Method 447.0

Determination of Chlorophylls *a* and *b* and Identification of Other Pigments of Interest in Marine and Freshwater Algae Using High Performance Liquid Chromatography with Visible Wavelength Detection

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DETERMINATION OF CHLOROPHYLLS *a* AND *b* AND IDENTIFICATION OF OTHER PIGMENTS OF INTEREST IN MARINE AND FRESHWATER ALGAE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH VISIBLE WAVELENGTH DETECTION

1.0 Scope and Application

1.1 This method provides a procedure for determination of chlorophylls *a* (chl *a*) and *b* (chl *b*) found in marine and freshwater phytoplankton. Reversed-phase high performance liquid chromatography (HPLC) with detection at 440 nm is used to separate the pigments from a complex pigment mixture and measure them in the sub-microgram range. For additional reference, other taxonomically important yet commercially unavailable pigments of interest are identified by retention time.

1.2 This method differs from previous descriptions of HPLC methods in several respects. Quality assurance/quality control measures are described in Sect. 9.0, sample collection and extraction procedures are described in Sect. 8.0 and reference chromatograms of pure pigments and reference algae are provided.

This method has also been evaluated in a multilaboratory study along with EPA Methods 445.0 and 446.0. Estimated detection limits, precision and bias are reported in Section 13.

Analyte	Chemical Abstracts Service Registry Number (CASRN)
Chlorophyll *a*	479-61-8
Chlorophyll *b*	519-62-0

1.3 Instrumental detection limits (IDLs) of 0.7 ng chl *a*, and 0.4 ng chl *b* in pure solutions of 90% acetone were determined by this laboratory. Method detection limit (MDL) determinations were made by analyzing seven replicate unialgal samples containing the chl *a* and *b*. Single-laboratory MDLs were chl *a* - 7 ng and chl *b* - 4 ng. A multilaboratory estimated detection limit (EDL) (in mg/L of extract is reported in Section 13.

1.4 Most taxonomically important pigments are not commercially available, therefore, a laboratory must be willing to extract and purify pigments from pure algal cultures to quantify and qualitatively identify these very important pigments. This method contains chromatographic information of select pure pigments found either in marine or freshwater algae. The information is included to aid the analyst in qualitatively identifying individual pigments and possibly algal species in natural samples.

1.5 This method uses 90% acetone as the extraction solvent because of its efficiency for extracting chl *a* from most types of algae. (**NOTE:** There is evidence that certain chlorophylls and carotenoids are more thoroughly extracted with methanol[1-3] or dimethyl sulfoxide.)[4] Using high performance liquid chromatography (HPLC), Mantoura and Llewellyn[5] found that methanol led to the formation of chl *a* derivative products, whereas 90% acetone did not. Bowles, et al.[3] found that for chl *a* 90% acetone was an effective solvent when the steeping period was optimized for the predominant species present.)

1.6 One of the limitations of visible wavelength detection is low sensitivity. It may be preferable to use fluorometry[6-8] or HPLC[913] with fluorometric detection if high volumes of water (>4 L) must be filtered to obtain detectable quantities of chl *a* or *b*.

1.7 This method is for use by analysts experienced in handling photosynthetic pigments and in the operation of HPLC or by analysts under the close supervision of such qualified persons.

2.0 Summary of Method

2.1 The HPLC is calibrated with a chl *a* and *b* solution that has been spectrophotometrically quantified

according to EPA Method 446. Chlorophyll-containing phytoplankton in a measured volume of sample water are concentrated by filtration at low vacuum through a glass fiber filter. The pigments are extracted from the phytoplankton into 90% acetone with the aid of a mechanical tissue grinder and allowed to steep for a minimum of 2 h, but not exceeding 24 h, to ensure thorough extraction of the pigments. The filter slurry is centrifuged at 675 g for 15 min (or at 1000 g for 5 min) to clarify the solution. An aliquot of the supernatant is filtered through a 0.45 µm syringe filter and 50 to 200 µL is injected onto a reversed-phase column. Following separation using a ternary gradient, concentrations are reported in µg/L (ppb) or mg/L (ppm) in the whole water sample. This method is based on the HPLC work of Wright, et. al.[9]

3.0 Definitions

3.1 Calibration Standard (CAL) -- A solution prepared from dilution of a stock standard solution. The CAL solution is used to calibrate the instrument response with respect to analyte concentration or mass.

3.2 Calibration Check Standard (CALCHK) -- A mid-point calibration solution that is analyzed periodically in a sample set to verify that the instrument response to the analyte has not changed during the course of analysis.

3.3 Field Replicates -- Separate samples collected at the same time and placed under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of field replicates give a measure of the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.

3.4 Instrument Detection Limit (IDL) -- The minimum quantity of analyte or the concentration equivalent that gives an analyte signal equal to three times the standard deviation of a background signal at the selected wavelength, mass, retention time, absorbance line, etc.

3.5 Laboratory Reagent Blank (LRB) -- An aliquot of reagent water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, reagents, or apparatus. For this method the LRB is a blank filter that has been extracted as a sample.

3.6 Material Safety Data Sheet (MSDS) -- Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.7 Method Detection Limit (MDL) -- The minimum concentration of an analyte that can be identified, measured and reported with 99% confidence that the analyte concentration is greater than zero.

3.8 Quality Control Sample (QCS) -- A solution of method analytes of known concentrations that is used to fortify an aliquot of LRB or sample matrix. Ideally, the QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

4.0 Interferences

4.1 Any compound extracted from the filter or acquired from laboratory contamination that absorbs light at 440 nm may interfere in the accurate measurement of the method analytes.

4.2 Proper storage and good sample handling technique are critical in preventing degradation of the pigments.

4.3 Precision and recovery for any of the pigments is related to efficient extraction, i.e. efficient maceration of the filtered sample and to the steeping period of the macerated filter in the extraction solvent. Precision improves with increasing steeping periods, however, a drawback to prolonged steeping periods is the possibility of pigment degradation. The extracted sample must be kept cold and in the dark to minimize degradation.

4.4 Sample extracts must be clarified by filtration through a 0.45 µm filter prior to analysis by HPLC to prevent column fouling.

4.5 All photosynthetic pigments are light and temperature sensitive. Work must be performed in subdued light and all standards, QC materials, and filtered samples must be stored in the dark at -20°C or -70°C to prevent rapid degradation.

5.0 Safety

5.1 Each chemical used in this method should be regarded as a potential health hazard and handled with caution and respect. Each laboratory is responsible for maintaining a current awareness file of Occupational Safety and Health Administration (OSHA) regulations regarding the safe handling of the chemicals specified in this method.[15-18] A file of MSDS also should be made available to all personnel involved in the chemical analysis.

5.2 The grinding of filters during the extraction step of this method should be conducted in a fume hood due to the volatilization of acetone by the tissue grinder.

6.0 Apparatus and Equipment

6.1 Centrifuge, capable of 675 g.

6.2 Tissue grinder, Teflon pestle (50 mm X 20 mm) with grooves in the tip with 1/4" stainless steel rod long enough to chuck onto a suitable drive motor and 30-mL capacity round-bottomed, glass grinding tube.

6.3 Filters, glass fiber, 47-mm or 25-mm nominal pore size of 0.7 µm unless otherwise justified by data quality objectives. Whatman GF/F filters were used in this work.

6.4 Petri dishes, plastic, 50 X 9-mm, or some other solid container for transporting and storing sampled filters.

6.5 Aluminum foil.

6.6 Laboratory tissues.

6.7 Tweezers or flat-tipped forceps.

6.8 Vacuum pump or source capable of maintaining a vacuum up to 6 in. Hg (20 KPa).

6.9 **Labware** -- All reusable labware (glass, polyethylene, Teflon, etc.) that comes in contact with chlorophyll solutions should be clean and acid free. An acceptable cleaning procedure is soaking for 4 h in laboratory grade detergent and water, rinsing with tap water, distilled deionized water and acetone.

6.9.1 Assorted Class A calibrated pipets.

6.9.2 Graduated cylinders, 500-mL and 1-L.

6.9.3 Volumetric flasks, Class A calibrated, 10-mL, 25-mL, 50-mL, 100-mL and 1-L capacity.

6.9.4 Glass rods or spatulas.

6.9.5 Pasteur Type pipets or medicine droppers.

6.9.6 Filtration apparatus consisting of 1 or 2-L filtration flask, 47-mm fritted glass disk base and a glass filter tower.

6.9.7 Centrifuge tubes, polypropylene or glass, 15-mL capacity with nonpigmented screw-caps.

6.9.8 Polyethylene squirt bottles.

6.9.9 Amber 2-mL HPLC autosampler vials with screw or clamp caps.

6.9.10 Glass syringe, 1 or 2-mL capacity.

6.9.11 HPLC compatible, low-volume, acetone resistant glass fiber or PTFE syringe filters.

6.10 Liquid Chromatograph

6.10.1 This method uses a ternary gradient thus requiring a programmable gradient pump with at least three pump inlets for the three different mobile phases required. A Dionex Model 4500 chromatograph equipped with a gradient pump, UV/VIS detector (cell path length, 6 mm, volume 9 µL) and PC data analysis (Dionex AI450 software, Version 3.32) system was used to generate data for this method. Tubing was made of polyether ether ketone (PEEK). A Dionex degas module was used to sparge all eluents with helium.

6.10.2 Helium or other inert gas for degassing the mobile phases OR other means of degassing such as sonication under vacuum.

6.10.3 Sample loops of various sizes (50-200 µL).

6.10.4 Guard Column -- A short column containing the same packing material as the analytical column placed before the analytical column to protect it from fouling by small particles. The guard column can be replaced periodically if it is noticed that system back pressure has increased over time.

6.10.5 Analytical Column -- A C_{18} reversed-phase column with end capping. A J.T. Baker 4.6 mm X 250 mm, 5 µm pore size column was used to generate the data in this method.

6.10.6 A visible wavelength detector with a low volume flow-through cell. Detection is at 440 nm.

6.10.7 A recorder, integrator or computer for recording detector response as a function of time.

6.10.8 Although not required, an autosampler (preferably refrigerated) is highly recommended.

7.0 Reagents and Standards

7.1 Acetone, HPLC grade, (CASRN 67-64-1).

7.2 Methanol, HPLC grade, (CASRN 67-56-1). Prepare ELUENT A, 80% (v/v) methanol/20% 0.5 M ammonium acetate, by adding 800 mL of methanol and 200 mL of the 0.5 M ammonium acetate (Sect. 7.5) to an eluent container.

7.3 Acetonitrile, HPLC grade, (CASRN 75-05-8). Prepare ELUENT B, 90% (v/v) acetonitrile/10% water, by adding 900 mL of acetonitrile and 100 mL of water (Sect. 7.7) to an eluent container.

7.4 Ethyl acetate, HPLC grade, (CASRN 141-78-6). ELUENT C, 100% ethyl acetate.

7.5 Ammonium acetate, ACS grade (CASRN 631-61-8). Prepare a 0.5 M solution by dissolving 38.54 g in approximately 600 mL of water in a 1-L volumetric flask. After the ammonium acetate has dissolved, dilute to volume with water.

7.6 Chl *a* free of chl *b* and chl *b* substantially free of chl *a* may be obtained from a commercial supplier such as Sigma Chemical (St. Louis, MO).

7.7 **Water** -- ASTM Type I water (ASTM D1193) is required. Suitable water may be obtained by passing distilled water through a mixed bed of anion and cation exchange resins.

7.8 **Aqueous Acetone Solution** -- 90% acetone/10% ASTM Type I water. Carefully measure 100 mL of the water into the 1-L graduated cylinder. Transfer to a 1-L flask or storage bottle. Measure 900 mL of acetone into the graduated cylinder and transfer to the flask or bottle containing the water. Mix, label and store.

7.9 **Chlorophyll Stock Standard Solution (SSS)** -- Chl *a* (MW = 893.5) and chl *b* (MW = 907.5) from a commercial supplier is shipped in amber glass ampules that have been flame sealed. The dry standards must be stored at -20 or -70°C in the dark. Tap the ampule until all the dried pigment is in the bottom of the ampule. In subdued light, carefully break the tip off the ampule. Transfer the entire contents of the ampule into a 25-mL volumetric flask. Dilute to volume with 90% acetone: (1 mg in 25 mL = 40 mg chl *a*/L) and (1 mg in 25 ml = 40 mg chl *b*/L), label the flasks and wrap with aluminum foil to protect from light. When stored in a light- and air-tight container at -20 or -70°C, the SSS is stable for at least six months. Dilutions of the SSS should always be confirmed spectrophotometrically using EPA Method 446.

7.10 **Laboratory Reagent Blank (LRB)** -- A blank filter that is extracted and analyzed just as a sample filter. The LRB should be the last filter extracted of a sample set. It is used to assess possible contamination of the reagents or apparatus.

7.11 **Quality Control Sample (QCS)** -- Since there are no commercially available QCSs, dilutions of a stock standard of a different lot number from that used to prepare calibration solutions may be used.

8.0 Sample Collection, Preservation and Storage

8.1 **Water Sample Collection** -- Water may be obtained by a pump or grab sampler. Data quality objectives will determine the depth and frequency[21] at which samples are taken. Healthy phytoplankton, however, are generally obtained from the photic zone (region in which the illumination level is 1% of surface illumination). Enough water should be collected to concentrate phytoplankton on at least three filters so that precision can be assessed. Filtration volume size will depend on the particulate load of the water. Four liters may be required for open ocean water where phytoplankton density is usually low, whereas 1 L or less is generally sufficient for lake, bay or estuary water. All apparatus should be clean and acid-free. Filtering should be performed in subdued light as soon as possible after sampling since algal populations, thus pigment concentrations, can change in relatively short periods of time. Aboard ship filtration is highly recommended.

Assemble the filtration apparatus and attach the vacuum source with vacuum gauge and regulator. Vacuum filtration should not exceed 6 in. Hg (20 kPa). Higher filtration pressures may damage cells and result in loss of chlorophyll. Care must be taken not to overload the filters. Do not increase the vacuum during filtration.

Prior to drawing a subsample from the water sample container, gently stir or invert the container several times to suspend the particles. Pour the subsample into a graduated cylinder and accurately measure the volume. Pour the subsample into the filter tower of the filtration apparatus and apply a vacuum (not to exceed 20 kPa). Typically, a sufficient volume has been filtered when a visible green or brown color is apparent on the filter. Do not suck the filter dry with the vacuum; instead slowly release the vacuum as the final volume approaches the level of the filter and completely release the vacuum as the last bit of water is pulled through the filter. Remove the filter from the fritted base with tweezers, fold once with the particulate matter inside, lightly blot the filter with a tissue to remove excess moisture and place it in the petri dish or other suitable container. If the filter will not be immediately extracted, wrap the container with aluminum foil to protect the phytoplankton from light and store the filter at -20°C or -70°C. Short term storage (2 to 4 h) on ice is acceptable, but samples should be stored at -20°C or -70°C as soon as possible.

8.2 Preservation -- Sampled filters should be stored frozen (-20°C or -70°C) in the dark until extraction.

8.3 Holding Time -- Filters can be stored frozen at -20°C for as long as 3½ weeks without significant loss of chl *a*.[20]

9.0 Quality Control

9.1 Each Laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the continued analysis of laboratory reagent blanks, field replicates, QCSs, and CALCHKs as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data generated.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance (IDLs) and laboratory performance (MDLs, extraction proficiency, and analyses of QCSs) prior to sample analyses.

9.2.2 Instrumental Detection Limit (IDL) -- After a low level calibration (Sect. 10), prepare a standard solution that upon injection into the chromatograph yields an absorbance of 0.002-0.010. If using an autosampler, variable volumes may be injected and the micrograms (µg) injected calculated by multiplying the known concentration (µg/µL) of the standard by the volume injected (µL). A practical starting point may be to inject 0.05 µg (that would be a 50 µL injection of a 1.0 mg/L standard solution) and reduce or increase the mass injected according to the resulting signal. Avoid injecting really small volumes (< 10 µL). After the quantity of pigment has been selected, make three injections and calculate the IDL by multiplying the standard deviation of the calculated mass by 3.

9.2.3 Method Detection Limit (MDL) -- At least seven natural phytoplankton samples known to contain the pigments of interest should be collected, extracted and analyzed according to the procedures in Sects. 8 and 11, using clean glassware and apparatus. Mass of the pigment injected into the chromatograph should be 2 to 5 times the IDL. Dilution of the sample extract solution to the appropriate concentration or reducing the volume of sample injected may be necessary. Calculate the MDL (in micrograms) as follows.[19]

$$MDL = (t) \times (S)$$

where, t = Student's t-value for n-1 degrees of freedom at the 99% confidence level. t = 3.143 for six degrees of freedom.

S = Standard deviation of the replicate analyses.

9.2.4 Quality Control Sample (QCS) -- When beginning to use this method, on a quarterly basis or as required to meet data quality needs, verify instrument performance with the analysis of a QCS (Sect. 7.11). If the determined value is not within $\pm$10% of the spectrophotometrically determined value, then the instrument should be recalibrated with fresh stock standard and the QCS reanalyzed. If the redetermined value is still unacceptable then the source of the problem must be identified and corrected before continuing analyses.

9.2.5 Extraction Proficiency -- Personnel performing this method for the first time should demonstrate proficiency in the extraction of sampled filters (Sect. 11.1). Fifteen to twenty natural samples should be obtained using the procedure outlined in Sect. 8.1 of this method. Sets of 10 samples or more should be extracted and analyzed according to Sect. 11. The percent relative standard deviation (%RSD) should not exceed 15% for samples that are at least 10X the IDL.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory Reagent Blank (LRB) -- The laboratory must analyze at least one blank filter with each sample batch. The LRB should be the last filter extracted. LRB data are used to assess contamination from the laboratory environment. LRB values that exceed the IDL indicate contamination from the laboratory environment. If the LRB value constitutes 10% or more of the analyte level determined in a sample, fresh samples or field replicates must be analyzed after the contamination has been corrected and acceptable LRB values have been obtained.

9.3.2 Calibration Check Standard (CALCHK) -- The laboratory must analyze one CALCHK for every ten samples to verify calibration. If the CALCHK is not ±10% of the spectrophotometrically determined concentration, then the instrument must be recalibrated.

10.0 Calibration and Standardization

10.1 Allow the visible wavelength detector (440 nm) to warm up for at least 15 min before calibration. Prepare ELUENTS A - C and degas by sparging with an inert gas for 10 minutes or sonicating under vacuum for 5 minutes. Prime the pump for each eluent taking care to remove all air that may be in the liquid lines. Equilibrate the column for ten minutes with 100% of ELUENT A.

10.2 Remove the SSS from the freezer and allow it to come to room temperature. Add 1 mL of the SSS to a 10-mL volumetric flask and dilute to 10 mL with 90% acetone. Prepare the chl *a* and *b* separately and determine the concentrations according to EPA Method 446 using the monochromatic equations for chl *a* determination. After the concentration of the SSS is determined, add 1 mL of the chl *a* SSS plus 1 mL of the chl *b* SSS to a separate 10-mL flask and dilute to volume. Store the calibration standard in a light tight glass bottle.

10.3 Program the pump with the following gradient:

Time	Flow	%1	%2	%3	Condition
0.0	1.0	100	0	0	Injection
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
20.0	1.0	0	31	69	Linear Gradient
22.0	1.0	0	100	0	Linear Gradient
25.0	1.0	100	0	0	Linear Gradient
30.0	1.0	100	0	0	Equilibration

Flow is in mL/min.

10.4 The first analysis is a blank 90% acetone solution followed by calibration. Calibrate with at least three concentrations, covering no more than one order of magnitude, and bracketing the concentrations of samples. If an autosampler is used, variable volumes ranging from 10 - 100% of the sample injection loop volume are injected to give a calibration of detector response versus mass of pigment. If doing manual injections, variable solution concentrations are made and a fixed sample loop volume is injected for standards and samples. Calibration can be either detector response versus mass or detector response versus concentration (mg/L or µg/L). Linearity across sensitivity settings of the detector must be confirmed if samples are analyzed at a different sensitivity settings from that of the calibration.

10.5 Construct a calibration curve of analyte response (area) versus concentration (mg/L in solution) or mass (µg) of pigment and perform a linear regression to determine the slope and y-intercept. A typical coefficient of determination is > 0.99.

10.6 Calibration must be performed at least weekly although it is not necessary to calibrate daily. Daily mid-point CALCHKs must yield calculated concentrations ±10% of the spectrophotometrically determined concentration.

11.0 Procedure

11.1 Extraction of Filter Samples

11.1.1 For convenience, a 10-mL final extraction volume is described in the following procedure. A smaller extraction volume may be used to improve detection limits.

11.1.2 If sampled filters have been frozen, remove them from the freezer but keep them in the dark. Set up the tissue grinder and have on hand laboratory tissues and wash bottles containing water and acetone. Workspace lighting should be the minimum that is necessary to read instructions and operate instrumentation. Remove a filter from its container and place it in the glass grinding tube. You may also tear the filter into smaller pieces and push them to the bottom of the tube with a glass rod. With a volumetric pipet, add 3 mL of the aqueous acetone solution (Sect. 7.6) to the grinding tube. Grind the filter until it has become a slurry. (**NOTE:** Although grinding is required, care must be taken not to overheat the sample. Good judgement and common sense will help you in deciding when the sample has been sufficiently macerated.) Pour the slurry into a 15-mL screw-cap centrifuge tube and, using a 7-mL volumetric pipet, rinse the pestle and the grinding tube with the aqueous acetone. Add the rinse to the centrifuge tube containing the filter slurry. Cap the tube and shake it vigorously. Place it in the dark before proceeding to the next filter extraction. Before placing another filter in the grinding tube, use the acetone and water squirt bottles to thoroughly rinse the pestle, grinding tube and glass rod. To reduce the volume of reagent grade solvents used for rinsing between extractions, thoroughly rinse the grinding tube and glass rod with tap water prior to a final rinse with ASTM Type I water and acetone. The last rinse should be with acetone. Use a clean tissue to remove any filter residue that adheres to the pestle or to the steel rod of the pestle. Proceed to the next filter and repeat the steps above. The last filter extracted should be a blank. The entire extraction with transferring and rinsing takes approximately 5 min. Approximately 500 mL of acetone and water waste are generated per 20 samples from the rinsing of glassware and apparatus.

11.1.3 Again, shake each tube vigorously before placing them to steep in the dark at 4°C. Samples should be allowed to steep for a minimum of 2 h but not to exceed 24 h. Tubes should be shaken at least once, preferably two to three times, during the steeping period to allow the extraction solution to have maximum contact with the filter slurry.

11.1.4 After steeping is complete, centrifuge samples for 15 min at 675 g or for 5 min at 1000 g. Draw approximately 1 mL into a glass syringe, attach a 0.45 µm syringe filter, filter the extract into an amber autosampler vial, cap and label the vial. Protect the filtered samples from light and heat. If using a refrigerated autosampler, chill to 10°C.

11.2 Sample Analysis

11.2.1 Draw into a clean syringe 2-3 times the injection loop volume and inject into the chromatograph. If using an autosampler, load the sample tray, prepare a schedule and begin analysis. A typical analyses order might be: (1) blank 90% acetone, (2) CALCHK, (3) 10 samples, (4) CALCHK, (5) QCS.

11.2.2 If the calculated CALCHK is not $\pm$10 of the spectrophotometrically determined concentration then recalibrate with fresh calibration solutions.

12.0 Data Analysis and Calculations

12.1 From the chl *a* or *b* area response of the sample, calculate the mass injected or concentration (C_E) of the solution that was analyzed using the calibration data. Mass injected must be converted to concentration in extract by dividing mass by volume injected (µL) and multiplying by 1000 to give concentration in mg/L (mg/L = µg/mL). Concentration of the natural water sample may be reported in mg/L by the following formula:

$$\frac{C_E \text{ X extract volume (L) X DF}}{\text{sample volume (L)}}$$

where:

C_E = concentration (mg/L) of pigment in extract.
DF = any dilution factors.
L = liters.

12.2 LRB and QCS data should be reported with each data set.

13.0 Method Performance

13.1 Single Laboratory Performance

13.1.1 An IDL was determined by preparing a mixed chl *a* (0.703 ppm) and chl *b* (0.437 ppm) standard. The injected mass yielded 0.004 AU for chl *a* (0.035 µg) and 0.003 AU for chl *b* (0.022 µg). Seven replicate 50 µL injections were made and the standard deviation of the calculated concentration was multiplied by three to determine an IDL. The IDL determined for chl *a* was 0.76 ng and 0.44 ng for chl *b*. The %RSDs for chl *a* and chl *b* was 0.45 and 0.67, respectively.

13.1.2 MDLs for chl *a* and chl *b* were determined by spiking seven replicate filtered samples of Pycnacoccus, extracting and processing according to this method. An

injection volume of 100 µL yielded an MDL for chl *a* of 7.0 ng and 4.0 ng for chl *b*. The RSDs were 5.1% for chl *b* and 4.7 % for chl *a*.

13.1.3 Recoveries of chl *a* and chl *b* from filtered samples of phaeodactylum were determined by spiking three filters with known amounts of the pigments, extracting, processing and analyzing the extraction solution according to the method, along with three unspiked filtered samples (to determine the native levels in the algae). The spiked levels were 1.1 ppm chl *a* and 0.53 ppm chl *b* in the 10 mL extraction volume. Chl *a* was 87% recovered and chl *b* was 94% recovered.

13.1.4 Figures 1-7 are chromatograms of seven reference unialga cultures processed according to this method.

13.1.5 Table 1 is a list of pure pigments with retention times obtained using this method. Purified pigments were prepared under contract to EPA by Moss Landing Marine Laboratory, Moss Landing, CA.

13.1.6 Table 2 contains single lab precision data for seven reference algal suspensions.

13.2 Multilaboratory Testing - A Multilaboratory validation and comparison study of EPA Methods 445.0, 446.0 and 447.0 for chlorophyll *a* was conducted in 1996 by Research Triangle Institute, Research Triangle Park, N.C. (EPA Contract No. 68-C5-0011). There were 8 volunteer participants in the HPLC methods component that returned data. The primary goals of the study were to determine estimated detection limits and to assess precision and bias (as percent recovery) for select unialgal species, and natural seawater.

13.2.1 The term, pooled estimated detection limit (p-EDL), is used in this method to distinguish it from the EPA defined method detection limit (MDL). The statistical approach used to determine the p-EDL was an adaptation of the Clayton, et. al.[21] method that does not assume constant error variances across concentration and controls for Type II error. The approach used involved calculating an estimated DL for each lab that had the desired Type I and Type II error rates (0.01 and 0.05, respectively). The median DLs over labs was then determined and is reported in Table 3. It is referred to as Pooled-EDL (p-EDL).

The p-EDL was determined in the following manner. Solutions of pure chlorophyll *a* in 90% acetone were prepared at three concentrations (0.11, 0.2 and 1.6 ppm) and shipped with blank glass fiber filters to participating laboratories. Analysts were instructed to spike the filters in duplicate with a given volume of solution and to process the spiked filters according to the method. The results from these data were used to determine a p-EDL for each method. Results (in ppm) are given in Table 3. The standard fluorometric and HPLC methods gave the lowest p-EDLs while the spectrophotometric (monochromatic equations) gave the highest p-EDLs.

13.2.2 To address precision and bias in chlorophyll *a* determination for different algal species, three pure unialgal cultures (Amphidinium, Dunaliella and Phaeodactylum) were cultured and grown in the laboratory. Four different "concentrations" of each species were prepared by filtering varying volumes of the algae. The filters were frozen and shipped to participant labs. Analysts were instructed to extract and analyze the filters according to the respective methods. The "true" concentration was assigned by taking the average of the HPLC results for the highest concentration algae sample since chlorophyll *a* is separated from other interfering pigments prior to determination . Pooled precision data are presented in Tables 4-6 and accuracy data (as percent recovery) are presented in Table 7. No significant differences in precision (%RSD) were observed across concentrations for any of the methods or species. It should be noted that there was considerable lab-to-lab variation (as exhibited by the min and max recoveries in Table 7) and in this case the median is a better measure of central tendency than the mean.

In summary, the mean and median concentrations determined for Amphidinium carterae (class dinophyceae) are similar for all methods. No method consistently exhibited high or low values relative to the other methods. The only concentration trend observed was that the spectrophotometric method-trichromatic equations (SP-T) showed a slight percent increase in recovery with increasing algae filtration volume.

For Dunaliella tertiolecti (class chlorophyceae) and Phaeodactylum tricornutum (class bacillariophyceae) there was generally good agreement between the fluorometric and the spectrophotometric methods, however, the HPLC method yielded lower recoveries with increasing algae filtration volume for both species. No definitive explanation can be offered at this time for this phenomenon. A possible explanation for the Phaeodactylum is that it contained significant amounts of chlorophyllide *a* which is determined as chlorophyll *a* in the fluorometric and spectrophotometric methods. The conventional fluorometric method (FL-STD) showed a slight decrease in chlorophyll *a* recovery with increasing Dunaliella filtration volume. The spectrophotometric-

trichromatic equations (SP-T) showed a slight increase in chlorophyll *a* recovery with incresing dunaliella filtration volume. The fluorometric and athe spectrophotometric methods both showed a slight decrease in chlorophyll *a* recovery with increasing Phaeodactylum filtration volume.

Results for the natural seawater sample are presented in Table 8. Only one filtration volume (100 mL) was provided in duplicate to Participant labs.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The USEPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation (e.g., Sect. 11.1.2). When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction*, available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036, (202) 872-4477.

15.0 Waste Management

15.1 The U.S. Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult *The Waste Management Manual for Laboratory Personnel*, available from the American Chemical Society at the address listed in the Sect. 14.2.

16.0 References

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

Table 1. Pure Pigments and Retention Times

PIGMENT	RETENTION TIME
19' butanoyloxyfucoxanthin	8.13
2,4-divinylpheoporphrin a_5	8.60
Peridinin	8.69
Fucoxanthin	8.75
19' hexanoyloxyfucoxanthin	8.90
Neoxanthin	10.07
Chlorophyll C_3	10.27
Chlorophyll C_2	10.40
Prasinoxanthin	11.20
Violaxanthin	12.00
Diadinoxanthin	15.20
Chlorophyll *b*	15.60
Myxoxanthophyll	17.00
Aphanaxanthin	17.20
Chlorophyll *a*	17.80
Monadoxanthin	17.93
Lutein	18.00
Alloxanthin	18.07
Nostaxanthin	18.70
Diatoxanthin	19.07
Zeaxanthin	19.40

Table 2. Single Lab Precision for Seven Pure Unialgal Cultures

Algae		Chlorophyll *a*	Chlorophyll *b*
Pycnacoccus provasolii	N[1]	3	3
	Mean (mg/L)[2]	2.15	1.47
	STD DEV	0.114	0.065
	% RSD	5.31	4.45
Rhodomonas salina	N[1]	3	3
	Mean (mg/L)	4.0	ND[3]
	STD DEV	0.014	ND
	% RSD	0.28	ND
Selenastrum capricornitum	N[1]	3	3
	Mean (mg/L)	4.25	0.483
	STD DEV	0.199	0.058
	% RSD	4.68	12.01
Amphidinium carterae	N[1]	3	3
	Mean (mg/L)	2.38	ND
	STD DEV	0.176	ND
	% RSD	7.40	ND
Dunaliella tertiolecti	N[1]	3	3
	Mean (mg/L)	6.68	1.42
	STD DEV	0.635	0.0412
	% RSD	9.51	2.90
Emiliania huxleyi	N[1]	3	ND
	Mean (mg/L)	1.03	ND
	STD DEV	0.008	ND
	% RSD	0.79	ND
Phaeodactylum tricornutum	N[1]	3	ND
	Mean (mg/L)	1.09	ND
	STD DEV	0.072	ND
	% RSD	7.07	ND

(1) N = Number of filtered samples.
(2) Mean concentration in extract solution.
(3) ND = none detected.

TABLE 3. POOLED ESTIMATED DETECTION LIMITS FOR CHLOROPHYLL *A* METHODS[1]

Method[2]	N[3]	p-EDL[4] (mg/L)
FL -Mod[5]	8	0.096
FL - Std	9	0.082
HPLC	4	0.081
SP-M	15	0.229
SP-T	15	0.104

(1) See Section 13.2.1 for a description of the statistical approach used to determine p-EDLs.

(2) FL-Mod = fluorometric method using special interference filters.

FL-Std = conventional fluorometric method with pheophytin *a* correction.

HPLC = EPA method 447.0

SP-M = EPA method 446.0, monochromatic equation.

SP-T = EPA method 446.0, trichromatic equations.

(3) N = number of labs whose data was used.

(4) The p-EDL was determined with p = 0.01 and q (type II error rate) = 0.05.

(5) Due to the large dilutions required to analyze the solutions by fluorometry, the fluormetric p-EDLs are unrealistically high.

TABLE 4. Measured Chlorophyll *a* (mg/L) in Dunaliella Samples

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
HPLC	5	5	0.172	0.064	36.8
	10	5	0.276	0.074	26.8
	50	5	0.757	0.344	45.4
	100	5	1.420	0.672	47.3

(1) Not all participants labs followed the EPA method exactly.

(2) N = Number of volunteer labs whose data was used.

TABLE 5. Measured Chlorophyll *a* (mg/L) in Amphidinium Samples

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
HPLC	5	5	0.104	0.043	56.8
	10	5	0.172	0.083	37.5
	50	5	0.743	0.213	17.4
	100	5	1.394	0.631	14.5

(1) Not all participants labs followed the EPA method exactly.
(2) N = number of volunteer labs whose data was used.

TABLE 6. Measured Chlorophyll *a* in Phaeodactylum Samples

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
HPLC	5	5	0.193	0.074	38.4
	10	5	0.317	0.114	36.1
	50	5	1.024	0.340	33.2
	100	5	1.525	0.487	29.9

(1) Not all participants labs followed the EPA method exactly.
(2) N = number of volunteer labs whose data was used.

TABLE 7. Minimum, Median, and Maximum Percent Recoveries by Genera, Method, and Concentration Level

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
Amphidinium	Minimum	FL-MOD	70	73	75	76
		FL-STD	66	91	91	90
		HPLC	82	85	87	88
		SP-M	36	48	68	64
		SP-T	21	63	71	70
	Median	FL-MOD	105	112	105	104
		FL-STD	109	107	111	109
		HPLC	102	106	112	105
		SP-M	99	101	101	101
		SP-T	95	96	106	107
	Maximum	FL-MOD	121	126	143	146
		FL-STD	156	154	148	148
		HPLC	284	210	131	116
		SP-M	141	133	126	125
		SP-T	115	116	119	117
Dunaliella	Minimum	FL-MOD	162	159	157	156
		FL-STD	179	171	165	164
		HPLC	165	109	64	41
		SP-M	120	188	167	164
		SP-T	167	169	166	165
	Median	FL-MOD	206	246	227	223
		FL-STD	250	228	224	210
		HPLC	252	177	89	80
		SP-M	240	247	247	243

Table 7. Cont'd.

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
		SP-T	225	244	256	256
Dunaliella	Maximum	FL-MOD	295	277	287	288
		FL-STD	439	385	276	261
		HPLC	392	273	172	154
		SP-M	342	316	296	293
		SP-T	291	283	283	283
Phaeodactylum	Minimum	FL-MOD	216	183	157	154
		FL-STD	189	220	223	219
		HPLC	150	119	84	75
		SP-M	161	138	156	160
		SP-T	203	195	216	244
	Median	FL-MOD	292	285	250	245
		FL-STD	296	263	254	254
		HPLC	225	203	114	90
		SP-M	287	274	254	253
		SP-T	286	281	277	274
	Maximum	FL-MOD	357	337	320	318

Table 8. Chlorophyll *a* Concentrations in mg/L Determined in Filtered Seawater Samples

Method	Con.(1)	No. Obs	No. Labs	Mean	Std. Dev	RSD (%)	Minimum	Median	Maximum
FL-MOD	100	14	7	1.418	0.425	30.0	0.675	1.455	2.060
FL-STD	100	15	8	1.576	0.237	15.0	1.151	1.541	1.977
HPLC	100	10	5	1.384	0.213	15.4	1.080	1.410	1.680
SP-M	100	38	19	1.499	0.219	14.6	0.945	1.533	1.922
SP-T	100	36	18	1.636	0.160	9.8	1.250	1.650	1.948
All Methods	100	113	57	1.533	0.251	16.4	0.657	1.579	2.060

(1) Con = mLs of seawater filtered.