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Note

Rapid method for the separation of chlorophylls *a* and *b* by high-pressure liquid chromatography

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The quantitative determination of chlorophyll *a* and *b* from aquatic algae requires efficient extraction followed by separation of the chlorophylls from each other and from compounds that interfere with chlorophyll analysis. These interfering compounds are typically pheophytins (chlorophyll which has lost its magnesium), chlorophyllides (chlorophyll which has lost its long-chain alcohol, either phytol or farnesol), or pheophorbides (chlorophyllides which have lost their magnesium). These degradation products of chlorophyll may be formed in the natural environment from decomposing algae or may be formed during inadequate or prolonged storage of a collected sample[1,2]. The literature is replete with time consuming separations utilizing thin layer chromatography[3–7]. Evans *et al.*[8] have separated two chlorophyll derivatives with high-pressure liquid chromatography (HPLC), and Eskins *et al.*[9] have described a preparative HPLC procedure too lengthy for routine analysis. The HPLC method described here is rapid and sensitive and has a high degree of precision. It can be used with larger amounts of chlorophyll than most thin layer methods. The HPLC method is also compatible with the use of dimethyl sulfoxide (DMSO), a more efficient chlorophyll extractant than acetone for green algae[10].

Algae were filtered through a glass-fiber filter, rolled and placed into a glass tissue grinder. DMSO, at 20–25% of the grinder volume, was added and the sample ground with a PTFE pestle for 3 min at 500 rpm. The sample was transferred to a screw-cap graduated centrifuge tube. The pestle and grinding vessel were rinsed with DMSO and this rinse was also added to the sample. An equal volume of diethyl ether was added, the cap was screwed on, and the sample shaken vigorously for 10 sec. After waiting an additional 10 sec, the sample was shaken vigorously for another 10 sec. The cap was removed and distilled water equal to 25% of the total volume was added slowly (drop by drop). As the water was added, the DMSO–diethyl ether solution separated into two immiscible liquids, the DMSO–water solution on the bottom and the diethyl ether layer containing the green chlorophylls above. The tube was capped and shaken well so that all the chlorophyll migrated into the diethyl ether layer. The sample was centrifuged at 1000 *g* for 10 min to cleanly separate the two layers and to sediment the glass fiber filter and algal cell debris. After centrifugation the upper diethyl ether layer containing all the chlorophyll was pipetted off and placed into a 60-ml separatory funnel. A volume of distilled water equal to half the volume of the diethyl ether was added to the separatory funnel. The funnel was shaken,

vented and allowed to sit five minutes to allow the layers to separate. The lower aqueous layer was drained from the separatory funnel and discarded. The diethyl ether chlorophyll portion was transferred out of the top of the separatory funnel, shaken with sodium sulfate to remove excess water, placed in a conical tube, and evaporated to 0.2–0.4 ml by gently blowing nitrogen over the ether surface. The sample was not evaporated to dryness. This chlorophyll solution was routinely diluted with acetone to a constant volume, usually 1 ml. Less acetone was added if the concentration of chlorophyll was low. This final volume was recorded immediately before injection of the sample into the chromatograph.

The chromatograph was equipped with a 25-cm Whatman* Partisil PXS 1025 ODS-2 column, and equilibrated with 5% water and 95% methanol as a solvent. A flow-rate of 4 ml/min was used. A variable wavelength spectrophotometric detector was set at 654 nm. Fig. 1 shows the retention times for a mixture of highly purified preparations of chlorophyll *a* and *b*.

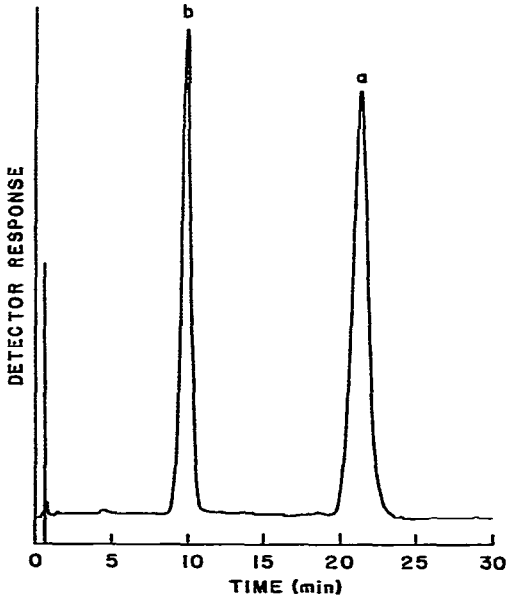
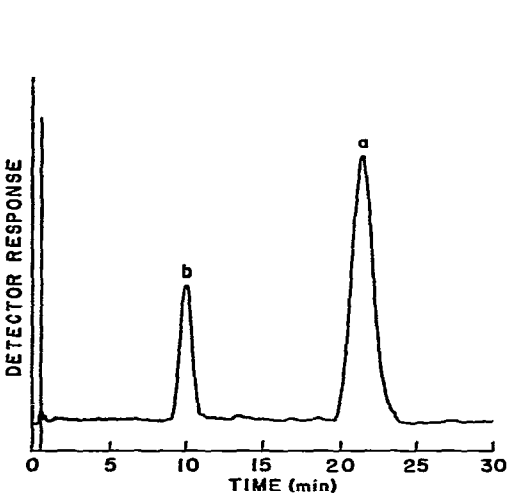


Fig. 1. Chromatography of chlorophyll standards.

The common degradation products (pheophytins *a* and *b*) were prepared from the purified chlorophyll solutions by acidifying with HCl and observing the absorbance at 664 nm for chlorophyll *a* (647 nm for chlorophyll *b*) until the absorbance decreased to a constant value. The solution was then extracted several times with water to remove excess acid. A small quantity of pH 7.4 buffer (morpholinopropane sulfonic acid) was added to assure a near neutral pH. Pheophytins *a* and *b* had much longer retention times than the chlorophylls and were usually flushed off with a stronger solvent (100% methanol).

* The use of the brand name in this report is for identification purposes only and does not imply endorsement by the U.S. Geological Survey.

Chlorophyll samples analyzed by HPLC yielded a variety of chromatographic patterns. The sample shown in Fig. 2 contained only chlorophylls *a* and *b*. Other peaks (presumably chlorophyll degradation products other than the pheophytins) were observed in a number of samples, but no peaks overlapped the chlorophyll *a* or *b* peaks. The resolution obtained with a sample containing chlorophylls *a* and *b* with several chlorophyll degradation product peaks is illustrated in Fig. 3.



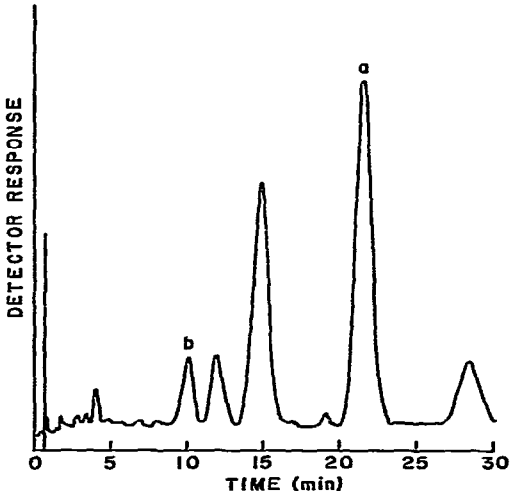


Fig. 2. Chromatography of a lake sample without chlorophyll degradation products.

Fig. 3. Chromatography of a lake sample containing several chlorophyll degradation products.

In summary, the HPLC method is more rapid than thin-layer methods and has a high degree of sensitivity and precision. It can be used with many solvents including methanol, acetone, or the more efficient chlorophyll extracting solvent, DMSO. The method can be semi-automated with the use of automatic injectors and computing integrators. Chlorophyll *a* and *b* are accurately determined by this method without interference from degradation products.

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