

Chapter 13. Measurement of Algal Chlorophylls and Carotenoids by HPLC

Updated by: C. Rathbun (April 1997), A. Doyle and C. Rathbun (April 1996); A. Doyle (June 1994); T. Waterhouse (February 1993)

Prepared by: J. Sorensen (April 1992)

1.0 Scope and field of application

Many individual algal pigments or pigment combinations and ratios are taxon-specific. Therefore, analysis of the chlorophylls and carotenoids present in a seawater sample can reveal the taxonomic composition of natural algal populations. This technique, using absorbance spectroscopy as analysed by high performance liquid chromatography (HPLC), allows for the rapid separation of phytoplankton pigments with detection limits for chlorophylls and carotenoids on the order of 1 ng (Bidigare, 1991). The HPLC method described here is a modified version of Wright et al. (1991), provided by Bidigare (1991). The method described here has been adopted as BATS protocol since BATS 70 (July 1994). This method uses less solvent than the previously used method and gives improved peak separation and better resolution at lower concentrations.

2.0 Definition

The concentration of all pigments is given as ng kg^{-1} in seawater.

3.0 Principle of Analysis

The reverse phase HPLC method described here separates all of the phytoplankton pigments listed in Table 2, in order of polarity upon passage through a column. The most polar pigments are removed earlier than the less polar pigments.

Table 2. HPLC Pigments.

Chlorophyllide *a*
Chlorophyll *c*₃
Chlorophyll *c*₁+ *c*₂ and Chlorophyll Mg 3,8DVP *a*₅
Peridinin
19' - Butanoyloxyfucoxanthin
Fucoxanthin

19' - Hexanoyloxyfucoxanthin
Prasincoxanthin
Diadinoxanthin
Alloxanthin
Diatoxanthin
Lutein
Zeaxanthin
Chlorophyll *b*
Chlorophyll *a*
 α Carotene
 β Carotene

Picoplanktonic prochlorophytes are abundant in tropical and subtropical seas and oceans. They contain divinyl-chlorophyll *a* and divinyl-chlorophyll *b* (more appropriately called 8-desethyl, 8-vinyl Chlorophyll), both of which co-elute with "normal" chlorophyll *a* and *b* with this reverse phase liquid chromatography technique.

4.0 Apparatus

- 4.1 *Filtration System* and Whatman[®] 47 mm GF/F filters
- 4.2 *Liquid nitrogen* and freezer for storage and extraction
- 4.3 *Glass centrifuge tubes* for extraction, 15 ml
- 4.4 High pressure liquid chromatograph capable of delivering three different solvents at a rate of 1 ml/minute.
- 4.5 *High-pressure injector valve* equipped with a 500 μ l sample loop.
- 4.6 *Column inlet filter* (0.5 μ m) to protect column from particulates.
- 4.7 *Guard Column* (50 x 4.6 mm, ODS-2 C18 packing material, 5 μ m particle size) for extending life of primary column.

4.8 *Reverse phase HPLC Column* (250 x 4.6 mm, 5 µm particle size, ODS-2 Spherisorb C18 column).

4.9 *Absorbance detector* capable of monitoring at 436 nm.

4.10 *Data recording device*: computer equipped with hardware and software for chromatographic data analysis. (Dyanmax[®] MacIntegrator Version 1.3.1. Rainin Instrument Co. Inc.)

4.11 *Glass syringe*, 1000 µl

5.0 Eluents

Eluent A (80:20, v:v, methanol: 0.5 M ammonium acetate, aq., pH 7.2).

Eluent B (90:10, v:v, acetonitrile:water).

Eluent C (ethyl acetate).

HPLC-grade solvents are used. Eluents are filtered through a 47 mm GF/F filter and degassed with helium before use.

The gradient program is listed in Table 3.

Table 3. HPLC solvent system program.

Time	Flow Rate	%A	%B	%C	Conditions
0.0	1.0	100	0	0	Linear gradient
2.0	1.0	0	100	0	Linear gradient
2.6	1.0	0	90	10	Linear gradient
13.6	1.0	0	65	35	Linear gradient
18.0	1.0	0	31	69	Hold
23.0	1.0	0	31	69	Linear gradient
25.0	1.0	0	100	0	Linear gradient
26.0	1.0	100	0	0	Hold
32.0	1.0	100	0	0	Inject

6.0 Sample Collection and Storage

Water samples are collected from OTE bottles into 4 liter polypropylene bottles equipped with a 1/4" outlet and Tygon[®] tubing at the base. Filtration is "in line"; each bottle is connected via the tubing to a polycarbonate filter holder (Gelman) holding a 47mm GF/F

filter, and a vacuum system. Samples are promptly filtered using a vacuum of less than 100 mm Hg. Filters are folded in half and lengthwise in half again, wrapped in aluminum foil and labeled, and stored in liquid nitrogen until on-shore analysis. Alternatively, if analysis is to be carried out onboard ship, filters can be placed in 100% acetone for immediate pigment extraction. Samples collected for HPLC analysis can also be used in the measurement of chlorophyll *a* and phaeopigments by fluorometric analysis.

Filtration volume will vary with sampling location. For oligotrophic waters, 4 liters are filtered, whereas in coastal regions a smaller volume (0.5-1.0 liters) may be appropriate. In this case, a 25 mm GF/F filter is recommended.

7.0 Procedure

- 7.1 After removal from liquid nitrogen, the pigments are extracted by placing the filters in 5.0 ml 100% acetone. For 47 mm GF/F filters, approximately 0.8 ml of water is retained on the filter, which adjusts the final extraction solution to approximately 90% acetone and the final extraction volume to 5.8 ml. The samples are covered with Parafilm, sonicated (0°C, subdued light) and allowed to extract overnight in the dark at -20°C. Following extraction, samples are vortexed; then the filters are pressed to the bottom of the tube with a stainless steel spatula and the samples centrifuged for 10 minutes to remove cellular debris.
- 7.2 The addition of 5.0 ml acetone for pigment extraction is necessary to completely submerge 47 mm GF/F filters in 15 ml centrifuge tubes. However, this volume can be altered depending on the sizes of the filter and the extraction tube.
- 7.3 The HPLC system is set up and equilibrated with Solvent System A at a flow rate of 1 ml minute⁻¹.
- 7.4 An external standard of chlorophyll *a* is run before each sample set for daily HPLC calibration. An aliquot of 1 ml of standard is mixed with 300 µl of Milli-Q water in a 2 ml vial. Samples are prepared for injection by mixing a 1 ml aliquot of the pigment extract with 400 µl of Milli-Q water in a 2 ml vial. These sub-samples and the standards are shaken and allowed to equilibrate in the dark for 5 minutes prior to injection.
- 7.5 Approximately 1000 µl of sample or standard are injected into the 500 µl sample loop. The three-step solvent program is initiated upon closure of the injection valve. The chromatogram produced is collected on a recording device.

- 7.6 The identities of the peaks from the sample extracts are determined by comparing their retention times with those of pure standards and algal extracts of known pigment composition. Peak identities can be confirmed spectrophotometrically by collecting eluting peaks from the column outlet.
- 7.7 The HPLC system is calibrated with pigment standards obtained commercially and/or by preparative scale HPLC standards (purified HPLC fractions eluted with standard solvents). Concentrations of pigment standards are determined in the appropriate solvent using a monochromator-based spectrophotometer, prior to the calibration of the HPLC system. The recommended extinction coefficients for most of the common algal pigments are provided in Table 4 (Bidigare 1991).

Table 4: Extinction coefficients of pigments separated by reverse phase HPLC.

Pigment	Wavelength (solvent)	E 1 cm(l g ⁻¹ cm ⁻¹)
Chlorophyll <i>a</i>	664 nm (90% acetone)	87.67
Chlorophyll <i>b</i>	647 nm (90% acetone)	51.36
Chlorophyll <i>c</i> ₁ + <i>c</i> ₂	631 nm (90% acetone)	42.6
Chlorophyllide <i>a</i>	664 nm (90% acetone)	128.0
Fucoxanthin	449 nm (EtOH)	160.0
19' - Hexanoyloxyfucoxanthin	447 nm (EtOH)	160.0
19' - Butanoyloxyfucoxanthin	446 nm (EtOH)	160.0
Lutein	445 nm (EtOH)	255.0
Zeaxanthin	450 nm (EtOH)	254.0
Prasinoxanthin	454 nm (EtOH)	160.0
Alloxanthin	453 nm (EtOH)	262.0
Peridinin	472 nm (EtOH)	132.5
Diadinoxanthin	446 nm (EtOH)	262.0
Diatoxanthin	449 nm (EtOH)	262.0
β Carotene	453 nm (EtOH)	262.0
Phaeophorbide <i>a</i>	665 nm (90% acetone)	69.8

8.0 Standards

- 8.1 Standards stored under nitrogen in the dark at -20°C are stable for approximately one month.

8.2 Pigment standard concentrations are calculated as follows:

$$C_s = \left(\frac{(A_{max} - A_{750nm})}{E \cdot l} \right) \left(\frac{1000mg}{1g} \right)$$

Where:

C_s	=	pigment concentration (mg l ⁻¹)
A_{max}	=	absorbance maximum (Table 4)
A_{750nm}	=	absorbance at 750 nm to correct for light scattering
E	=	extinction coefficient (l g ⁻¹ cm ⁻¹ , Table 4)
l	=	cuvette path length (cm)

8.3 After the concentrations of each pigment standard are determined, they are injected onto an equilibrated HPLC system to calculate standard response factors (RF).

The response factors (RF) are calculated as follows:

$$RF = \frac{(C_s)(IV)}{A}$$

Where:

RF	=	standard response factor (ng unit area ⁻¹)
C_s	=	pigment standard concentration (ng μl ⁻¹)
IV	=	injection volume (μl)
A	=	integrated peak area

9.0 Calculation and Expression of results

Concentration of the individual pigments in the sample are calculated using the following formula:

$$C_i = A(RF) \left(\frac{1}{IV} \right) (EV) \left(\frac{1}{SV} \right) (D)$$

Where:

- C_i = individual pigment concentration (ng l^{-1})
 A = integrated peak area
 RF = standard response factor (ng unit area^{-1})
 IV = injection volume (ml)
 EV = extraction volume (ml)
 SV = sample filtration volume (l)
 D = dilution factor

$$D = \frac{\left(\frac{IV}{V_o}\right)}{\left(\frac{IV}{V_s}\right)}$$

Where:

- IV = injection volume of standard or sample (μl)
 V_o = Total standard solution volume (μl)
 V_s = Total sample solution volume (μl)

The units of ng kg^{-1} can be obtained by dividing the calculated pigment concentrations by the density of the seawater.

10.0 References

- Bidigare, R. (1991). in Spencer and Hurd (eds.). The analysis and characterization of marine particles. American Geophysical Union, Washington D.C.
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