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Note

Rapid, quantitative separation of chlorophylls and their degradation products by high-performance liquid chromatography

PAUL G. FALKOWSKI* and JAMIE SUCHER
Oceanographic Sciences Division, Department of Energy and Environment, Brookhaven National Laboratory, Upton, NY 11973 (U.S.A.)
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The quantitative determination of chlorophylls and their degradation products is one of the most frequently performed analyses in aquatic ecology. The most convenient and widely used method is based on the difference in fluorescence yields between chlorophyll *a* and its phaeopigment derivatives (phaeophytin *a* and phaeophorbide *a*)[1]. It is subject to error if significant concentrations of chlorophyll *b* are present[2], but it is not possible to determine whether chlorophyll *b* is present using the fluorometeric technique alone. Chlorophylls can also be determined by absorbance spectroscopy[3], but the method is not sufficiently sensitive and is subject to error if phaeopigments are present. The quantitative separation of all chlorophylls and phaeopigments has been achieved by thin-layer chromatography (TLC)[4]. The technique is time consuming, impractical on shipboard, and may produce artifacts[5]. Finally, a number of high-performance liquid chromatographic (HPLC) procedures have been described for the quantitative separation of chlorophylls and/or their degradation products[6–9]. None of the HPLC methods thus far described combines rapid separation with methodological simplicity, sensitivity, and precision. The major objective of this report is to describe such an HPLC system for the determination of the major chlorophylls and their degradation products, including: chlorophylls *a*, *b*, and *c*, chlorophyllide *a*, phaeophorbide *a*, and phaeophytins *a* and *b*.

EXPERIMENTAL

Reagents

All solvents used for pigment extraction were reagent grade, and all chromatographic solvents were HPLC grade.

Pigments extraction

Pigments were obtained from cultures of the marine diatom, *Skeletonema costatum* (chlorophylls *a* and *c*, and chlorophyllide *a*) and the marine chlorophyte *Dunaliella tertiolecta* (chlorophylls *a* and *b*). Algae were grown in an enriched natural seawater media as previously described[10] and harvested by continuous-flow centrifugation. Pigments were extracted with methanol–water (90:10) at 0 to 4°C by grinding frozen cells (1 to 3 g fresh weight) in a glass tissue grinder with a PTFE pestle. A glass fiber filter (Gelman AE) was added during grinding to promote cell breakage. The

brie was filtered through a glass fiber filter and the pigment extracts were chromatographed on icing sugar columns[11] or by TLC[4], eluted, and stored in 100% methanol in the dark at −20°C. Immediately prior to use the standards were purified by TLC and brought to desired concentrations in methanol–water (80:20). The pigment standards were calibrated with an Aminco DW-2a spectrophotometer, using accepted absorbtivity coefficients[4]. Chlorophyllide *a* was obtained from *S. costatum* by extraction in acetone–water (90:10) at room temperature; conditions which are favorable for the enzymatic hydrolysis of phytol.

For extraction of field samples, 100 to 200 ml of sea water (0.1 to 0.5 μg chlorophyll *a*) were filtered on 25 mm glass-fiber filters. The filters were ground in a tissue grinder with 2 ml of absolute methanol and brie filtered through a glass-fiber filter into a graduated centrifuge tube. The ground glass filters were reextracted on the filter manifold with an additional 1 ml of methanol. A 25-μl volume of pooled pigment extracts was applied to the HPLC column for analysis.

Liquid chromatography

A two-step solvent program was used to resolve chlorophyll *c* from chlorophyllide *a* and phaeophorbide *a* and at the same time allow phaeophytin *a* to elute in a were delivered with an Eldex A-30S pump at 1.5 ml/min. Samples (5–25 μl) were applied to the column with a Valco CV-6-UH Pa-N60 sample injection valve fitted with a 50-μl loop. The detection system consisted of a Farrand Optical Co. A-4 fluorometer equipped with a 10-μl quartz flow cell. Two Corning 5543 filters ($\lambda_{max.}$ 420 nm) were used with an 85 W mercury source to provide excitation energy, and single Corning 2030 was placed on the emission side. The analogue output was recorded directly and also digitized with a Hewlett-Packard (HP) 3437A high-speed digital volt meter. The digital output was accessed and processed with an HP-85 desktop computer.

A two-step solvent program was used to resolve chlorophyll *c* from chlorophyllide *a* and phaeophoribde *a* and at the same time allow phaeophytin *a* to elute in a reasonable time (*ca.* 18 min). Methanol–water (90:10) was applied until phaeophorbide *a* was eluted, followed by methanol–water (98:2). Solvent delivery was automatically programmed with a single-step solenoid valve.

RESULTS AND DISCUSSION

The major chlorophylls and their degradation products were separated in 18 min (Fig. 1). Using the Farrand A-4 fluorometer, less than 0.1 pmole of chlorophyll *a* can be detected (Fig. 2). Fluorescence output is linear with pigment concentration over three orders of magnitude and the coefficient of variation for the estimation of chlorophyll *a* was ±1.3% ($n = 13$, $r^2 = 0.998$).

The HPLC system described provides a rapid and sensitive method for the determination of chlorophylls and their degradation products. It should be considered as a supplement to the simple fluorometric method. For general water analysis, the simple fluorometric method is rapid and precise. In some applications, however, noteable artifacts prevent its sole use. For example, in the analysis of sediment samples, those sediments rich in humic and other organic acids may have a large background fluorescence which is not acid labile. Using the simple fluorometric method alone phaeopigment concentrations would be grossly overestimated. The physical separation of phaeopigements by HPLC prevents such artifacts from occurring.

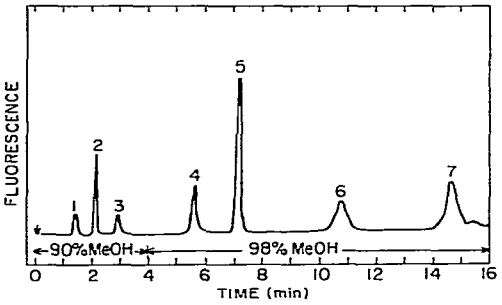

Fig. 1. Elution profile of chlorophylls and degradation products prepared from algal extracts. A 15-cm RP-8 column was used with 1.5 ml/min solvent flow-rate. Peaks: 1 = chlorophyll *c*; 2 = chlorophyllide *a*; 3 = phaeophorbide *a*; 4 = chlorophyll *b*; 5 = chlorophyll *a*; 6 = phaeophytin *b*; 7 = phaeophytin *a*.

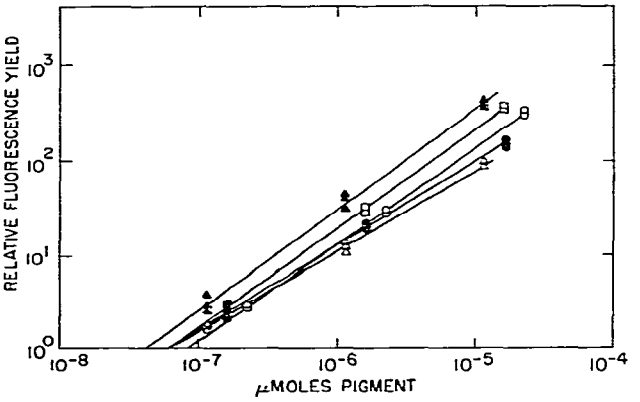

Fig. 2. Calibration curves for five plant pigments using the HPLC system described. ▲ = Chlorophyll *a*; △ = phaeophytin *a*; ● = chlorophyll *b*; ○ = phaeophytin *b*; □ = chlorophyll *c*.

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