An Improved Method for the Extraction and Thin-Layer Chromatography of Chlorophyll a and b from Spinach

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The chromatographic separation of plant pigments has both historical *(1)* and practical *(2)* importance and as a result has remained a popular and well-studied experiment in the chemical laboratory curriculum. Moreover, this experiment remains popular because students can easily relate to the materials used and the pigments involved, including chlorophyll a and b as well as β-carotene, that are easily observed without the need for UV lamps or post-chromatographic chemical or physical treatment of the TLC plates. Several improvements have been reported in the literature that address difficulties with both the sample preparation *(3–5)* and the chromatographic conditions *(6–8)* used. For example, the extraction of relatively nonpolar pigments into a suitable organic solvent is complicated by the presence of water in the raw spinach leaf (or other pigment source). Also many of the chromatographic solvent systems reported for this separation do not adequately resolve chlorophyll a and chlorophyll b from each other or from other pigments present in the sample. A further complication exists in that the pigments to be isolated are susceptible to degradation *(7–11)* resulting in the observation of materials that are not representative of the pigments in the plant. We present significant improvements in both the sample preparation and the mobile phase for the extraction and separation of plant pigments. This simple and fast method clearly resolves chlorophyll a and b from spinach leaves on analytical TLC plates while minimizing the appearance of chlorophyll degradation products.

Experimental Procedure

General

Solvents and reference samples were purchased from Aldrich Chemical Company and were used without additional purification. The TLC plates were also purchased from Aldrich and these experiments were optimized using silica gel plates on a polyester support containing a fluorescent indicator. Comparable results were obtained on TLC plates without the fluorescent indicator. The aluminum backed Merck TLC plates can be substituted but give R_f values approximately 15% lower than the corresponding polyester backed plates. The sand used was obtained from Ward's (Ottawa Sand) and is much larger in particle size than sand commonly used for chromatographic applications. A full description of the catalog numbers and CAS registry numbers of all materials used as well as the laboratory handout used by our students is available in the Supplemental Material.[W]

Sample Preparation

Fresh or frozen spinach (0.5 grams) was combined with 0.5 grams of anhydrous magnesium sulfate and 1.0 gram of sand. The mixture was ground in a mortar and pestle until a light green powder was obtained (5–10 minutes). The light green solid was transferred to a small test tube containing 2.0 mL of acetone. This heterogeneous mixture was agitated to ensure complete mixing of the acetone and the solid. This mixture was allowed to stand for 10 minutes and the green acetone solution was removed by pipette and transferred to a microcentrifuge tube. The acetone extract was kept sealed when not being actively used. Alternately a coffee grinder was used to prepare larger quantities of solid material for subsequent acetone extraction. In this case 5.0 grams of fresh or frozen spinach was combined with 5.0 grams of anhydrous magnesium sulfate and 10 grams of sand in a commercial coffee mill and ground for 5 seconds. The leaf pulp from the top and sides of the mill container was scraped back to the bottom of the mill and ground for an additional 30 seconds. The resulting powder was extracted as described above (2.0 grams of solid with 2.0 mL of acetone).

TLC Separation

TLC plates (3.5 cm × 9.0 cm) were cut from the commercially available sheets. The acetone extract was transferred in the standard manner and the plates were eluted in a closed chamber with the following mobile phase: 60% petroleum ether (bp 35–60 °C)/16% cyclohexane/10% ethyl acetate/10% acetone/4% methanol. The elution order using this elution solvent system was β-carotene (R_f = 0.95), chlorophyll a (R_f = 0.44), chlorophyll b (R_f = 0.32), and xanthophyll (R_f = 0.16). Commercial purified samples of β-carotene, chlorophyll a, and chlorophyll b were used as controls.

Demetalation of the Spinach Extract

Dowex 50WX8 H^+ resin (100 mg) was combined with 100 μL of the acetone spinach extract described above. This mixture was allowed to stand for 3 minutes and a small sample was removed using a TLC applicator. This sample was chromatographed as previously described. This short treatment with ion exchange resin resulted in the complete loss of the chlorophyll a band and the appearance of both pheophytin a (R_f = 0 .60) and pheophytin b (R_f = 0.49).

Hazards

There are no unusual hazards associated with this experiment. The proper handling and disposal of the solvents used should be observed. The organic solvents used in this experiment are flammable and should be isolated from ignition sources such as the coffee grinder described in the experiment.

Results and Discussion

The extraction and separation of plant pigments is a common introduction to chromatography and is found in many laboratory manuals *(12–14)*. The direct extraction of

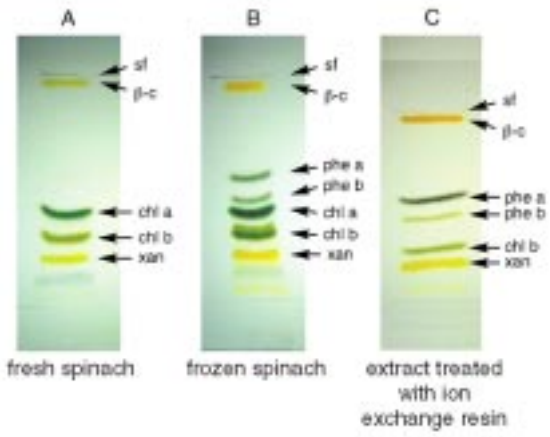

Figure 1. TLC plates of spinach extracts: (A) fresh spinach, (B) frozen spinach, (C) extract treated with ion exchange resin; xan is xanthophyll, chl is chlorophyll, c is carotene, phe is pheophytin, and sf is solvent front.

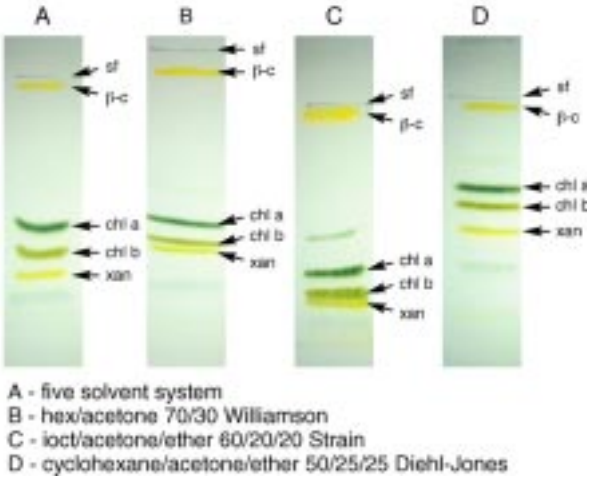

Figure 2. Comparison of solvent systems used on the TLC plates: (A) five-solvent system, (B) hexane / acetone (70 / 30) *(14),* (C) *i*-octane / acetone / ether (60 / 20 / 20) *(11),* (D) cyclohexane / acetone / ether (50 / 25 / 25) *(6).*

the plant pigments from spinach is complicated by the presence of water. Since the chromatographic separation will be affected by even small quantities of water, a bulk separation of the extract using a separatory funnel (or comparable microscale techniques) followed by drying of the organic layer with an appropriate drying agent is often required *(2, 3, 6, 11–14).* A further complication arises in this separation owing to the well-documented limited stability of the chlorophyll pigments *(7–10).* This can be confusing to the student since the sample can produce both the expected pigments and colored degradation products with similar R_f values. One of the most common degradation products is the loss of the magnesium ion from chlorophyll resulting in the formation of the colored pheophytin at an R_f higher than the original chlorophyll. Pheophytin a and b are observed at R_f values consistent with literature reports *(7)* in addition to the corresponding chlorophyll a and b when dried or frozen spinach was used to prepare extract (Figure 1). These degradation products are also found in commercially available purified chlorophyll a and b samples. The demetalation of chlorophyll using strong acids has been previously described *(7–10)* and we have found that it can be easily effected by a brief treatment of the pigment extract with a strong ion exchange resin. Treatment of the spinach extract with the ion exchange resin resulted in complete conversion of chlorophyll a to pheophytin a and partial demetalation of chlorophyll b as shown in Figure 1C. This procedure can be used to confirm the presence of the demetalated pigment in partially degradated samples.

Extraction methods that rely on drying the spinach leaves or storing the organic extract solution for prolonged periods results in some pigment degradation that complicates the TLC analysis. In addition these procedures require a substantial quantity of time be allocated to the pigment extraction prior to chromatographic analysis. In contrast, the method described here is fast, reliable, and does not require a separate extraction or drying step. The student grinds the fresh spinach leaf with sand and drying agent followed by extraction directly into acetone. The sand helps to lyse the cells and acts as an inert material, which improves the extraction process. The anhydrous magnesium sulfate, in the quantities used, is sufficient to remove all of the water present (0.5 grams of $MgSO_4$ can remove an equal mass of water yielding the heptahydrate). This results in a fast extraction into a dry solvent, which is desirable for the chromatographic analysis. Anhydrous sodium sulfate can also be used with comparable results. TLC analysis using this procedure shows the primary observed spots corresponding to β-carotene, chlorophyll a, chlorophyll b, and xanthophyll as shown in Figure 1A and B. It should be noted that some pigments, especially beta carotene, fade over time and that the students should record their observations soon after the separation is complete. The observation of the common degradation products of the chlorophylls (removal of the Mg ion to form the pheophytins or subsequent hydrolysis products) is minimized using this method. Furthermore this method only requires 30 minutes of preparation time by the student to prepare an acetone extract of the pigments suitable for analysis. We have found that using a commercial coffee grinder is an excellent way to prepare larger quantities of the sand/spinach/drying agent solid for acetone extraction. A tenfold greater quantity (enough for 15–20 students working in pairs) can be prepared in just a few minutes. In addition this solid could be prepared before the laboratory session, which reduces the total time for the experiment to about 45 minutes, which corresponds to the students performing just the acetone extraction and TLC analysis. If the instructor desires the students to perform just the TLC analysis resulting in a 30-minute experiment, then the instructor just prior to the laboratory session can perform the extraction. Of these options we have favored the manual grinding of the materials in a mortar and pestle to give the students an appreciation of how the pigments are isolated directly from the plant. With this procedure we have had students perform the extraction and

chromatographic analysis, using purified commercial samples of the pigments as standards, in less than two hours.

The solvent systems reported in the literature often give acceptable resolution between chlorophyll a and b but poor resolution between chlorophyll b and xanthophyll (Figure 2). Many of the solvent systems reported in the literature show the chlorophyll b and the xanthophyll (a yellow band of lower R_f) as overlapping bands. We evaluated several elution solvents and found that the separation of the yellow xanthophyll from chlorophyll b was improved by the addition of small quantities of alcohols for which methanol was found to be superior to ethanol or isopropanol. This resulted in our adoption of the five-solvent mobile phase described in the experimental section. The results are shown in Figure 2A, where this mobile phase shows the complete and reproducible resolution of chlorophyll a, chlorophyll b, and xanthophyll without the use of ether. We have used this solvent system for two years in the undergraduate laboratory and students have successfully observed the separation of these three pigments.

The extraction method that we describe appears to be general and could be used to perform other natural product extractions where water is normally removed by a separate treatment. The use of spinach is meant to be representative and this method could also be applied to other extractions such as the separation of carotene from tomato paste, which is another common undergraduate experiment. We have performed this extraction with other green plants and have obtained excellent results using kale (*Brassica oleracea acephala*) although spinach is a cheap and convenient source of these pigments.

Conclusions

The use of an inert material and drying agent in the extraction of pigments from spinach has provided a simple, fast, and reproducible method of obtaining samples suitable for TLC analysis. This method avoids the common complication arising from degradation products in the pigment extract and can be used on a variety of plants. The demetalation of chlorophyll can be observed by treating the pigment extract with a strong ion exchange resin. An improved mobile phase for the TLC analysis of spinach extract that allows for the complete resolution of the common plant pigments found in green plant leaves is also presented.

W Supplemental Material

A copy of our laboratory handout and a list of chemicals and materials used in this experiment are available in this issue of *JCE Online*. We have also attached copies of figures that show how this method works for a number of green vegetables and extracts from dried and fresh spinach stored for extended periods of time to show the pigment degradation products.

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