Standard Operating Procedure for:

Spectrophotometric Determination of Corrected and Uncorrected Chlorophyll *a* and Pheophytin

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1. SCOPE AND APPLICATION

1.1. This method is based on Standard Methods 10200H and EPA Method 446.0. This method is used to determine the amount of chlorophyll *a* and pheophytin *a* in marine and freshwater algae by visible spectrophotometry. Uncorrected chlorophyll *a* is calculated using the trichromatic equation. Corrected chlorophyll *a* and pheophytin are calculated using the monochromatic equation. The absorption-peak-ratio (chlorophyll/pheophytin) is also determined.

1.2. This procedure is used for samples with LIMS test IDs of CHL-CORR-W and CHLSUITE-W.

1.3. Method Detection Limits (MDL) at 500 mL filter volume with 8 mL extract volume.

1.3.1. Chlorophyll *a* corrected for pheophytin is 0.55 µg/L.

1.3.2. Uncorrected chlorophyll *a* is 0.40 µg/L.

1.3.3. Pheophytin *a* is 0.40 µg/L.

1.3.4. The practical quantitation limit (PQL) is calculated at 3 times the MDL.

2. SUMMARY OF METHOD

2.1. A sample is vacuum filtered onto a glass fiber filter. The filter is then macerated with a tissue grinder and steeped in 90% acetone to extract chlorophyll from the algal cells. The sample is clarified through centrifugation. The absorbance of the clarified extract is then measured on a spectrophotometer at 750,665,664,647 and 630 nm wavelengths before and after a 90 second HCl acidification step.

2.2. Interferences

2.2.1. Chlorophyll pigment is sensitive to heat and light. To avoid degradation, extraction and spectrophotometer readings are performed in a dark room using subdued lighting only. The grinding tube is kept on ice to minimize heat from friction during extraction. Also, chlorophyll samples are stored in the freezer after filtration.

2.2.2. Any compound that absorbs light between 630 and 665 nm may interfere with chlorophyll measurement. The absorbance measurement at 750 nm is subtracted from the sample's other measured absorbance values (665, 664, 647, and 630 nm) to account for the turbidity of the clarified sample. If the absorbance at 750 nm is greater than 0.005 AU, recentrifuge the sample to further clarify the extract.

2.2.3. The spectral overlap of chlorophyll *a*, *b*, *c* and pheophytin can cause over or underestimation of chlorophyll and/or pheophytin. The amount of chlorophyll *b* and *c* in a sample is dependent on the taxonomic composition of the

phytoplankton it contains. In the trichromatic equation, chlorophyll *a* may be overestimated in the presence of pheophytin. In the monochromatic equation chlorophyll *a* may be slightly overestimated in the presence of chlorophyll *b* and pheophytin may be overestimated in the presence of carotenoids.

2.3. Definitions

2.3.1. Subdued Lighting – Only red bulbs are used in subdued lighting. All other lights are turned off. This helps minimize chlorophyll degradation from exposure to light.

2.3.2. Absorbance – A measurement of the amount of light at a specific wavelength absorbed by a liquid. Although, absorbance has no true units, AU (Absorbance Units) is used for clarity in this SOP.

2.3.3. Path length – The path length is the width of the cuvette cell (length between optical non-frosted sides). For this method, 1 and 4 cm path length cuvettes are used.

2.3.4. Trichromatic equation – Also known as, Jeffrey and Humphrey's Trichromatic Equations, they require absorbance values at 664, 647, and 630 nm to calculate the amount of uncorrected chlorophyll *a* in a sample. Chlorophyll *b* and *c* pigments can also be calculated. No acidification is required and pheophytin cannot be calculated from this equation.

2.3.5. Monochromatic equation – Also known as Lorenzen's modified monochromatic equation, it requires the absorbance values of 664 and 665 nm before and after a 90 second acidification step to calculate the amount of chlorophyll *a* and pheophytin in a sample. The chlorophyll *a* is reported as corrected for pheophytin. Chlorophyll *b* and *c* cannot be calculated from this equation.

3. EQUIPMENT AND SUPPLIES

3.1. Filtration

3.1.1. Vacuum filtration system (See Figure 12.1)

3.1.2. Whatman GF/C 55mm Glass Fiber Filters

3.1.3. Small roll of aluminum foil, cut in 3 inch squares

3.1.4. 500 mL graduated cylinder

3.1.5. Extra fine tip black marker

3.1.6. 2 Rinse bottles (1 small, 1 large)

3.2. Extraction

3.2.1. BD Falcon 15 mL Centrifuge tubes

3.2.2. Test tube rack

3.2.3. Scissors

3.2.4. Eberbach Tissue Grinder motor

3.2.5. Wheaton Tissue Grinder with 30 mL tube

3.2.6. Small container with ice

3.2.7. Rinse bottle

3.2.8. Fume hood in dark room

3.3. Spectrophotometric Analysis

3.3.1. Perkin Elmer Lambda 35 UV/Vis Spectrophotometer

3.3.2. 4 cm path length UV quartz glass cuvette

3.3.3. 1 cm path length UV quartz glass cuvette

3.3.4. Mini cuvette stirrer

3.3.5. PC with Perkin Elmer software

3.3.6. Small beaker

3.3.7. Finpipette 1-5 mL pipette with tips

3.3.8. Hamilton 100 µL pipette and tips

4. REAGENTS AND STANDARDS

4.1. Deionized Water

4.2. Acetone, HPLC grade

4.3. Saturated Magnesium Carbonate Solution

4.3.1. Add 10 g of $MgCO_3$ (ACS grade) to 1 L of deionized water. Record preparation in Reagent Solutions Log.

4.4. Aqueous Acetone Solution with Magnesium Carbonate

4.4.1. Add 400 mL of filtered saturated magnesium carbonate solution to 3600 mL of Acetone (HPLC grade). Record preparation in Reagent Solutions Log. Store in Biology lab refrigerator. May keep 1 L at a time at room temperature in the chlorophyll room for daily use.

4.5. Aqueous Acetone Solution (90% acetone)

4.5.1. Add 900 mL of Acetone (HPLC grade) to 100 mL of deionized water. Record preparation in reagent solutions log.

4.6. 0.1 N Hydrochloric Acid

4.6.1. Add 0.85 mL of concentrated HCl (ACS grade) to 100 mL of deionized water. Record in reagent solutions log.

4.7. Sigma C5753 Chlorophyll *a* from spinach, 5 mg

4.8. Chlorophyll *a* Stock Standard

4.8.1. Prepare under subdued lighting. Tap the ampoule of chlorophyll *a* spinach until all the powder is settled to the bottom. Break open the tip and empty contents into a Class A 25 mL volumetric flask containing about 10 mL of 90% acetone. If necessary, rinse ampoule thoroughly with 90% acetone into the volumetric flask. Dilute to volume with 90% acetone. Add a small stir bar and mix thoroughly. Transfer standard to a dark glass bottle and store in Biology lab freezer.

Note: Be careful when preparing stock solution. The entire contents of the spinach ampoule must be emptied into the flask. Any spill can alter the concentration of the stock solution.

4.8.2. Record preparation in LIMS Standard Preparation Tracker, print label and attach to bottle.

4.8.3. Concentration of stock standard is 200 mg/L. The holding time is 9 months from the date of preparation.

4.9. Chlorophyll *a* Working Standard

4.9.1. In subdued lighting, add 0.20 mL of chlorophyll stock standard to a 200 mL Class A graduated cylinder. Bring up to volume with 90% Acetone. Transfer to a dark glass bottle. Store in biology lab freezer.

4.9.2. Record preparation in LIMS Standard Preparation Tracker, print label and attach to bottle.

4.9.3. Concentration of working standard is 0.20 mg/L. The holding time is 3 months from the date of preparation.

5. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

5.1. See FDEP Field SOP FS 1000 for sample collection and preservation.

6. SAMPLE PREPARATION

6.1. Refer to SOP BB-030 for sample custody, labels and worksheet instructions.

6.2. Chlorophyll Filtration

6.2.1. Shake sample bottle to ensure sample is thoroughly mixed. Use a graduated cylinder to measure 500 mL of sample. Filter through a GF/C glass fiber filter. If sample is highly turbid and difficult to filter do not filter the entire 500 mL volume. A smaller volume may be used as long as the filter is sufficiently loaded with algae. Add 2 mL of $MgCO_3$ suspension when there is 25-50 mL of sample left to filter through the funnel.

6.2.2. Once the sample is completely filtered, rinse the funnel with deionized water (about 10-20 mL). When nearly dry remove and fold the filter in half with the algae facing in, using the filtrate edge as a guide (not the filter edge). Then, fold the filter in half again while lining up the filtrate edge. Wrap the folded filter in aluminum foil. Place the "A" prep label on the foil and write the filter volume on it.

6.2.3. If there is a "B" label for a sample, repeat steps 6.2.1 and 6.2.2 for the "B" label. Use equal volumes for "A" and "B" aliquots of replicate samples.

6.2.4. Repeat steps 6.2.1.-6.2.3 for each sample.

6.2.5. When all samples are complete, record filter volumes, analysis date/time, and analyst on prep worksheet. Rubber band the samples together and place in a plastic bag. Label the plastic bag with the date and store in the biology lab freezer. Holding time for frozen samples is 28 days from time of filtration until extraction. At the bottom of the prep worksheet record the time that the samples are placed in the freezer.

6.2.6. Discard unused sample in the sink unless it is suspected of being hazardous waste. If suspected of being hazardous waste, notify the safety officer and supervisor for proper disposal of the sample. Rinse sample containers with water and arrange them on a cart with the filter date visible for storage purposes.

6.3. Chlorophyll Batch Preparation

6.3.1. Using the prep worksheet, create batches of 20 samples each for analysis, by drawing a line between every 20 samples. Combine worksheets from different days as necessary. In the margin, number batches in a repeating sequence of 1-10.

6.3.1.1. If there are priority 1 samples, place them in the first available batch. Otherwise, create batches in the order they appear in prep worksheets.

6.3.2. Copy the prep worksheet batches into the Chlorophyll Run Log.

6.3.2.1. Write the batch number at the top of the Run Log page.

6.3.2.2. Write "BLK" in the column for Field ID for Tube #1.

6.3.2.3. Write the Sample IDs in the Sample # column for Tube #2-21.

6.3.2.4. Write "dup" in the margin for Tube #22. Complete this row after extracting a "B" sample.

6.3.2.5. Enter filter volumes in the column for w/p#. Fill in the column for Job IDs.

6.3.2.6. Write "B" volumes under Comments in the corresponding row (i.e. B=500).

6.3.3. Using the Chlorophyll Run Log, retrieve corresponding samples from the freezer to create packets for extraction.

6.3.3.1. For each batch, place a laboratory "A" blank at the front, then "A" samples, followed by any "B" samples from that batch. Wrap in foil and label the with batch number.

7. SAMPLE ANALYSIS

7.1. Extraction

7.1.1. Retrieve the chlorophyll packet associated with the first available batch in the Chlorophyll Run Log from the biology lab freezer. Record date/time/analyst in the Chlorophyll Run Log.

7.1.2. In subdued lighting, unwrap sample filter from foil and make sure sample label information matches the Chlorophyll Run Log. Trim the filter above the filtrate line to remove excess filter (See Figure 12.2). To avoid losing sample, do not cut too close to the filtrate line. Roll up filter and place in the grinding tube. Add 2-3 mL of aqueous acetone-$MgCO_3$ solution.

7.1.3. Grind the filter long enough to convert it to a slurry without allowing the sample to overheat. This may take up to 1 minute. Grooves may be cut into the bottom of the pestle to facilitate grinding. During extraction keep the grinding tube in ice water when not in use to minimize overheating from friction. Rinse the pestle and the walls of the tube with 1-2 mL of aqueous acetone-$MgCO_3$ solution.

7.1.4. Transfer mixture to the corresponding numbered centrifuge tube. Use aqueous acetone-$MgCO_3$ solution to rinse any remaining sample in the grinding tube into the centrifuge tube. Bring up to 8 mL volume and cap. If rinsing makes tube volume greater than 8 mL, bring final volume up to the next available whole number (a maximum of 14 mL). Record acetone volume in the Chlorophyll Run Log.

7.1.5. Repeat steps 7.1.2-7.1.4 for each sample.

7.1.6. Cover centrifuge tube rack to avoid light exposure. Allow samples to steep at 4 °C for a minimum of 2 hours. Do not exceed 24 hours.

7.2. Instrument Setup

7.2.1. Turn on the spectrophotometer and let warm up for at least 1 hour. After warm up, turn spec off and on again. Allow about 5 minutes for the spec to re-run initial tests with warm lamps before opening the computer program.

7.2.2. Turn the computer on, open the Perkin Elmer program. Select analyst name from the drop-down menu and click **OK**.

7.2.3. Select the "Chlorophyll Suite" method in the shortcut or method list.

7.2.4. Fill in the sample table in the Perkin Elmer program with the correct Sample ID, Volume Filtered, and Volume Extracted information.

7.2.5. Click the **Start** button.

7.2.6. Fill the reference and sample cuvettes with 90% Acetone. The reference cuvette (1 cm) should be completely filled and capped. The sample cuvette (4 cm) should be at least 2/3 full and does not need to be capped. Wipe the optical sides of the cuvettes with a Kim-wipe. Place in the machine and click **OK** to perform an auto zero. After this, the program will be ready for the first sample. The reference cuvette stays in the spec compartment throughout the entire analysis.

7.3. Spectrophotometric Analysis

7.3.1. Centrifuge samples for 15 minutes at 2500 rpm. Decant supernatant from each sample into a clean centrifuge tube with matching tube number.

7.3.2. When the spectrophotometer prompts for "Measurement 1" of a sample, add 3 mL of the sample to the cuvette. Place in the spectrophotometer and close the compartment door. Click **OK** to begin reading.

7.3.3. If the absorbance at 664 nm is 0.007 AU or less, do not acidify the sample. When the program prompts for "Measurement 2" Click **OK** and skip to Step 7.3.6.

7.3.4. If the absorbance at 664 nm is above 0.007 AU but less than 1.5 AU, add 0.1 mL of 0.1 N HCl and stir with the mini-mixer. Wait 90 seconds then click **OK**. Skip to Step 7.3.6.

Note: Be careful not to touch the optical sides of the cuvette with the stirrer because this will scratch the cell and affect absorbance readings.

7.3.5. If the absorbance at 664 nm is greater than or equal to 1.5 AU, the sample must be diluted. Click **OK**. When the spectrophotometer is done reading, click **Cancel**. Highlight the row below the sample and click **Insert**. Fill in the new row with the correct information. Note the dilution in the *Chlorophyll Run Log*. Click the **Start** button and run diluted sample as prompted (Return to Step 7.3.2.).

7.3.5.1. If reading is less than or equal to 2.5, make a 50% dilution, by adding 3 mL of sample and 3 mL of 90% aqueous acetone solution. The new volume filtered is 50% of the original sample.

7.3.5.2. If reading is greater than 2.5, make a 25% solution, by adding 1 mL of sample and 3 mL of 90% aqueous acetone. The new volume filtered is 25% of the original sample.

7.3.6. When the spectrophotometer is finished reading, it will prompt for "Measurement 1" of the next sample. Remove the sample cell and empty it into the waste flask. If acidified, rinse the cuvette with 100% Acetone.

7.3.7. Repeat Steps 7.3.2.-7.3.6 until all samples (including blanks and standards) have been read.

8. DATA ARCHIVAL

8.1. Save the file by selecting **File/Save As/Task** from the menu. Collect the sample table printout from the wet lab printer. Also, write the Run ID in the *Chlorophyll Run Log*.

8.1.1.1. Name the file with the Run ID. The format is YYYY-MM-DD-A. The last character is an A for the first run of the day, B for the next run and so forth.

8.1.1.2. Saving the task will automatically save an output data file in the proper directory and print a copy of the sample table.

8.2. Refer to SOP BB-031 for QC Manager data uploading and verification instructions. Output data files are named "Sample Replicates Table.csv" in the Run ID folder and are located in the following directory: \\Tlhlab3\Biology\Chlorophyll\PerkinElmer. Use extract date/time as analysis time in QCM.

8.3. After upload and verification, refer to SOP BB-032 for LIMS uploading and data authorization.

8.4. Calculations for Phytoplankton samples:

Pheophytin-corrected chlorophyll (µg/L or mg/m^3):

$$\text{Chlorophyll } a \text{ corrected (mg/m}^3) = \frac{26.7(664_B - 665_A) \times V_1}{V_2 \times L}$$

Pheophytin (µg/L or mg/m^3):

$$\text{Pheophytin } a \text{ (mg/m}^3) = \frac{26.7[1.7(665_A) - 664_B] \times V_1}{V_2 \times L}$$

Uncorrected chlorophyll (µg/L or mg/m^3):

$$\text{Chlorophyll } a \text{ uncorrected (mg/m}^3) = \frac{[11.85(664_B) - 1.54(647_B) - 0.08(630_B)] \times V_1}{V_2 \times L}$$

Chlorophyll/Pheophytin Ratio:

Absorption peak ratio: $664_B / 665_A$

where: 664_B = Subtract 750 nm value (turbidity correction) from absorbance at 664 nm before acidification
665_A = turbidity-corrected absorbance at 665 nm after acidification
647_B = turbidity corrected absorbance at 647 nm before acidification
630_B = turbidity corrected absorbance at 630 nm before acidification
V_1 = volume of extractant (mL)
V_2 = volume of sample filtered (L)
L = path length (cm)

9. QUALITY CONTROL

9.1. Negative Control

9.1.1. Filter 500 mL of deionized water as a laboratory blank after every 20 samples filtered.

9.1.2. Analyze the laboratory blank at the beginning of each batch analyzed.

9.1.3. The acceptance limit is ≤ 0.007 AU at all wavelengths.

9.1.4. If a blank failure occurs at the start of a batch, re-zero the spectrophotometer and reread the blank before continuing. If this does not correct the problem, run a 90% acetone sample to help determine whether the problem is caused by a spectrophotometer malfunction or blank contamination.

9.2. Positive Control

9.2.1. As a laboratory control sample (LCS), analyze the chlorophyll *a* working standard at the beginning of every batch during spectrophotometric analysis.

9.2.2. Record the working standard serial number at the top of the Run Log page.

9.2.3. Calculate percent recovery of LCS as follows:

$$\%\,\mathrm{Rec} = \frac{26.7\left(664_B - 665_A\right)}{4 \times 0.2} \times 100\%$$

9.2.4. The acceptance limit for recovery is 85-115%.

9.2.5. As a quick check of the standard during sample analysis, the difference between 664_B and 665_A should be between 0.025-0.034 AU to pass recovery limits. If the difference exceeds these limits, re-zero the spec and re-run the blank and standard.

9.2.6. If standard has repeated low failures, it is possible that the standard was exposed to heat or light over time and has degraded. Make a new batch of working standard.

9.3. Sample Duplicates

9.3.1. Analyze a laboratory replicate for 5% of samples. The acceptance limit is $\leq$25% RPD.

9.3.2. If absorbencies for sample duplicates do not appear similar, re-run both duplicates at the end of the batch.

9.4. Refer to Quality Manual for method performance, corrective actions and handling out-of-control data.

10. SAFETY/HAZARDOUS WASTE MANAGEMENT

10.1. Do not pour acetone or any reagent/standard containing acetone down the sink. Pour any waste acetone in the waste flask located in the chlorophyll room sink. When this is full, empty the flask into an unfilled acetone bottle and label it with "Hazardous Waste", waste content, amount, date generated and initials. Follow the Hazardous Waste Management manual to dispose of full waste acetone bottles.

10.2. Refer to the Laboratory Safety Manual and Hazardous Waste Management.

10.3. Read MSDS information for reagent handling and disposal.

11. REFERENCES

11.1. Standard Methods Online Edition SM10200H(2001)

11.2. EPA Method 446.0, Revision 1.2

11.3. Perkin Elmer Lambda 35 UV/Vis Spectrophotometer Manual

11.4. NELAC Quality Systems Manual

11.5. FDEP Biology Section Quality Manual
http://www.floridadep.org/labs/library/progplan.htm#qamp

11.6. Laboratory Safety Manual http://depnet/burlabs/safety.htm

11.7. Hazardous Waste Management Manual http://depnet/burlabs/safety.htm

12. TABLES, DIAGRAMS

Figure 12.1. Vacuum Filtration System

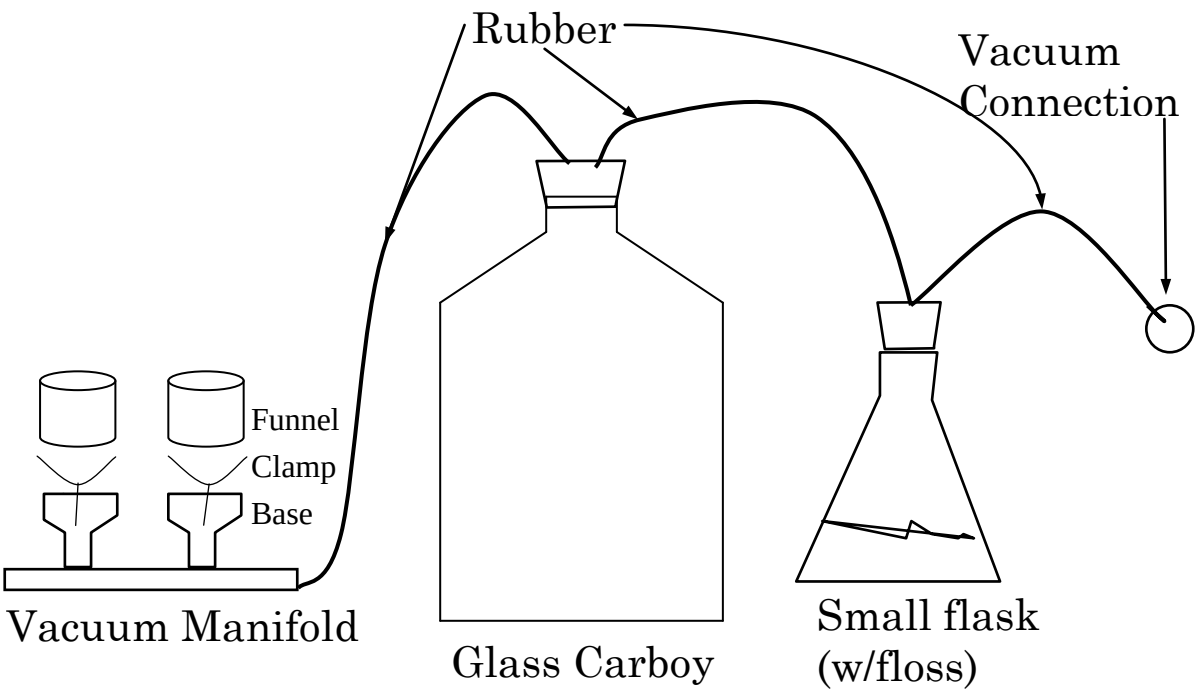
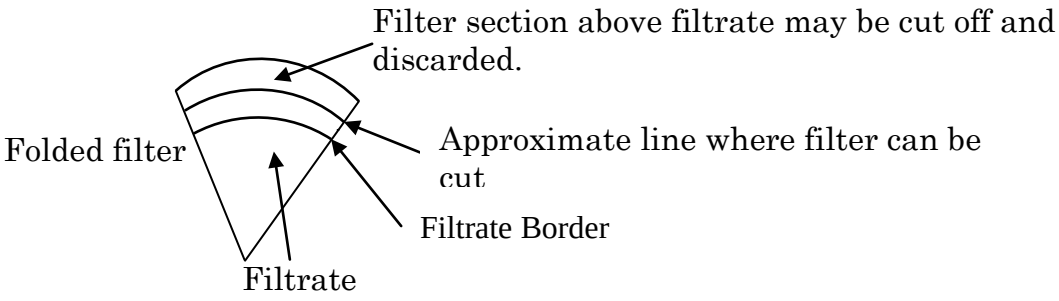


Figure 12.2 Filter trimming for extraction



Appendix of Changes

1/16/2009 Updated SOP by editing sections 6.2.6 and 9.2.2. cf
1/13/2010 Updated MDL section 1.3. Updated laboratory blank information (one blk/20 samples instead of historical 2 blks). Added steps to record filter freeze time and bottle rinsing procedure. cf