful chemicals and lengthy procedures. The experiment introduces students to the concepts of extraction and separation of compounds. Combined with absorption and fluorescence spectroscopy using a plate reader, this laboratory experiment demonstrates the concept of light absorption by different pigments that are involved in photosynthesis as well as their photoluminescence properties. This new approach allows students to perform multilaboratory techniques within a 3 h laboratory session. The experiment has been used successfully in an introductory level course, but it may be easily adapted to second-year courses such as organic chemistry laboratory where these methods are widely used.

KEYWORDS: First-Year Undergraduate/General, Laboratory Instruction, Hands-On Learning/Manipulatives, Photosynthesis, UV−vis Spectroscopy, Fluorescence Spectroscopy, Thin Layer Chromatography

Separation and characterization of green plant pigments have been mainstays of both biology and chemistry laboratory experiments for many years. Early methods typically involved the use of solvent extraction procedures followed by paper chromatography to separate pigments such as chlorophylls a and b, beta carotene, and xanthophylls. Later methods introduced the use of less hazardous solvents for extraction and thin layer/column chromatography to improve separation. Every new method provided a significant improvement of extraction and separation procedures ranging from drying the leaves before homogenization, using different organic solvents for extraction, adding drying agents such as magnesium sulfate, and another step of extraction with nonpolar organic solvents such as hexane, to using metal chelators such as Dowex.2−6

Visible absorption spectroscopy has been used extensively to characterize photosynthetic pigments.7,8 Absorption spectra of these molecules illustrate their ability to capture light energy and perform their function in plants. Preparation of individual pigment molecules for spectroscopic analysis required large amounts of plants, solvents, and tedious separation methods, such as performing column chromatography, to get pure compounds.9−11 Single beam instruments used in these methods required large amounts of sample, were cumbersome, and required time-consuming data acquisition. A variety of new, low-cost UV−vis spectrometers have become available that make it possible to acquire and download data easily and provide increased sensitivity at the same time.12

Fluorescence properties of compounds are rarely introduced in the introductory level laboratory experiments even though the theory of luminescence is often discussed in the lecture. When a molecule is excited by irradiation with a certain wavelength, it absorbs energy and emits light at a different wavelength that is usually a longer wavelength due to dissipation of some of the energy absorbed.13 Therefore, each molecule that shows fluorescence has absorption and emission wavelengths. Fluorescence spectra can be taken immediately following the absorption spectra on the same samples, which therefore allows students to observe light absorption patterns as well as the fluorescence properties of both chlorophyll a and b. Although extraction, TLC, and absorption spectroscopy have been described many times in the literature, a laboratory experiment where all of these techniques are used and that involves analyzing individual pigments has not been reported. This experiment introduces many laboratory concepts to students with respect to the physical and photochemical properties of molecules about which they learn in the lecture. Extraction and separation are performed based on the physical properties of the molecules. By using spectroscopic methods, students can measure the wavelengths molecules absorb and relate them to the process of photosynthesis in plants.
EXPERIMENTAL PROCEDURES

Students work in groups of two or four, and each group develops two TLC plates. Fern (*Polystichum acrostichoides*) is obtained from a local store and maintained at 25 °C under fluorescent lamps. Detailed procedures for the experiment are in the Supporting Information.

Sample Extraction

Fresh fern (1 g) is cut into small pieces (approximately 1 cm × 1 cm). The pieces are ground in a mortar (1 min), acetone (6 mL) is added, and the mixture is homogenized 1–3 min or until a green solution is obtained. The solution is filtered through glass wool.

Thin-Layer Chromatography

The acetone extract is spotted onto a TLC plate (3.5 cm × 9.0 cm) using a capillary tube, and the plate is developed in a closed chamber with acetone/petroleum ether mixture (40:60).

Visible Spectrophotometric Analysis

After recording the TLC analysis, each pigment band (chlorophyll *a*, chlorophyll *b*, and xanthophyll) is removed separately from the TLC plate by scraping with a spatula into a small vial; acetone (20 drops) is added, and the mixture is stirred (1 min) and then filtered through glass wool. All extracts should be used immediately to lessen degradation of the chlorophylls. The pigment sample (200 μL) is added to a multitiier base plate with 0.5 mL (9.0 mm × 17 mm) flat bottom glass inserts; pure acetone is used as a blank. The 96-well plate should be covered to prevent evaporation of the solvent. Visible spectra are recorded in the range of 400–720 nm at 5 nm intervals on a spectrophotometer with a built-in plate reader. If a plate reader is not available, the TLC samples from at least 10 or all groups in a lab section can be combined, extracted using a minimum amount of acetone, placed into a standard size 1 cm cuvette, and analyzed in a commonly available UV–vis spectrophotometer.

Fluorescence Spectra

Fluorescence spectra are recorded using the same samples to obtain visible spectra. Two excitation wavelengths (440 and 460 nm) are used to observe fluorescence properties of chlorophyll *a* and *b*, respectively, in the spectral range of 600–800 nm at 10 nm intervals and a cutoff wavelength at 495 nm.

HAZARDS

Acetone and petroleum ether are flammable and should be isolated from open flames and ignition sources. Proper handling and disposal of the solvents used should be enforced. These chemicals, when in contact, may cause irritation to skin, eyes, and respiratory system. Safety goggles should be worn at all times, and standard lab safety procedures should be followed.

RESULTS

A representative student’s data for separation of the pigments from fern by TLC are shown in Figure 1 using acetone/petroleum ether (40:60) as the mobile phase. The primary spots observed corresponded to β-carotene, chlorophyll *a*, chlorophyll *b*, and xanthophyll. The separation order was the same order as previously reported results.1,11,14 Average *R* values with standard deviation (Std. Dev.) of the four pigments from a section of 30 students are in Table 1. Relative standard deviation (RSD (%)) was calculated to determine the precision of this method. Small RSD values suggest that student data were consistent and that the experimental data were reproducible across the students.

A fast extraction and separation method provided fresh pigment samples suitable for spectroscopic analyses. UV–vis spectra were obtained using a spectrophotometer with an integrated plate reader. The overlaid absorption spectra of three plant pigments (chlorophyll *a*, chlorophyll *b*, and xanthophyll) from freshly harvested fern are shown in Figure 2. While the absorption maxima for chlorophyll *a* were at 440 and 665 nm, chlorophyll *b* showed shifted absorptions at 460 and 650 nm. Xanthophyll gave two absorption maxima at 450 and 470 nm. The spectra of these pigments showed the same absorption pattern compared to the spectra of pure compounds.15,16 Beta-

<table>
<thead>
<tr>
<th>Pigments</th>
<th>Average <em>R</em></th>
<th>Std. Dev.</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-carotene</td>
<td>0.96</td>
<td>0.010</td>
<td>1.0</td>
</tr>
<tr>
<td>chlorophyll <em>a</em></td>
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<td>0.056</td>
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</tr>
<tr>
<td>chlorophyll <em>b</em></td>
<td>0.71</td>
<td>0.061</td>
<td>8.6</td>
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<tr>
<td>xanthophyll</td>
<td>0.63</td>
<td>0.068</td>
<td>11</td>
</tr>
</tbody>
</table>

Figure 1. TLC separation of fern extract. Silica gel coated TLC plate was use as a stationary phase and acetone/petroleum ether (40:60) mixture as mobile phase.

Figure 2. Visible light absorption spectra of chlorophyll *a* (solid line), chlorophyll *b* (dash line), and xanthophyll (dotted line) from fern. The bands on TLC plates were individually removed and extracted with acetone.
Carotene was excluded from spectroscopic analysis due to a very weak absorption signal. Although solvents used for extraction and separation procedure in this study were adequate for TLC, pure acetone cannot extract the more hydrophobic beta-carotene in sufficient amounts for spectroscopic analysis.17

Fluorescence spectra were obtained using two excitation wavelengths, 440 and 460 nm (Figure 3a,b). Maximum emission wavelengths of chlorophyll a and chlorophyll b were 670 and 650 nm, respectively. These spectra exhibit the emission wavelengths corresponding to the literature values.18 During excitation at 440 nm, chlorophyll a would be expected to fluoresce more compared to chlorophyll b since this wavelength is the maximum absorption wavelength ($\lambda_{\text{max}}$) of chlorophyll a found on the visible light absorption. Overlaid spectra in Figure 3, panel a show that the fluorescence intensity of chlorophyll a exceeded that of chlorophyll b. With the excitation wavelength set at 460 nm, chlorophyll b demonstrated a higher emission due to its absorption at this wavelength. If time permitted, students could see the effect of the excitation wavelength to the emission spectra by varying the excitation wavelengths and observing the intensity of corresponding emission spectra. The fluorescence spectrum of xanthophyll was not acquired because the carotenoid structure that xanthophyll possesses generally gave a low quantum yield for emission and very weak fluorescence.19

**DISCUSSION**

Several methods have been reported for extraction of photosynthetic pigments from plant leaves. Most used spinach leaves, and the methods required either harsh chemicals such as hexane for extraction and column chromatography for separation of these pigments.11 Extraction of pigments from fern has recently been reported with the use of ultrasonic instrument with more than 2 h of sample preparation and extraction time.20 The microscale extraction method used in this study is ultrafast, and no other processing is required prior to TLC analysis. This method has been used in a first-year undergraduate chemistry course with an average of 30 students in each section, two sections per semester, for three semesters. The results have been similar and reproducible, although data shown here are representative data from only one section. The students also tried different types of plants including spinach (Spinacia oleracea), water pennywort (Hydrocotyle sp.), and other leaves picked from the garden. Most of them showed similar results in the order of separation by TLC, although some gave better separation than the others. Ferns gave the best extraction and separation results of all the leaves tested by students. The absorbance data obtained for the pigments matched the spectra of xanthophyll, chlorophyll a, and chlorophyll b found in commonly used texts and laboratory manuals.21,22 Comparison between the spectra from fern and other leaves revealed that the absorbance patterns of the same pigments looked the same.

Visible and fluorescence spectra of these pigments are available in the literature. However, these papers focused on the extraction and the spectrum of the whole extract rather than individual pigments.10,20,23 While this kind of spectrum may show the overall absorption of all pigments that cover most of the visible light region, it is more useful for students to see the absorption spectrum of each pigment and determine what the combined absorption spectrum would be for a mixture of these molecules. In this experiment, students recorded the absorption spectra of individual pigments and compared them to the available literature spectra for pure compounds. More importantly, acquiring the fluorescence spectra of individual pigments and exciting them at different wavelengths gave students a better understanding of how the excitation wavelength affected the emission spectrum of different compounds.

Although this experiment has been used in an introductory level course, it can also be easily implemented in second-year courses where UV−vis and fluorescence spectroscopy can be explained in depth. With the use of a plate reader, the number of samples required for analysis was reduced significantly, which, in turn, reduced student exposure to chemicals as well as the cost of solvents and waste management in the long run. As another advantage, student data for a section were collected simultaneously within 10 min. The ability to perform all steps rapidly is important because these pigments degrade with time.24,25 Main concepts taught through this experiment were light absorption pattern in relation to the color and photoluminescence properties of different molecules. Students also learned concepts of solvent extraction and chromatography as well as computer competency in making the graphs using Microsoft Excel. During the experiment, the order of pigment separation and differences in $R_f$ values may be discussed in relation to the chemical structure. Students may write lab reports containing (a) interpretation of their results based on the methods they learned and (b) relation of the results to the properties and function of the molecules. Successful completion of the experiment by students was assessed during the laboratory session and through their postlab reports. A set of questions relating to the concepts from this experiment was included in the lab exam to quantitatively access the outcome.

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The scores for this set of questions were compared with those for the rest of the questions on the test. Quantitative data, 74% for this experiment and 76% for the other questions, suggests that there was no significant difference between this experiment and the other topics taught in the course.

**CONCLUSIONS**

A quick and easy approach combined with modern laboratory equipment has made it possible to complete the extraction, separation, and characterization of photosynthetic molecules from fern in a typical 3 h laboratory session.

**ASSOCIATED CONTENT**

2 Supporting Information

Student handout and instructor notes. This material is available via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**


