Introduction

The separation of chlorophyll a and chlorophyll b from the other similar plant-derived organic materials (such as the xanthophylls) and from each other is a good example of the utility and necessity of chromatography. Please read or review chapters 10 and 16 of *Macroscale and Microscale Organic Experiments* by K. Williamson [1] to be sure you understand the theory and practice of both column chromatography and high pressure liquid chromatography (HPLC).

The structures of chlorophylls a and b are shown below. The separation of chlorophylls a and b are a good example of the power of chromatography. Because the two chemicals are so similar (the only difference is that one has a methyl substituent where the other has a methanaldehyde substituent), they could not possibly be separated by crystallization. However, this slight difference in structure is enough to allow separation by chromatography.

Chlorophylls a and b are readily available from plants and are extremely important biologically due to their use by green plants and most algae to absorb light and produce food--photosynthesis. This process is the basis of the food chain as we know it.

While tons of chlorophylls and related molecules are synthesized by plants globally each day, it took R. B. Woodward and a small regiment of organic chemists many years to synthesize a few milligrams of chlorophyll a in the lab[6]. Thus, the source of chlorophyll in this experiment is a natural one, spinach leaves.

The extraction of chlorophyll a, chlorophyll b, and β-carotene from spinach is carried out using a SEP-PAK™ cartridge procedure [5]. A thin layer chromatographic procedure for separating the chlorophylls, xanthophylls, carotenoids, etc. using thin layer chromatography is
presented by Williamson [1], pp 146-148 and should be tried if the HPLC method fails. The HPLC procedure is an adaptation of one in the literature [5].

A good explanation of why conjugated double bonds (such as those found abundantly in the compound mentioned here) are UV active and how UV/Vis spectroscopy works can be found on the next three pages and also in the text by John McMurry [4].

**Experimental**

**Spectral Measurements**

UV/Vis spectra should be recorded using a Hewlett Packard 5842 Diode Array Spectrophotometer in 10% 2-propanol in isoctane. Directions on the use of the instrument will be found near it.

**Cautions**

Chlorophylls a and b are delicate organics that tend to decompose by air oxidation and light- or heat-induced reactions with time; within 24 hours, the solutions will fade, so spectra must be run soon after being eluted from the column.

**Extraction of Chlorophylls, etc.**

Add 10 mL of 2-propanol to 8-10 g spinach and stir/crush with a large mortar and pestle for one minute. The 2-propanol serves to to eliminate the water from the disrupted cells. Decant the liquid extract into a small beaker pressing the plant material to squeeze out as much solvent as possible. Add 5 to 10 mL of isoctane, stir vigorously with a magnetic stirrer and bar for a minute and decant the green isoctane extract into a 100-mL RB flask with a 19/22 ground glass joint. Remove all of the isoctane on a rotary evaporator. Prewet the packing material (silica) in the SEP-PAK™ cartridge by pushing 2-5 mLsoctane through it with a Luer-type syringe (use the gas-tight syringe from the Schlenk line kit in the stockroom). Dissolve the chlorophyll extract in 3 mL of isoctane and push this through the SEP-PAK cartridge using the same syringe. Flush 3 mL of isoctane through the cartridge, discarding the eluant. Now use the syringe to flush the cartridge with 5 mL of 2-propanol, collecting only the yellow and green bands in separate 20-mL vials. Cap the vials and cover with foil to minimize photochemical decomposition reactions. Concentrate the solutions to about 1/10 their original volume by blowing them down with a gentle stream of nitrogen.

**High Performance Liquid Chromatography (HPLC) Analysis**

Inject 5-10 μL of the extract into the GOW-MAC HPLC column while eluting with 10% 2-propanol in isoctane. The β-carotene should elute first followed by chlorophyll a and then chlorophyll b. Other compounds will also elute (see ref [5] for an example chromatogram). Collect the eluant for each component as it elutes in separate vials and immediately take UV/Vis spectra.

**Characterization**

Chlorophylls a and b can be characterized by their UV/Vis spectra:

- Literature $\lambda_{\text{max}}$ and extinction coefficients ($\epsilon$):
  - Chlorophyll a: 430 nm ($\epsilon = 215,000$), 660 nm ($\epsilon = 150,000$)
  - Chlorophyll b: 460 nm ($\epsilon = 380,000$), 645 nm ($\epsilon = 115,000$)

- Chlorophyll a: $\lambda_{\text{max}}$ at 409, 429, 498, 531, 577, 613, 660 nm
- Chlorophyll b: $\lambda_{\text{max}}$ at 427, 453, 545, 565, 593, 642 nm

**References**


Plant Pigments, Column Chromatography, and Electronic Absorption Spectroscopy
Reprinted from "Experimental Organic Chemistry" by Marjorie C. Caserio

Introduction
The orange and red pigments in fruits and vegetables such as tomatoes and carrots are hydrocarbons known as carotenoids. The two commonest carotenoids, which incidentally are precursors to Vitamin A, are lycopene and β-carotene, the structures of which are shown below.

Vitamin A C_{20}H_{30}O

lycopene C_{40}H_{56} - m.p. 173°C (all trans)

β-carotene C_{40}H_{56} - m.p. 183°C (all trans)

The carotenoid pigments also occur in the leaves of plants but they are not obvious because of the presence of other pigments.

The green pigments in leaves are principally chlorophyll-a and chlorophyll-b, which have the structures shown in experiment 404.

Chlorophyll-b differs from chlorophyll-a in that it has a formy1 group in place of a methyl group (CH2), at the position shown in the above structure.

Thin Layer Chromatography
One of the major problems the chemist faces is the analysis of complex mixtures of organic compounds, and many of the techniques of organic chemistry involve methods for determining the number of different compounds in a mixture and the percentage composition of the mixture. A common approach is to physically separate the mixture into its components by adsorption chromatography, and the first experiment in this manual utilizes a technique known as thin layer chromatography to separate organic mixtures on an analytical scale.

Thin layer chromatography simply involves applying a very small amount of a solution of the sample to be analyzed to a spot on the surface of some solid adsorbent, usually silica gel (hydrated SiO2) or alumina (Al2O3), spread as a thin layer on a glass plate. When the solvent has evaporated, leaving the sample deposited on the adsorbent, the coated plate is placed in a jar containing a small amount of some solvent or solvent mixture such that the lower end of the plate dips 1-2 cm below the surface of the solvent. With the jar covered, the solvent is drawn vertically up the coated surface by capillary action and travels past the spot at which the sample was applied. The components in the sample move with the solvent up the coated surface but at different rates depending on a number of factors such as the nature of the compounds in the sample, the nature of the solvent, and the activity of the solid adsorbent. Thus, a separation of the components is achieved by virtue of the fact that they move (i.e. they are eluted) at different rates.

The origin of this effect lies in the interaction of the sample components and the solvent with the surface of
the adsorbent. An active solid adsorbent has a large surface area exposing a large number of polar sites that can reversibly bind or adsorb small concentrations of added substances by electrostatic forces of attraction. As the solvent travels up the surface of the adsorbent, it competes with the sample for the adsorbent and with the adsorbent for the sample and so displaces the sample reversibly and continuously in the direction of travel of the solvent “front.” This process can be thought of as a three-way competition between the sample, the solvent, and the adsorbent, as expressed in the following equilibria:

\[ \text{sample-adsorbent} \leftrightarrow \text{solvent-adsorbent} \leftrightarrow \text{sample-solvent} \]

The rate of elution of the sample components will depend in part on the nature of the components. Polar or polarizable compounds, such as alcohols ROH, carboxylic acids RCO2H, amides RCONH2, and amines RNH2, are adsorbed more strongly and eluted less readily than less polar or polarizable compounds, such as halogen compounds RCl, aldehydes and ketones RCHO and R2CO, ethers ROCH2, and hydrocarbons RH.

The activity of the solid adsorbent will also determine the rate at which the components are eluted. In the case of silica gel, which is the adsorbent most commonly used, its activity (or affinity for the sample components) will be greatly affected by its water content. The more water in the coating, the lower will be its activity. In preparing a coating, a slurry of the gel in water is spread evenly over a glass plate, and the coating is then activated by drying the coated plate in an oven for 30-60 minutes at about 100-120°C.

The solvent used to elute the sample will also affect the rate of elution. The more polar the solvent, the more rapidly the components will move. It follows therefore that the choice of solvent will be dictated by the natures of the components to be separated, polar solvents being used for strongly adsorbed components and nonpolar solvents for weakly adsorbed components. Some commonly used solvents are listed below in order of increasing eluting power which parallels the order of increasing polarity.

Separation by Column Chromatography
To separate the carotenoid pigments from the chlorophyll pigments in weighable amounts, column chromatography is the method of choice. The principles of column chromatography are essentially the same as in thin layer chromatography. The solid adsorbent, commonly alumina (Al2O3), is packed as evenly as possible into a glass column filled with a suitable organic solvent. A concentrated solution of the sample to be separated is then added at the top of the column such that the adsorbent or packing remains undisturbed. The column is then "developed" by allowing solvent to pass slowly through the column, the packing being covered with solvent at all times. The components in the sample are continuously and reversibly adsorbed on the alumina then desorbed by the solvent while passing slowly down the length of the column. For a chromatographic separation to be successful, the components in the sample must be adsorbed unequally such that they move down the column at different rates and emerge separately from the column. The rate at which a given compound elutes from the column depends on the polarity of the compound, the activity of the alumina, and the nature of the eluting solvent. Nonpolar compounds such as the carotenoid hydrocarbons are only weakly adsorbed by alumina and are readily eluted with hydrocarbon solvents. But compounds such as chlorophyll-a and chlorophyll-b which have several polar sites per molecule are strongly adsorbed and can only be eluted with relatively polar solvents (e.g. acetone, chloroform).

Electronic Absorption Spectroscopy
Irradiation of a compound with electromagnetic radiation of suitable wavelengths can cause transitions of the bonding electrons from their normal (i.e. ground) states to higher excited electronic states. For example, irradiation of lycopene with visible light in the region 400-500 mM causes excitation of the p
electrons to p* antibonding levels, and this is evident as an absorption of the exciting radiation (Figure below). There are actually several absorption peaks in the electronic spectrum of lycopene, but the peak of maximum intensity corresponds to a wavelength near 473 mM. B-Carotene behaves similarly on irradiation, but the peak of maximum absorption is found at shorter wavelengths, around 448 mM, the exact position of the maximum depending somewhat on the solvent.

![Electronic absorption spectrum of lycopene and β-carotene](image)

The optical system of a simple absorption spectrophotometer is shown diagrammatically below.

![Schematic diagram of a simple UV/Vis spectrophotometer](image)

The light source is either a hydrogen lamp, which emits radiation in the region 180-400 mM, or a tungsten lamp, which emits in the region 325-800 mM. The emitted radiation is focused and directed through a prism of the monochromator system, and is thereby refracted according to wavelength. Any desired wavelength may be selected by rotating the angular position of the prism in the light beam. The resulting monochromatic light (i.e. light of a specific wavelength) is then focused and directed by a system of mirrors and lenses through two silica cells, one containing the sample of interest dissolved in some non-absorbing solvent, and the other containing just the solvent. Any change in absorption between the sample and solvent cells is detected and amplified by a photomultiplier tube, the signal being detected and recorded as absorbance or transmission of radiation of known wavelength.

The carotenoid pigments separated from spinach, tomato paste or carrots may be identified from their absorption spectra. Record the spectra of your extracts using ligroin as the solvent in both the sample and the blank runs. Since the intensity of electronic transitions in the carotenoid hydrocarbons is very large, only very small quantities are required for an absorption spectrum. If, therefore, your solutions absorb so strongly that they deflect the recorder trace off scale, dilute your samples until the absorption maxima remain on scale.
HPLC Analysis of Chlorophyll a, Chlorophyll b, and β-Carotene in Collard Greens

A Project for a Problem-Oriented Laboratory Course

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The theory and application of high performance liquid chromatography should be an integral part of college chemistry curricula since many students will be utilizing this technique in their profession. A popular experiment (1, 2) in undergraduate organic laboratories is the separation of a carotenoid, such as β-carotene, from the chlorophylls in vegetables (e.g., collard greens, spinach, etc.) using column or thin layer chromatography (TLC). We have expanded this traditional experiment by utilizing high performance liquid chromatography (HPLC) to separate and quantitate β-carotene, chlorophyll a, and chlorophyll b originating from collard greens. This experiment clearly illustrates to the student the versatility of HPLC as a powerful analytical technique. A project undertaken by SUNY Oswego students who have previously (3–5) been involved in project-oriented laboratories, is described.

Experimental

Instrumentation

The instrument used was a Varian 5020 liquid chromatograph equipped with a Model UV-10 variable wavelength detector and a Model 917-02 dual pen, strip-chart recorder. The 4-mm × 30-cm prepacked analytical column for this separation was a MicroPak Si-5Silica. A 4-mm × 30-cm bonded reverse-phase column (monomeric octodecylsilane) MicroPak MCH-10 also was used. For a comprehensive discussion of reverse-phase chromatography, see reference (6). Each of these analytical columns was preceded by its appropriate 4.6-mm × 3-cm pre-column. The mobile phase was a nonlinear, step-and-hold gradient of isocyanataneisopropanol (99:1 V/V) to a final composition of 85:15 (V/V) (isocyanataneisopropanol). All solvents used were Omnisolv™ glass-distilled.1

The solvent flow rate was 1 ml/min. The detector was fixed at 0.8 nm and 0.005 AUFS. The sample size was 10 μl (10–2) and was introduced by a Valco Model AH-CV-0-UHPa-N60 air-activated sample injector. The chart speed was 1 cm/min.

1 Information about Omnisolv™ glass-distilled solvents may be obtained from MCB Manufacturing Chemists, Inc., 2909 Highland Ave., Cincinnati, OH 45212.

Presented in part at the American Chemical Society, 11th Northeast Regional Meeting, October 18–21, 1981, Rochester, NY.

Literature Cited

Sample Preparation

Separatory Funnel Extraction Method. Crush approximately 15 g of fresh or frozen collard greens in 50 ml methanol with a mortar and pestle. This is to remove as much water from the plant cells as possible. Decant and discard the methanol solution and press the vegetable as free of solvent as possible. Re-grind the collard greens with 15 ml methanol and 25 ml isooctane. Filter the mixture through a glass funnel plugged with absorbent cotton into a 125-ml separatory funnel. Again, re-grind the vegetable with 15 ml methanol and 25 ml isooctane. Filter as previously and place in the separatory funnel.

Discard the lower methanol phase and add 50 ml water to the separatory funnel and shake gently. If an emulsion appears, add sodium chloride. Repeat as previously with another 30 ml water.

Place the isooctane solution into a 125-ml Erlenmeyer flask, and dry by adding 1-2 g anhydrous sodium sulfate. Decant the liquid into a sample bottle with a screw top. Fill to the top with additional isooctane as needed. This minimizes contact of the sample with oxygen. Strong light and oxygen hasten the decomposition of components.

To exclude light, the sample bottle is wrapped tightly with aluminum foil. Save the sample for HPLC analysis.

SEP-PAK™ Extraction Procedure (7). Add 10 ml 2-propanol to 5-10 g of collard greens and stir with a mortar and pestle for 1 min. The 2-propanol serves to eliminate the water from the disrupted cells (i.e., same as methanol). Press the vegetable as free of solvent as possible and decant the mixture.

Extract the pigments by adding 5-10 ml isooctane and stirring. Decant the green colored mixture. Evaporate the liquid almost to dryness using a steam bath. Add 2-5 ml isooctane through the silica SEP-PAK™ with a Luer-type syringe. Immediately dissolve the extracted residue in 2 ml isooctane and pump it onto the precoated silica SEP-PAK cartridge with the Luer-type syringe. Flush 3 ml isooctane through the cartridge and discard the eluate. With 5 ml 2-propanol elute the yellow and green bands, collecting only the colored bands. Place this in a screw-top sample bottle, fill to the top with 2-propanol and cover with giallum foil.

Results and Discussion

A typical chromatogram obtained by students showing the separation of β-carotene from chlorophyll a and chlorophyll b is shown in the figure. The entire HPLC analysis can be accomplished in about 25 min. Interestingly, both the silica and the octadeylsilane column gave comparable class results, with the more polar compounds being eluted last. Therefore, in the octadeylsilane run, one is actually observing a form of "reverse-reverse phase" (i.e., normal) chromatography. The HPLC class results from either the longer separatory funnel extraction method or the shorter SEP-PAK™ preparative procedure also gave comparable HPLC results.

Average HPLC Student Results of β-carotene, Chlorophyll a, and Chlorophyll b from Collard Greens

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Occurrence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Carotene</td>
<td>4.7 X 10⁻⁴ g</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>1.6 X 10⁻⁴ g</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>1.2 X 10⁻⁴ g</td>
</tr>
</tbody>
</table>

* Values are expressed as per gram fresh weight of collard greens. Standards were purchased from Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178.

The selectivity (α) class value for chlorophyll a and b was 1.4, and the resolution was Li1; this indicates that if one wished to collect the samples, one would obtain approximately 99.2% of each component. The average HPLC student results, expressed as per gram fresh weight of collard greens for β-carotene, chlorophyll a, and chlorophyll b are summarized in the table. The other, unidentified compounds appearing in the chromatogram are typically phophytins (chlorophyll which has lost its magnesium), chlorophyllides (chlorophyll which has lost its long-chain alcohol, either phytol or farnesol), pheophorbides (chlorophyllides which have lost their magnesium), or phoephtins which have lost their phytol moiety.

The literature is replete with time-consuming separations utilizing column or thin layer chromatography (8-11). Evans et al. (12) have separated two chlorophyll derivatives with high-pressure liquid chromatography and Ekins et al. (13) have described a preparative HPLC procedure too lengthy for routine analysis. Shoaf (14) has separated chlorophyll a and b from aquatic algae utilizing dimethyl sulfoxide as the extracting solvent and water-methanol as the HPLC mobile phase and Braumann and Grimme (15) have done a single-step separation of photosynthetic pigments with HPLC. The chromatographic separation of β-carotene, chlorophyll a, and chlorophyll b by TLC and open-column methods is time-consuming and causes pigment decomposition by exposing these labile compounds to oxygen, light, solvents, and adsorbents for relatively long periods of time.

The project we have described demonstrates to the student that high performance liquid chromatography is useful for rapid quantitative identification and analysis of such compounds as plant pigments. Because the separation is rapid and requires only a few, inexpensive standards, it can be integrated easily into existing laboratory curricula.

Acknowledgment

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Literature Cited