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Normal-Phase Open Column Versus Reversed-Phase High Performance Liquid Chromatography

Separation of Chlorophyll a and Chlorophyll b from their Diastereomers

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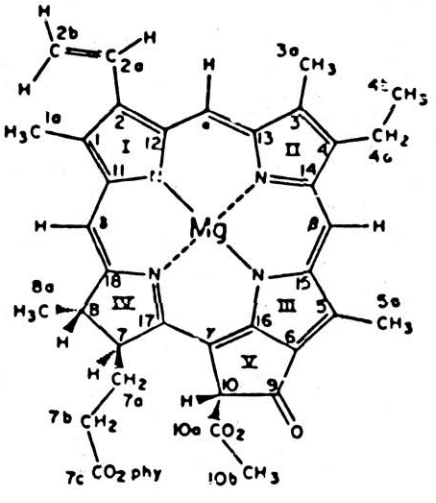
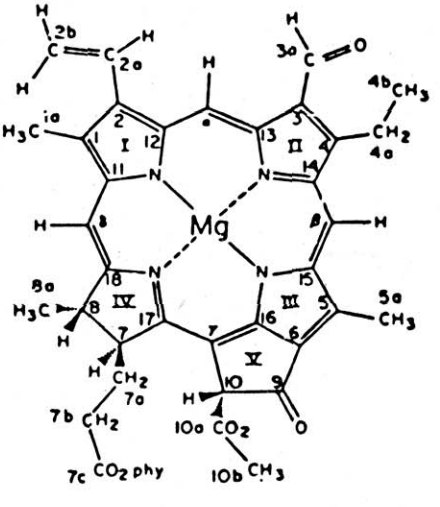
In view of the ever-expanding role of high performance liquid chromatography (HPLC) in modern analytical chemistry, it behooves the undergraduate chemistry student to become familiar with both the theory and application of this technique. Also important, however, is that students develop a firm awareness of the advantages HPLC offers over the more classical open-column methods. To illustrate these advantages experimentally, a system is needed wherein the speed and inherent superior resolving power of HPLC can easily be demonstrated. Here at Canisius College, such a student-oriented experiment has been developed and used in our senior level analytical course. The experiment involves initial separation and isolation of the plant pigments chlorophyll a (Chl a) and chlorophyll b (Chl b) from spinach leaves via normal-phase open-column chromatography on sugar (*1–2*). Separation of Chl a and Chl b from their diastereomers about C-10 (Chl a′ and Chl b′, respectively, Fig. 1), however, is much more difficult, but can be achieved with HPLC using a 5-μm reversed-phase C_{18} column. In the course of this experiment, students are required to utilize both normal-phase open-column and reversed-phase HPLC, identify species observed in HPLC chromatograms, calculate classical chromatographic parameters and answer several questions pertaining to the overall experiment. This project-oriented laboratory and typical student results is herein described.

Experimental

Sample Isolation

Initial Preparation and Extraction Procedure (*1–2*). Approximately 10 g of fresh spinach leaves (free of midribs) are placed in 50 mL of vigorously boiling water for 1–2 min. The water is quickly cooled by running in an excess of cold water and decanted. The leaves are collected, rinsed once with an additional amount of cold water, and dried, first by hand squeezing and then between paper





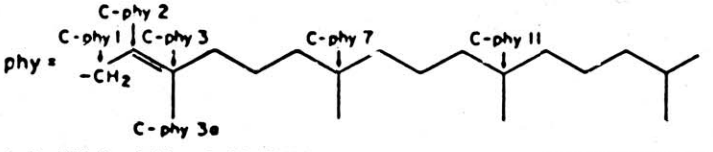


Figure 1. Molecular structures of chlorophyll a (Chl a) and chlorophyll b (Chl b). Phy indicates a phytyl alcohol chain. Replacement of the Mg atom by 2H forms the corresponding pheophytin. The C-10 diastereomers of Chl a and Chl b are designated as Chl a′ and Chl b′, respectively.

towels. These procedures inactivate enzymes and facilitate extraction of the plant pigments (*3–4*).

The dried leaves are separated, placed in 20 mL of a methanol:petroleum ether (b.p. 20–40°C) solution (2:1 v/v), and allowed to stand, with occasional agitation, for approximately 30 min. Methanol helps remove water from the plant material and the petroleum ether takes up the pigments before they can undergo undesirable reactions such as hydrolysis, oxidation, allomerization, etc. (*5–9*).

Caution: Extreme care should be exercised when handling petroleum ether and other flammable solvents.

The resultant deep green extract is decanted through a pad of cotton into a 125-mL separatory funnel. The leaves are re-extracted once more with the same quanitity of methanol:petroleum ether solution and then with 5 mL of fresh petroleum ether. These extracts are likewise filtered into the separatory funnel as previously described. The combined methanol plus petroleum ether extract is diluted with 10 mL of a saturated salt (NaCl) solution and shaken. If an emulsion forms or if the plant pigments are not completely transferred to the petroleum ether layer, the aqueous methanol layer is re-extracted with 5 mL of a petroleum ether (b.p. 20–40°C): diethyl ether solution (1:1 v/v). The petroleum ether layer(s) is separated and evaporated to dryness on a rotary evaporator, at 40°C.

Normal-Phase Open Column Chromatography (on Sugar). The dried pigment extract from above is dissolved in 1 mL of diethyl ether. To this was added 4 mL of petroleum ether (b.p. 20–40°C). The entire sample is placed on the head of an open, normal-phase chromatographic column (2.5 × 50.0 cm). The column is prepared by pressing small portions of commercial powdered (or ground) sugar, containing 3% starch[1], into a chromatographic tube. Application of a slight vacuum while packing was found to produce the most uniform columns (Fig. 2). When the entire sample has been added, it is washed with fresh petroleum ether (~5 mL) and developed initially using petroleum ether containing 0.5% *n*-propanol. A mild vacuum is applied to speed the elution process (Fig. 2). As the column develops, the student may wish to increase the percent (%) *n*-propanol to accelerate the elution rate. However, it is *not* advisable to exceed 3% *n*-propanol.

The column is allowed to develop until an adequate separation between the Chl a (a′) and Chl b (b′) zones is obtained (~10–15 cm). It is then allowed to be sucked dry by the mild vacuum. The developed packing is removed, the Chl a (a′) and Chl b (b′) zones separated, repacked into fresh individual chromatographic tubes, and eluted with an ethanol:petroleum ether solution (1:1 v/v).

Isolation of Chl a (a′) and Chl b (b′). The chlorophyll containing ethanol plus petroleum ether solutions from above, are each washed once with 25 mL of saturated salt (NaCl) solution and then extracted with 3 × 25 mL portions of 70% methanol. The petroleum ether layers are washed three more times with fresh water (25 mL), dried over anhydrous magnesium sulfate ($MgSO_4$), filtered, and evaporated to dryness on a rotary evaporator, at 40°C. To prevent photodecomposition from light exposure, each product container is wrapped with aluminum foil and stored under a N_2 atmosphere.

Preparation of Sample for Reversed-Phase HPLC. The Chl a (a′) and Chl b (b′) isolated via open column chromatography on sugar (vide supra) are each dissolved in 2 mL of HPLC grade[2] acetonitrile. A 5–10 μL sample of each is removed and introduced onto a C_{18} reversed-phase column operated under the conditions indicated below.

Reversed-Phase HPLC

Instrumentation. All reversed-phase HPLC data were collected using a modified Varian Model 5000 Liquid Chromatograph (Varian Associates, Inc., Walnut Creek, CA). This system was equipped with a pneumatic actuated automatic sample injector (Model ACV-6-HPAX, Valco Inst., Houston, TX), fitted with a 10-μL loop. The detector was a variable wavelength visible-ultraviolet detector (Model VUV-10, Vari-Chrom, Varian, Walnut Creek, CA). Wavelength was set at 663 nm to detect both Chl a (a′) and Chl b (b′). Chromatograms were recorded on an OmniScribe, dual pen, strip chart X-Y recorder (Model B 5217-5 I; Houston Instruments, Austin, TX).

Stainless steel reversed-phase columns (30 × 0.4 cm), prepacked with monomeric octadecylsilane (C_{18}, ODS) bonded onto 5-μm silica gel, were used (MicroPak MCH-5; Varian, Walnut Creek, CA). The mobile phase consisted of an isocratic mixture of HPLC-grade acetonitrile and tetrahydrofuran (95:5 v/v). Columns were operated at ambient temperature with a flow rate of 1 mL/min. Chart speed was set at 1 cm/min. Detector band width was fixed at 8 nm and AUFS adjusted as necessary.

[1] Commercial powdered (or ground) sugar that already contains 3% starch to prevent caking is preferred.

Results and Discussion

The experiment was divided into two parts and run over consecutive 4 h "open" laboratory periods. In Part 1, students were given a refresher lecture on the theory and application of normal-phase and reversed-phase liquid chromatography and allowed to form small study groups of two or three. They were then required to isolate the plant pigments Chl a (a′) and Chl b (b′) using normal-phase open column techniques on sugar. Part 2 of the laboratory involved further separation of the isolated species using reversed-phase HPLC.

Part 1

A good and relatively inexpensive source of the chlorophylls is fresh spinach. The canned or frozen variety can also be used, but they, in general, contain a rather large quantity of alteration products (*3*). Students were given an ample supply of spinach (~10 g) and a handout describing the isolation procedures and were instructed on the proper methods for safely handling flammable solvents. Sugar columns were dry packed as described in the experimental section. Slurry packing has been suggested but often results in column shrinkage or channeling when sucked dry (*10*).

The chlorophylls are the only green (or yellow-green) species observed on the column and thus are easily recognized by the students. Since sugar is a normal stationary phase, the Chl a (a′) zone (less polar) elutes more quickly than the Chl b (b′) zone (Fig. 2). The chlorophylls were easily isolated, weighed, and stored as described. Typical weights are given in Table 1.

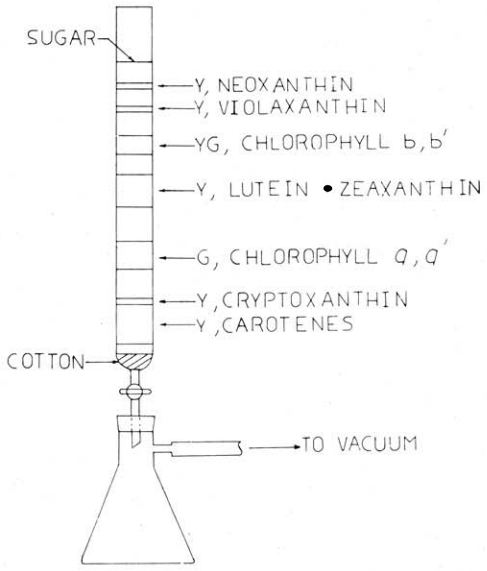


Figure 2. Typical apparatus used to separate green plant (spinach) pigments. The column consists of dry packed, commercial-grade powdered (or ground) sugar. This ideal adsorption chromatogram results from development with 0.5% *n*-propanol in petroleum ether (G, green; YG, yellow-green; Y, yellow).

Table 1. Typical Recovery Range for Chl a (a′) and Chl b (b′) on Sugar Columns

Pigment	Weight[a]
Chl a (a′)	$3.5–5.0 \times 10^{-4}$ g
Chl b (b′)	$1.5–2.5 \times 10^{-4}$ g

[a] Weights are expressed per gram of fresh spinach. No additional attempts were made to dry the pigments further.

This portion of the laboratory required approximately 30 min to describe and administer proper precautionary procedures. With an ample supply of the necessary solvents provided, isolation procedures were successfully accomplished by the average student in 2.5 to 3.0 h.

Part 2

The following laboratory period, students were provided with another handout listing the appropriate instrumental parameters and given instruction on the operation of the Varian LC-5000. Before proceeding, each student team was required to prove competency on the system by going through a check-out procedure using a standard sample.

The extracted chlorophyll residues were then removed from storage and redissolved in HPLC grade acetonitrile.[2] A small portion of each solution was removed and injected onto the head of a 5-μm, C_{18} reversed-phase column (Varian MCH-5, ODS). Both pigment samples were eluted using a 95:5 (v/v) acetonitrile: tetrahydrofuran isocratic mixture. If HPLC chromatograms were off-scale or too small, students were required to adjust AUFS and/or sample size.

Figure 3 gives typical HPLC chromatograms obtained by students; each takes less than 30 min to run. Of special interest is the clean separation achieved between Chl a and a′; Chl b and b′ and the predominance of the more stable parent (unprimed) stereoisomer (*11*). Also note that under identical conditions, the chlorophylls elute in the expected order on reversed-phase columns (i.e., Chl b (b′) (more polar) elutes more quickly than Chl a (a′)). A recent report in THIS JOURNAL (*12*), however, indicates the opposite order using a step-and-hold isopropanol:isooctane gradient and a Varian MicroPak MCH-10 reversed-phase column (C_{18}, ODS); the so-called "reversed-reversed-phase" behavior.

Students were given samples of authentic Chl a and Chl b[3] and asked to identify the major components in each chromatogram. About one-half of the students chose the method of retention-time analysis, the remainder chose the superior method of co-chromatography. All students were easily able to identify the appropriate species.

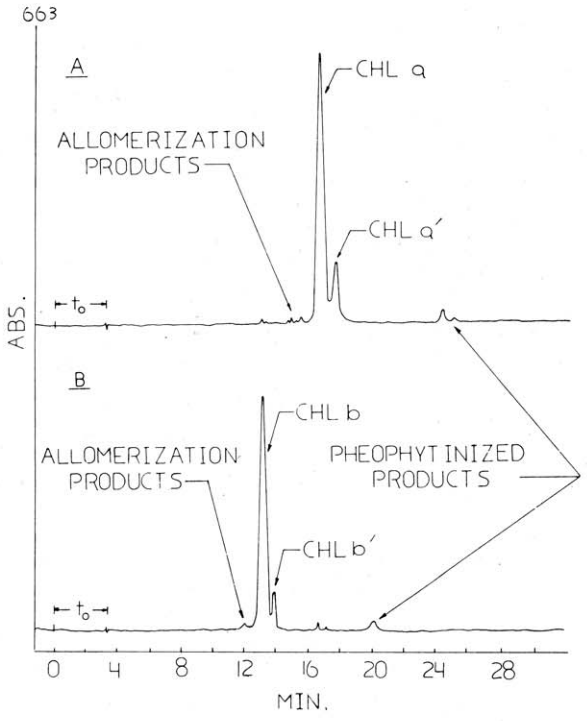


Figure 3. HPLC chromatograms of the chlorophylls isolated from fresh spinach on sugar columns. *A*: Chl a and a′. *B*: Chl b and b′. For both chromatograms, the mobile phase consisted of acetonitrile:tetrahydrofuran (95:5, v/v), flow rate was 1 mL/min, and detection was set at 663 nm.

Table 2. Selected Parameters Calculated from HPLC Chromatograms[a,b]

Pigment	Retention Time (t_r)	Capacity Factor[c] (k')	Selectivity Factor (α)	Resolution (R_s)
Chl a	16.60 min	3.81		
			1.09	1.48
Chl a′	17.75 min	4.14		
Chl b	13.05 min	2.78		
			1.10	1.39
Chl b′	14.00 min	3.06		

[a] Chromatographic parameters were calculated using standard equations found in ref. (*15*).
[b] These data represent results obtained from a typical student experiment.
[c] The retention time for an unretained peak, t_0 = 3.45 min.

Often, small amounts of alteration products were observed in the chromatogram (Fig. 3). Some students even experienced cross-contamination between Chl a (a′) and Chl b (b′). Although much cross-contamination results from improper cleansing of the injection port, a small amount does result from the separation process on sugar. The more polar (fast eluting) alteration products are most likely the products of allomerization: 10-hydroxy chlorophyll and the 10-methoxylactone of chlorophyll. These species have been conclusively identified via TLC, HPLC co-chromatography and californium-252 plasma desorption mass spectroscopy (*13–14*). The less polar (slower eluting) alteration products are more prominent and are most likely pheophytinized (*3*) products (i.e., chlorophyll in which the central magnesium atom has been replaced by two hydrogens (Fig. 1)). Lively discussions relating to the origin and relative retention times of these alteration products lead to a surge of interest in chlorophyll chemistry. The average student took approximately 4 h to complete Part 2 of the experiment.

Finally, students were asked to calculate and report a number of classical chromatographic parameters, such as:

1) the capacity factors (k') for each major component in the chromatograms,
2) the selectivity factor (α) between Chl a and a′; Chl b and b′,
3) the resolution (R_s) between Chl a and a′; Chlb and b′ and
4) the number of theoretical plates (N) using the equation

$$N = 16\left(\frac{t_r}{W}\right)^2$$

where t_r is the retention time for the peak of interest and W is its width. Table 2 contains typical retention time (t_r), k', α, and R_s data.

Students were also asked to respond to a series of questions related to the experiment in general. A few examples are as follows:

1) What changes can be made to affect a better separation on the reversed-phase column?
2) Account for Chl b (b′) eluting faster than Chl a (a′) on the reversed-phase column but more slowly on sugar.
3) Why are Chl a and a′ easily separated on the reversed-phase column but not on the normal-phase column, etc.?

Conclusion

The laboratory project described was generally well received by the students. In addition to demonstrating the power and speed of HPLC it also provides the student with

[2] HPLC-grade solvents were obtained from Fisher Scientific Company, Fair Lawn, NJ.
[3] Sigma Chemical Company, St. Louis, MO.

practical experience. Since the solvents used and the normal-phase packing material are relatively inexpensive, and the separation process on the reversed-phase column is rapid, this experiment can easily be integrated into most laboratory curricula where HPLC capabilities exist.

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