

**STANDARD OPERATING PROCEDURE  
FOR THE DETERMINATION OF CHLOROPHYLLS AND RELATED PIGMENTS  
BY HPLC**

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**NELAC CERTIFICATION # E46077**

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## 1.0 Identification of Test Method

- 1.1 This is the Standard Operating Procedure for the determination of chlorophylls and related pigments in the laboratory of the South Florida Water Management District using high pressure liquid chromatography (HPLC). It is identified by the method number SFWMD-LAB-SOP-3180-001.
- 1.2 This method is based on EPA Method 447.0 (modified) and Van Heukelem & Thomas (2001) J. Chromatogr. A. 910:31-49
- 1.3 Principal modifications of this method from EPA Method 447.0 are the eluent composition and the column type. This method utilizes a binary gradient composed of tetrabutylammonium acetate and methanol and a C8 column as the stationary phase as per Van Heukelem & Thomas (2001) J. Chromatogr. A. 910:31-49. Additionally, a photodiode array (PDA) detector is used rather than a fixed wavelength detector so that the entire absorption spectrum of each peak can be recorded and compared to reference spectrum stored in the system software. The method is optimized for fast elution of Chlorophyll a, b and Pheophytin a and the chromatogram is recorded at 665 nm as absorption at this wavelength is limited to the compounds of interest. A typical chromatogram is shown in section 24.3 and reference spectra in section 24.4. The chromatographic conditions are shown in section 14.11.

## 2.0 Applicable Matrices

- 2.1 This method can be applied to the determination of chlorophyll a, chlorophyll b and pheophytin a in surface, rain, saline and ground waters collected within the South Florida Water Management District.

## 3.0 Detection Limit

- 3.1 The detection limit for this method was determined using seven replicate extractions of a typical surface water sample. Analysis was performed on dilutions of the extracts at 3 to 5 times the expected detection limit.
- 3.2 The method detection limits (MDL's) for this method were determined on October 6, 2010, and are shown in the table below.

Pigment	MDL( $\mu\text{g/l}$ )	PQL ( $\mu\text{g/l}$ )
Chlorophyll a	3	12
Chlorophyll b	5	20
Pheophytin a	9	36

To achieve the required sensitivity for this method, the procedure includes steps for extraction and concentration of the pigments. The reportable Chlorophyll pigment

concentrations and DL's of a given sample are calculated automatically in LIMS after data review using the following calculation:

$$\underline{RPC(\mu\text{g/l}) = AR \times DF \times CF}$$

$$\underline{RDL(\mu\text{g/l}) = DL \times DF \times CF}$$

Where;

RPC = Reportable Pigment Concentration

RDL = Reportable Detection Limit

AR = Instrument Analytical Result

DF = Instrument required Dilution Factor

CF = Concentration Factor (Extract volume / Initial Sample Volume)

- 3.3 A narrative file, along with the individual values and calculations for the determination of the method detection limits are kept on file in the office of the Laboratory Quality Assurance Officer.

#### **4.0 Scope and Application**

- 4.1 This method is based on EPA Method 447.0 and is applicable to surface, ground, and saline waters.
- 4.2 The concentration of chlorophyll is used extensively to estimate phytoplankton biomass, with chlorophyll *a* comprising 1-2% of the algal dry weight. Other pigments that occur in phytoplankton algae are chlorophyll *b* and *c*, xanthophylls, phycobilins and carotenes.
- 4.3 The algae are removed from water by filtration with a glass fiber filter, and then the pigments are extracted with an aqueous acetone solution. To achieve consistent complete extraction of the pigments the cells are disrupted using sonication or grinding.
- 4.4 Chlorophyll *a* is the dominant type of chlorophyll in the algae most commonly found in surface waters. Pheophytin is a breakdown product of chlorophyll and the ratio of chlorophyll to pheophytin provides information of the health of the algal population. During rapid growth, the proportion of pheophytin is low. During periods of decline, such as follows prolonged cloudy weather or exposure of the algae to toxic substances, the proportion of pheophytin is high.

#### **5.0 Summary of Test Method**

- 5.1 A well-mixed sample is filtered through a glass fiber filter. Pigments are extracted from the retained residue with an aqueous acetone solution and concentrations of various

chlorophylls and related pheopigments in the extract solution are determined using liquid chromatography. An appropriate concentration factor is determined for each sample based on the amount of sample filtered and the final extract volume. This concentration factor is applied to the concentrations determined in the extract to compute the pigment concentrations in the original sample.

- 5.2 The working range is up to the concentration of the highest standard for each pigment in the sample extract. The sample extract must be diluted if the concentration is greater than the highest standard for a given pigment. A 50  $\mu$ L injection volume is used for all samples and standards.

## **6.0 Definitions**

- 6.1 A comprehensive listing of terms and definitions in common use in the SFWMD laboratory can be found in the Laboratory Quality Manual glossary. All acronyms are defined upon first use in this document. Only terms specific to this procedure are defined in this section.

## **7.0 Interferences**

- 7.1 Conduct work with chlorophyll extracts in subdued light to avoid degradation. Light and heat cause the chlorophylls and related pigments to degrade and may produce inaccurate results. Use opaque containers or wrap with aluminum foil. The samples should be filtered as quickly as possible in order to minimize the time that samples are exposed to light and room temperature.

## **8.0 Safety**

- 8.1 Wear safety glasses and a full-length, long-sleeved laboratory coat.
- 8.2 Use acetone-resistant gloves during extraction and analysis.
- 8.3 All personnel conducting this method should be familiar with the SFWMD Chemical Hygiene Plan and should have reviewed Material Safety Data Sheets for all reagents and standards utilized in this method.
- 8.4 Avoid contact with skin and eyes while handling samples, sample extracts, and reagents.
- 8.5 Discard acetone waste into the assigned waste drum. Do not discard any acetone waste into the sink drains.
- 8.6 The electrical power should be disconnected before conducting any repairs inside the instrument on controllers, electrical wiring or any other components near sources of electricity.

8.7 Hearing protection is required while using the ultrasonic disruptor to extract pigments from sample filters.

## **9.0 Equipment and Supplies**

9.1 HPLC System including UV/VIS diode array detector (Shimadzu Prominence), Model LC-20AT.

9.2 Chromatography data system (Shimadzu LC Solution)/ 1.245 P1

9.3 C8 HPLC Column (Kromasil 100-3.5C8, 150 x 4.6 mm)

9.4 Centrifuge, temperature controlled, Eppendorf Model # 5810R

9.5 Mechanical Tissue Grinder with Teflon tip

9.6 Vacuum filtration assembly

9.7 Magnetic filter funnel

9.8 Graduated cylinder, 100-1000 mL capacity

9.9 Pipettes, glass class A

9.10 Eppendorf Reference pipette, 100  $\mu$ L

9.11 Glass Fiber filters, Whatman Type GF/F filter, 47mm, (or equivalent)

9.12 Forceps

9.13 Aluminum foil

9.14 .045  $\mu$ m filters compatible with the extraction solvent

9.15 Water-proof marker

9.16 Plastic transfer pipettes, disposable

9.17 Wet ice

9.18 Amber volumetric flasks, class A, 100 mL

9.19 Misionix Sonicator 3000 sonication apparatus with a microtip probe #420

9.20 Sarstedt tubes, item #62.515.006 (or equivalent)

9.21 Sonication probe holder

9.22 Hamilton Gastight syringes, 50,100, 200,250  $\mu$ L

## 10.0 Reagents and Standards

- 10.1 **Saturated Magnesium Carbonate Solution** - prepared from Fisher Scientific Cat. No M29-500 or equivalent. Add 1 g finely powdered  $\text{MgCO}_3$  into a 100 mL class A volumetric flask and dilute to the mark with deionized water. Shake the solution well and allow it to settle overnight.
- 10.2 **Aqueous Acetone Solution** - Prepare this solution in a hood. Mix 450 mL acetone (Fisher Scientific Cat No A929-04 or equivalent) with 50 mL saturated  $\text{MgCO}_3$  solution in a 500 ml volumetric flask. The acetone and saturated magnesium carbonate must be measured separately before combining because the total volume after mixing will be less than the sum of the individual volumes. Shake well, with frequent venting. Wait at least 1 hour before using the solution to allow settling of excess  $\text{MgCO}_3$  precipitate. Prepare this solution every 2 months or discard solution if additional precipitates begin to form.
- 10.3 **Chlorophyll a Stock Solution (~10 mg/L)** – Prepared from chlorophyll a powder, Sigma Cat. No. C5753, or equivalent. To prepare a 10 mg/L solution, weigh ~1 mg of chlorophyll a powder on a micro analytical balance. Record the amount of chlorophyll a in the Chlorophyll Reagents/QC Preparation Log. Transfer the chlorophyll using a glass funnel and glass transfer pipette into a 100 mL amber volumetric flask. Dilute to the mark with 90% aqueous acetone solution. Completely enclose the volumetric flask in aluminum foil and let steep at 4°C for at least 2 hours before using (solution must be stored at -20<sup>0</sup> C). To determine the final concentration of the solution, place well mixed sample of the stock solution into a clean 1cm cuvette and measure the absorbance of the solution at 664.3 nm after zeroing the spectrometer with the same cuvette filled with 90% acetone. Record the absorbance in Chlorophyll Stock Solution Concentration Verification sheet (calculation shown in section 15.4). Record true chlorophyll a concentration in Chlorophyll Reagent/QC preparation Log. This solution must be prepared every 28 days.
- 10.4 **Chlorophyll b Stock Solution (~10 mg/L)** – Prepared from chlorophyll b powder, Sigma Cat. No.25740. To prepare a 10 mg/L solution, weigh ~1 mg of chlorophyll b powder on a micro analytical balance. Record the amount of chlorophyll b in the Chlorophyll Reagents/QC Preparation Log. Transfer the chlorophyll using a glass funnel and glass transfer pipette into a 100 mL amber volumetric flask. Dilute to the mark with 90% aqueous acetone solution. Completely enclose the volumetric flask in aluminum foil and let steep at 4°C for at least 2 hours before using (solution must be stored at -20<sup>0</sup> C). To determine the final concentration of the solution, place well mixed sample of the stock solution into a clean 1cm cuvette and measure the absorbance of the solution at 646.8 nm after zeroing the spectrometer with the same cuvette filled with 90% acetone. Record the absorbance in Chlorophyll Stock Solution Concentration Verification sheet (calculation shown in section 15.5). Record true chlorophyll b concentration in Chlorophyll Reagent/QC preparation Log. This solution must be prepared every 28 days.

- 10.5 **Pheophytin a Stock Solution (~10 mg/L)** – To prepare the pheophytin a stock solution, pipette 20 ml of the chlorophyll a stock solution (10.3) into a 50 ml centrifuge tube using a class A volumetric pipette, add 400  $\mu$ L of Hydrochloric Acid Solution (Fisher SA54-1) using an Eppendorf pipette, mix well, and steep for 3 hours. To determine the final concentration of the solution, place a well mixed sample of the stock solution into a clean 1cm cuvette and measure the absorbance of the solution at 667.0 nm after zeroing the spectrometer with the same cuvette filled with 90% acetone. Record the absorbance in Chlorophyll Stock Solution Concentration Verification sheet (calculation shown in section 15.6). Record true pheophytin a concentration in Chlorophyll Reagent/QC preparation Log. This solution must be prepared every 28 days.
- 10.6 **28 mM Tetrabutylammoniumacetate (TBA) Solution** – Using an automated pipettor add 28 mL of 1M TBA (obtained from Sigma – Aldrich, Catalog#401803 or equivalent) into a 1000 mL volumetric flask and dilute to the mark with DI water.
- 10.7 **Mobile Phase 1 – 70:30 Methanol: 28mM TBA** – Using a 500 mL graduated cylinder add 300 mL of 28 mM TBA Solution into a 1L mobile phase bottle and then with a 1000 mL graduated cylinder add 700 mL of methanol.
- 10.8 **Mobile Phase 2 – 100% Methanol** – Fisher Optima or equivalent.
- 10.9 **Deionized Water (D.I. water)** - Laboratory grade.
- 11.0 Sample Collection, Preservation, Shipment and Storage**
- 11.1 Samples are collected using the procedures outlined in the SFWMD Field Sampling Manual or FDEP SOPs.
- 11.2 Samples are collected unfiltered, placed in amber 1 L HDPE bottle and stored at 2-6 °C.
- 11.3 Samples must be filtered within 48 hours from the time of collection (filters stored in a negative 20 C°  $\pm$  2 C° freezer). Samples must be analyzed within 24 hours from the time of extraction, and within 28 days from the date of collection.
- 12.0 Quality Control**
- 12.1 Since no commercial standard reference materials are available for chlorophylls and pheopigments, appropriate control of accuracy is achieved through the use of certified solutions obtained from a different source than the standards. These control samples are measured after calibration to verify instrument performance. If the determined value is not within +/- 10% of the certified value, then the instrument should be recalibrated with fresh stock standards (see section 10.0). If the re-determined value is still not acceptable then the source of the problem must be identified and corrected before continuing analyses. These control solutions, designated in LIMS as LCS1, LCS2 and LCS3 are measured on a weekly basis (following calibration) or as required to meet data quality objectives. The % Recovery for these solutions is calculated using the equation shown in

section 15.1. Note: Matrix spikes are not feasible for this method and are not used as a quality control measure.

- 12.2 **LCS1 (Chlorophyll a Control Solution)** – DHI Product ID PPS-CHLA. The concentration of this commercially obtained standard solution is approximately 1000 µg/L; 2.5ml of the standard supplied in a serum vial with a septum cap. The actual concentration is reported on the certificate of analysis. For use as LCS1, an appropriate volume must be withdrawn from the vial using a glass gas tight syringe equipped with a stainless steel needle and injected into an autosampler vial. An appropriate amount of acetone is then added to the autosampler vial using a gas tight glass syringe to dilute the concentration of the standard solution to approximately 1000 µg/L. All required information regarding the stock solution(Lot#, etc.) is recorded in the LIMS standard log (see SFWMD-LAB-SOP-5510-001).
- 12.3 **LCS2 (Chlorophyll b Control Solution)** – DHI Product ID PPS-CHLB. The concentration of this commercially obtained standard solution is approximately 1000 µg/L; 2.5ml standard is supplied in a serum vial with a septum cap. The actual concentration is reported on the certificate of analysis. For use as LCS2, an appropriate volume must be withdrawn from the vial using a glass gas tight syringe equipped with a stainless steel needle and injected into an autosampler vial. An appropriate amount of acetone is then added to the autosampler vial using a gas tight glass syringe to dilute the concentration of the standard solution to approximately 500 µg/L. All required information regarding the stock solution(Lot#, etc.) is recorded in the LIMS standard log (see SFWMD-LAB-SOP-5510-001).
- 12.4 **LCS3 (Pheophytin a Control Solution)** – DHI Product ID PPS-PHAE. The concentration of this commercially obtained standard solution is approximately 1000 µg/L ; 2.5ml of the standard is supplied in a serum vial with a septum cap. The actual concentration is reported on the certificate of analysis. For use as LCS3, an appropriate volume must be withdrawn from the vial using a glass gas tight syringe equipped with a stainless steel needle and injected into an autosampler vial. An appropriate amount of acetone is then added to the autosampler vial using a gas tight glass syringe to dilute the concentration of the standard solution to approximately 1000 µg/L. All required information regarding the stock solution(Lot#, etc.) is recorded in the LIMS standard log (see SFWMD-LAB-SOP-5510-001).
- 12.5 After the weekly calibration is completed and verified using the control solutions described above, a continuing calibration blank (CCB) is run at the beginning of each run and continuing calibration verification samples (CCV1, CCV2) are run at the beginning, every 10 samples and at the end of each run. If the CCB is not below the MDL and/or the CCV concentrations are not within +/- 10% of the expected value then the instrument must be recalibrated. The % Recovery for CCV1 and CCV2 is calculated using the equation shown in section 15.1.
- 12.6 **CCB (Continuing Calibration Blank for establishing the “zero point”)** – This is the 90% acetone solution (10.2). This sample is run at the very beginning of each run. If this

sample has a concentration greater than the MDL the run should be stopped immediately, corrective action taken (see section 24.1) to resolve the problem and the run restarted. Under no circumstances should a run where the results of this sample exceed the MDL be accepted.

- 12.7 **CCV1 (Chlorophyll a and b Continuing Calibration Verification)** – Using class A volumetric pipettes, add 10 mL of the chlorophyll a stock solution (10.3) and 2 mL of the chlorophyll b stock solution (10.4) into a 100 mL class A amber volumetric flask containing 75 mL of 90% acetone solution and dilute to the mark with the 90% acetone solution. This solution will be prepared every 28 days. (~1000  $\mu\text{g/L}$  chlorophyll a, ~200  $\mu\text{g/L}$  chlorophyll b in 90% acetone).
- 12.8 **CCV2 (Pheophytin a Continuing Calibration Verification)** - Using a class A volumetric pipette, add 5 mL of pheophytin a stock solution (10.5) into a 100 mL class A amber volumetric flask containing 75 mL of 90% acetone solution and dilute to the mark with the 90% acetone solution. This solution will be prepared every 28 days. (~500  $\mu\text{g/L}$  pheophytin a in 90% acetone).
- 12.9 To assess method precision, a duplicate sample should be extracted and analyzed for every 10 samples analyzed. If possible, choose a duplicate filter with intense coloration from the sample as it is more likely to yield concentrations above the PQL and therefore allow for accurate determination of precision. The relative percent difference (RPD) of the duplicate set is calculated using the equation shown in section 15.2. The acceptable RPD limit is specified in the Laboratory Quality Manual. If the RPD exceeds the current limit refer to section 24.1 for corrective actions. Note: In some cases the RPD will exceed the limit because the concentration of the analyte is near to or below the PQL, in these cases consult your supervisor before implementing any corrective action as they may be unnecessary.
- 12.10 To determine if samples were contaminated during sample processing and analysis, a method blank, produced by extracting a blank filter, is prepared along with each batch of samples and is analyzed at beginning of every run. The blank value must be less than the detection limit for all three pigments being measured. If the method blank concentration is not below the MDL, the sample extracts are suspect of contamination and must be discarded and the samples re-extracted. An investigation into the potential source(s) should be undertaken immediately and methods to eliminate or minimize contamination should be implemented prior to re-analysis of the affected samples. Any corrective actions should be documented and conveyed to the Laboratory Quality Assurance Officer as soon as possible.
- 12.11 All other quality control data must be checked against the currently established limits before entering sample data into the LIMS system. In the event of a QC failure refer to section 24.0 for corrective actions. If the problems cannot be resolved, consult with your supervisor to determine if the samples should be re-run, re-extracted or have appropriate qualifiers added to the result(s). Chronic failure of quality control samples or unusual

trends in quality control data should be investigated, documented, and reported to the Laboratory Quality Assurance Officer when they occur.

### 13.0 Calibration and Standardization

13.1 Initial calibration of the HPLC system must be carried out on a weekly basis or more frequently as needed to meet quality control requirements. A continuing calibration blank (zero point) is run at the beginning of each run and continuing calibration verification is performed at the beginning, after every 10 samples, and at end of each run. Only the initial calibration is used for quantification of unknown samples. Sample extracts with concentrations above the highest standard are diluted to approximately 50% of the working range and rerun. Samples with concentrations below the detection limit are qualified with a “U” and samples with concentrations above the MDL but below the PQL are qualified with an “I”.

13.2 Calibration must be completed individually for each pigment that will be determined. To calibrate the system, a series of 6 standards for chlorophyll a and pheophytin a and 5 standards for chlorophyll b are analyzed. The peak area from each of the standards is plotted against the concentration to determine the response factor for the individual pigments by linear regression. In order for the calibration to be acceptable, the correlation coefficient for the linear regression must be greater than 0.995. **Note: The concentration and total number of standards was determined based on the typical concentrations observed in the sample extracts and the desire to establish that the calibration is linear throughout the working range. The lowest concentration standard is at or less than the Practical Quantitation Limit (PQL) for each analyte.**

#### 13.3 Chlorophyll a Calibration Standards

13.3.1 **Standard 1 (~2000 µg/L Chlorophyll a)** – Using a class A volumetric pipette, add 20 mL of chlorophyll a stock solution (10.3) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix and bring it to volume with the acetone solution.

13.3.2 **Standard 2 (~1000 µg/L Chlorophyll a)** – Using a class A volumetric pipette, add 10 mL of chlorophyll a stock solution (10.3) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix and bring it to volume with the acetone solution.

13.3.3 **Standard 3 (~500 µg/L Chlorophyll a)** – Using a class A volumetric pipette, add 5 mL of chlorophyll a stock solution (10.3) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix and bring it to volume with the acetone solution.

13.3.4 **Standard 4 (~200 µg/L Chlorophyll a)** – Using a class A volumetric pipette, add 2 mL of chlorophyll a stock solution (10.3) into a 100 mL class A amber volumetric

flask containing 70 mL of 90% acetone solution, mix and bring it to volume with the acetone solution.

13.3.5 **Standard 5 (~100 µg/L Chlorophyll a)** – Using a class A volumetric pipette, add 10 mL of Standard 2 (13.3.2) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix and bring it to volume with the acetone solution.

13.3.6 **Standard 6 (~10 µg/L Chlorophyll a)** – Using a class A volumetric pipette, add 10 mL of Standard 5 (13.3.5) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix and bring it to volume with the acetone solution.

13.3.7 **Standard 7 (0.0 µg/L Chlorophyll a)** – This is the 90% acetone solution.

#### 13.4 Chlorophyll b Calibration Standards

13.4.1 **Standard 1 (~500 µg/L Chlorophyll b)** – Using a class A volumetric pipette, add 5 mL of chlorophyll b stock solution (10.4) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix, and bring it to volume with the acetone solution.

13.4.2 **Standard 2 (~300 µg/L Chlorophyll b)** – Using a class A volumetric pipette, add 3 mL of chlorophyll b stock solution (10.4) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix, and bring it to volume with the acetone solution.

13.4.3 **Standard 3 (~100 µg/L Chlorophyll b)** – Using a class A volumetric pipette, add 20 mL of chlorophyll b Standard 1 (13.4.1) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix, and bring it to volume with the acetone solution.

13.4.4 **Standard 4 (~50 µg/L Chlorophyll b)** – Using a class A volumetric pipette, add 10 mL of chlorophyll b Standard 1 (13.4.1) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix, and bring it to volume with the acetone solution.

13.4.5 **Standard 5 (~20 µg/L Chlorophyll b)** – Using a class A volumetric pipette, add 20 mL of chlorophyll b Standard 3 (13.4.3) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix, and bring it to volume with the acetone solution.

13.4.6 **Standard 6 (0.0 µg/L Chlorophyll b)** – This is the 90% acetone solution.

#### 13.5 Pheophytin a Calibration Standards

- 13.5.1 **Standard 1 (~1000 µg/L Pheophytin a)** – Using a class A volumetric pipette, add 10 mL of pheophytin a stock solution (10.5) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix, and bring it to volume with the acetone solution.
- 13.5.2 **Standard 2 (~500 µg/L Pheophytin a)** – Using a class A volumetric pipette, add 5 mL of pheophytin a stock solution (10.5) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix, and bring it to volume with the acetone solution.
- 13.5.3 **Standard 3 (~200 µg/L Pheophytin a)** – Using a class A volumetric pipette, add 2 mL of pheophytin a stock solution (10.5) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix, and bring it to volume with the acetone solution.
- 13.5.4 **Standard 4 (~100 µg/L Pheophytin a)** – Using a class A volumetric pipette, add 10 mL of pheophytin a standard 1 (13.5.1) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix, and bring it to volume with the acetone solution.
- 13.5.5 **Standard 5 (~50 µg/L Pheophytin a)** – Using a class A volumetric pipette, add 10 mL of pheophytin a standard 2 (13.5.2) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix, and bring it to volume with the acetone solution.
- 13.5.6 **Standard 6 (~10 µg/L Pheophytin a)** – Using a class A volumetric pipette, add 10 mL of pheophytin a standard 4 (13.5.14) into a 100 mL class A amber volumetric flask containing 70 mL of 90% acetone solution, mix, and bring it to volume with the acetone solution.
- 13.5.7 **Standard 7 (0.0 µg/L Pheophytin a)** – This is the 90% acetone solution.
- 13.6 The calibration plots and individual chromatographs for the weekly calibrations are stored in the calibration notebook for the HPLC. The response factors determined from the calibration are entered into the HPLC software in order to compute the concentration of unknown samples automatically. All data needed to reconstruct the calibration plot are stored in the notebook.
- 13.7 Once completed the calibration must be verified by running appropriate control solutions as described in section 12.0.
- 13.8 During each analytical run Continuing Calibration Verification (**CCV1, CCV2**) standards are run for each 10 samples to verify that the system remains properly calibrated (see section 12.0 for details).

## 14.0 Procedure

14.1 Create a batch (batch preparation) for the analyses to be conducted. Refer to the SFWMD-LAB-SOP-5000 (current version) "Use of Horizon LIMS".

14.2 Chlorophyll samples should be filtered as quickly as possible in order to minimize the time that samples are exposed to light and room temperature. Light and heat cause the chlorophyll to degrade, which may produce inaccurate results.

### 14.3 Filtration

14.3.1 The filtration procedure should be conducted in subdued light to avoid degradation, and should be carried out as quickly as possible in order to minimize the time that the samples are exposed to light and room temperature.

14.3.2 Enter the project name, date and time filtered, sample ID# and analyst initials in the Filtration and Extraction logbook. Also record any observations regarding sample composition.

14.3.3 Prepare filtration assembly and graduated cylinder. All glassware must be clean and acid-free.

A. If using the grinding method, pre-label petri dishes with LIMS #, date filtered and project.

B. If using the sonication method, pre-label the Sarstedt tubes with LIMS #, date filtered and project.

14.3.4 Assemble the filter funnel; place a glass fiber filter, rough side up, on top of the filter funnel. The vacuum manifold should be connected to 4 L filter flask and a small flask (to keep overflow water out of the vacuum system) via rubber tubing. Turn the vacuum on by pressing the switch located in front of vacuum pump.

14.3.5 Gently shake the sample container to homogenize the sample. The sample will be divided into two equal volumes of up to 500 mL each. If less than 1L of sample is available, or if the sample is high in solids filter as much sample as can pass through the filter in 10 minutes.

14.3.6 Turn the vacuum on. Pour a measured sample aliquot into the filter funnel and allow to filter completely. If the sample filters though quickly add more sample (up to half the total available sample) and allow to filter completely. If the sample begins to clog, discard the filter and prep a new one and start again using a smaller aliquot. Rinse the graduated cylinder and the funnel with approximately 20 mL of D.I. water. Record the volume of sample filtered.

14.3.7 **After the entire sample has been filtered, dispense 1 mL of MgCO<sub>3</sub> solution onto the filter.**

14.3.8 Dry the filter by maintaining the vacuum for an additional 30 seconds.

- A. If using the grinding extraction procedure, carefully remove the filter, fold it in half enclosing the algae and transfer into the petri dish. Record volume filtered in the logbook. Repeat steps 14.3.6 and 14.3.7 for the second aliquot. Label the two aliquots filtered for each sample "A" and "B". Place the petri dishes in aluminum bags, and freeze.
  
- B. If using the sonication extraction procedure, carefully remove the filter, roll filter into a loose tube, roughly the diameter of the centrifuge tube, with algae side inside and transfer into the bottom of the centrifuge tube. Place the centrifuge tube in a rack and record volume filtered. Repeat steps 14.3.6 and 14.3.7 for the second aliquot. Label the two aliquots filtered for each sample "A" and "B". After finishing filtration of all the samples, cover the rack with aluminum foil and store the filtered samples in a freezer.

#### 14.4 Pigment Extraction

A. If using a tissue grinder:

- 14.4.1 Remove the aluminum bags containing the filters from the freezer and place in a bucket containing ice. Allow the filters to thaw for a few minutes.
- 14.4.2 Fold the filter to make a small pellet, and transfer it into the bottom of the glass grinder tube.
- 14.4.3 Cover the sample with 2-3 mL of aqueous acetone solution. Insert the tube into a small plastic sleeve half-filled with ice. Grind with a motor driven Teflon pestle until the filter/acetone mixture has been reduced to a uniform slurry or up to one minute.
- 14.4.4 Carefully transfer the sample into a pre labeled 15 mL screw cap centrifuge tube. Rinse the grinder with 1-3 mL aqueous acetone solution, adding the rinsate into the centrifuge tube. Bring to a maximum of 15mL final volume (10 mL minimum) with the aqueous acetone solution. Record the extract volume in the logbook to the nearest 0.5 mL. Process all samples in this manner. Rinse the pestle and glass grinder tube with acetone between each sample and discard the wash.

14.4.5 Place the extract tubes in a rack and cover with aluminum foil to protect from light. Steep the sample extracts for a minimum of 2 hours, but not more than 24 hours at 4°C.

14.4.6 After the samples have steeped, filter the extract through a 0.45µm filter (pressure filter or centrifugation apparatus is acceptable).

B. If using a sonicator:

14.4.7 Remove the Sarstedt tubes containing the filters from the freezer and place in a cooler containing ice. Allow the filters to thaw for a few minutes.

14.4.8 Cover the filter with no more than 6.5 mL aqueous acetone solution. Leave tube uncapped and place in a holder surrounded by ice.

14.4.9 Lower the sonication probe into the centrifuge tube, leaving the tip of the probe above the conical bottom of the tube. The probe must not contact the tube or filter. Sonicate for 45 seconds at power range setting 8 using 2 seconds on 0.5 second off pulse. (The power output should be between 18 to 24 W).

14.4.10 Rinse the sonication probe with 1-3 mL aqueous acetone solution, adding the rinsate into the Sarstedt tube. Bring to 10mL final volume with aqueous acetone solution. Process all samples in this manner.

14.4.11 Shake the centrifuge tube before placing the tubes in a rack and place rack in a cooler to protect from light. Steep extracts for a minimum of 2 hours, but not more than 24 hours at 4°C.

14.4.12 After the samples have steeped, filter the extract through a 0.45µm filter (pressure filter or centrifugation apparatus is acceptable).

14.5 Analysis by HPLC

14.5.1 In the HPLC software create a sample table by cutting and pasting the sample ID's from the LIMS batch. Save the sample table using the Batch ID as the filename.

14.5.2 Using an Eppendorf and a clean tip for each sample, pipette ~400 µL of each filtered sample extract into an HPLC autosampler vial. Place the vials in the autosampler tray according to the sample table.

14.5.3 Open a new batch file in the HPLC software by clicking on "File" and "New" from the menu.

14.5.4 Cut and paste all sample ID's from the LIMS batch into the HPLC software batch file. Enter the appropriate dilution factors based on the volume of sample filtered and final extract volume according to the calculation found in section 15.3. Save

the newly created batch file using the Batch ID assigned by LIMS. Verify that the injection volume is set to 50 µL for all samples.

- 14.5.5 Hit the “Start” button in the HPLC software to begin the analysis. Reports for each sample will be printed into NuGenesis and parsed by the LIMS so that each set of sample results will appear in the autopost pipe.
- 14.5.6 When the batch is completed, remove the vials from the autosampler tray and dispose.
- 14.6 To edit a batch Refer SFWMD-LAB-SOP-5000 (current version) “Use of Horizon LIMS”.
- 14.7 To send the data to LIMS Refer SFWMD-LAB-SOP-5000 (current version) “Use of Horizon LIMS”.
- 14.8 To post data using the autopost pipe Refer SFWMD-LAB-SOP-5000 (current version) “Use of Horizon LIMS”. Prior to posting data, all chromatograms should be reviewed to ensure proper peak integration. In the event that a change to the peak integration is required, follow the procedures outlined in the document, Laboratory Policy for Manual Peak Integration. Any changes to the integration should be noted on the analytical run and/or annotated to the electronic copy of the run stored in NuGenesis.
- 14.9 Discard waste acetone into the assigned 55-gal drum outside the building or a satellite container inside the lab. DO NOT DISPOSE OF ANY ACETONE WASTE INTO THE SINK.
- 14.10 Consult your supervisor before making any major changes, adjustments and/or repairs to the instrumentation.
- 14.11 Chromatographic Conditions

<u>Time (min)</u>	<u>T (°C)</u>	<u>Flow (mL/min)</u>	<u>% 1</u>	<u>% 2</u>	<u>Condition</u>
0.01	60	2.0	50	50	Injection
5.00	60	2.0	2	98	Linear Gradient
6.00	60	2.0	2	98	Hold
6.50	60	2.0	50	50	Linear Gradient
10.00	60	2.0	50	50	Equilibration

## 15.0 Calculations

- 15.1 Recovery of the LCS is calculated as follows:

$$LCS \% Recovery = \frac{LCS\ Result}{LCS\ True\ Value} \times 100$$

- 15.2 The relative percent difference of the duplicate samples is calculated with the following formula:

$$RPD = \frac{\overbrace{Value1 - Value2}}{\text{Mean}} \times 100$$

- 15.3 The dilution factor for each sample is calculated as follows:

$$\text{Dilution Factor} = V(e)/V(s)$$

Where V(e) and V(s) are volume (in mL) of extract and sample respectively.

- 15.4 The concentration of the chlorophyll a stock solution is calculated according to the following equation:

$$[\text{Chlorophyll } a, \text{ mg/L}] = 1000 \times Abs_{664.3}/87.67$$

Where Abs<sub>664.3</sub> is the absorbance of the solution at 664.3 nm in a 1cm cuvette.

- 15.5 The concentration of the chlorophyll b stock solution is calculated according to the following equation:

$$[\text{Chlorophyll } b, \text{ mg/L}] = 1000 \times Abs_{646.8}/51.36$$

Where Abs<sub>646.8</sub> is the absorbance of the solution at 646.8 nm in a 1cm cuvette.

- 15.6 The concentration of the pheophytin a stock solution is calculated according to the following equation:

$$[\text{Pheophytin } a, \text{ mg/L}] = 1000 \times Abs_{667.0}/51.20$$

Where Abs<sub>667.0</sub> is the absorbance of the solution at 667.0 nm in a 1cm cuvette.

## 16.0 Method Performance

- 16.1 This method was validated through comparative analysis and through continuing participation in the QUASIMEME double blind study. Charts of comparative data, results from the blind studies, and data from the initial spiking study are maintained by the Laboratory Quality Assurance Officer and are located in his office. Detection limits are shown in section 3.2 and the supporting data are available from the Quality Assurance Officer.

## **17.0 Pollution Prevention**

- 17.1 Discard waste acetone into the assigned waste container outside the building. DO NOT DISPOSE OF ANY ACETONE WASTE INTO THE SINK.
- 17.2 Standards and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired standards and reagents to be disposed.

## **18.0 Data Assessment and Acceptance Criteria for Quality Control Measures**

- 18.1 Quality control data must meet acceptable quality control limits, otherwise appropriate qualifiers must be added to the result.
- 18.2 The sample holding time is 28 days from the date of collection. Notify the supervisor if any samples are out of the holding time.
- 18.3 Method blank results must be less than the current MDL. Refer to section 24.1 for corrective action.
- 18.4 Control solutions are analyzed after calibration to verify system performance. The percent recovery must be within current limits. If control solutions values fall outside the currently established acceptance limits, refer to section 24.1 for corrective action.
- 18.5 The RPD for the duplicate analysis must be within current limits. If the value is outside the currently established acceptance limits, refer to section 24.1 for corrective action.

## **19.0 Corrective Actions for Out-of-Control Situations**

- 19.1 See the table in section 24.1 for corrective actions.

## **20.0 Contingencies for Handling Out-of-Control or Unacceptable Data**

- 20.1 If the out-of-control situation cannot be resolved by the corrective actions described in section 24.0, the sample result(s) should have an appropriate qualifier code added. See the supervisor to enter these flags into the LIMS.
- 20.2 It may be necessary to obtain technical service to make repairs to instruments that are producing unacceptable data. See the supervisor for assistance in obtaining this service.

## **21.0 Waste Management**

- 21.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and

controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

21.2 The samples are disposed of according to Method 5030.1, Rev. #: 1.0.

## **22.0 Instrument Maintenance**

22.1 A maintenance logbook for each piece of instrumentation identified in this procedure shall be maintained by the analyst. The maintenance logbook must include the following items:

1. The name or laboratory designation of the instrument and the associated software;
2. The manufacturer's name, type identification, serial number or other unique identification, and the SFWMD inventory control number;
3. Initial calibration records or other checks to confirm that the instrument complies with the method requirements;
4. The current location of the instrument;
5. Manufacturer's instructions, owner's manuals, or reference to their location;
6. Dates, results and copies of reports and certificates of all calibrations, adjustments, acceptance criteria, and the due date of the next calibration;
7. The maintenance plan as appropriate, and documentation of all maintenance carried out to date, including all routine and non-routine maintenance activities and reference material verifications;
8. Records of any damage, malfunction modification or repair to the instrument;
9. The date received and condition when first placed into service.

22.2 Any condensation water has to be removed regularly from rotor chamber of centrifuge. Leave switch off the centrifuge after use, leave the lid open and empty drip tray

## **23.0 References**

23.1 SFWMD-LAB-SOP-5000 (current version) "Use of Horizon LIMS"

23.2 SFWMD Comprehensive Quality Assurance Manual, current version.

23.3 Van Heukelem & Thomas (2001) J. Chromatogr. A. 910:31-49

23.4 EPA Method 447.0 - Determination of Chlorophylls a and b and Identification of Other Pigments.

23.5 EPA Method 446.0 – In Vitro Determination of Chlorophyll a, b, c1+c2 and Pheopigments.

23.6 SFWMD-LAB-SOP-5510 (current version) "HORIZON Standards Log"

23.7 SFWMD-LAB-SOP-5030 (current version) "Sample Disposal"

## 24.0 Tables, Diagrams, Flowcharts and Validation Data

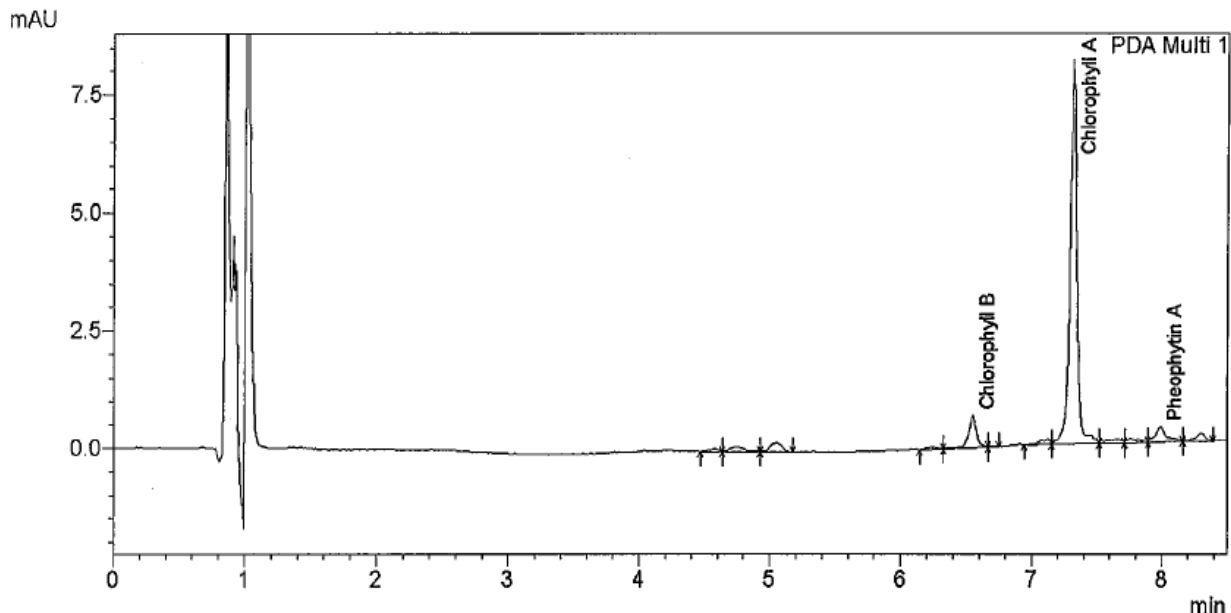
### 24.1 Corrective actions for out-of control laboratory quality controls.

Lab QC Type	Acceptance Criteria	Recommended Corrective Action
Initial Instrument Blank Method Reagent Blank	<MDL	Prepare new blank & restart analysis. If same response is obtained, determine cause of contamination (reagents, calibration standards, environment, equipment failure, etc) & eliminate the source of contamination.
Instrument Calibration performed weekly	Correlation coefficient greater than 0.995	If same response is obtained, consult your supervisor.
Laboratory Control Samples	Accuracy(%R) High/Low 90-110	Re-analyze LCS check standard. If that fails, check against an alternate QC source. Obtain approval from QA officer or staff. Discard unacceptable LCS once confirmed & document findings on QC result log.
Laboratory Duplicate/Duplicate Sample	Precision(RPD) High/low 0-20(chlorophyll a,b) 0-30(pheophytin a)	Determine & eliminate cause of problem (baseline drift, carryover, etc). Re-analyze all affected samples.

### 24.2 Corrective actions for out-of-control field quality control samples.

Field QC Type	Acceptance Criteria	Recommended Corrective Action
Equipment Blank(EB)	<MDL or less than established limits	Laboratory should re-analyze positive field blanks to confirm results.
Field Duplicates (FD) Replicate Samples (RS) Split Samples (SS)	Precision within limits See Quality Manual (only if values >PQL)	Laboratory should re-analyze failing field QC samples to confirm results.

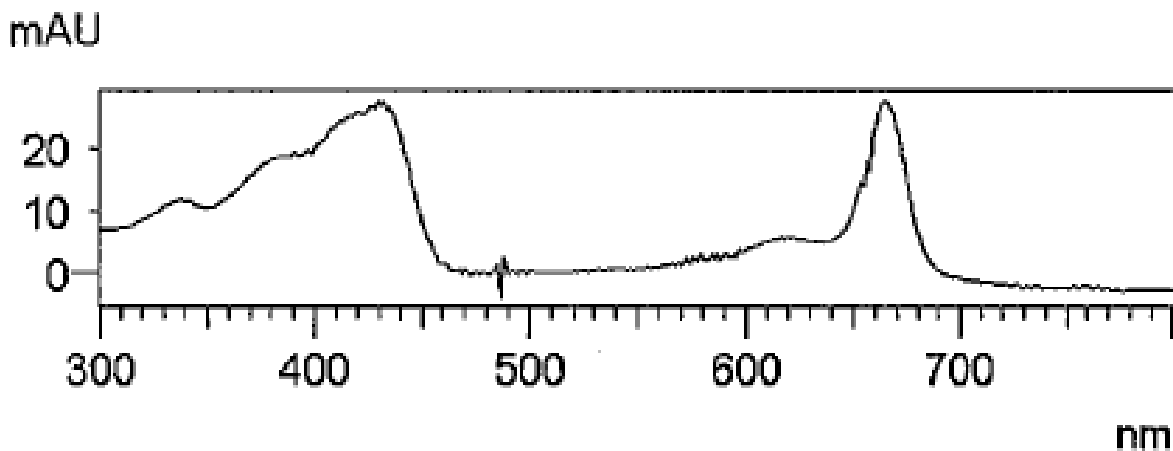
### 24.3 Typical Chromatogram



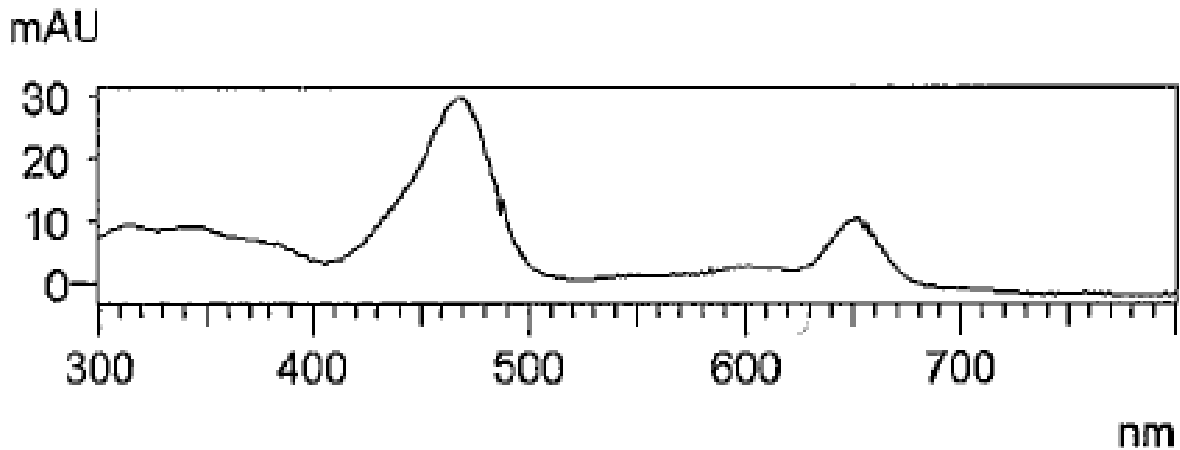
1 PDA Multi 1/665nm 20nm

### 24.4 Reference Spectra

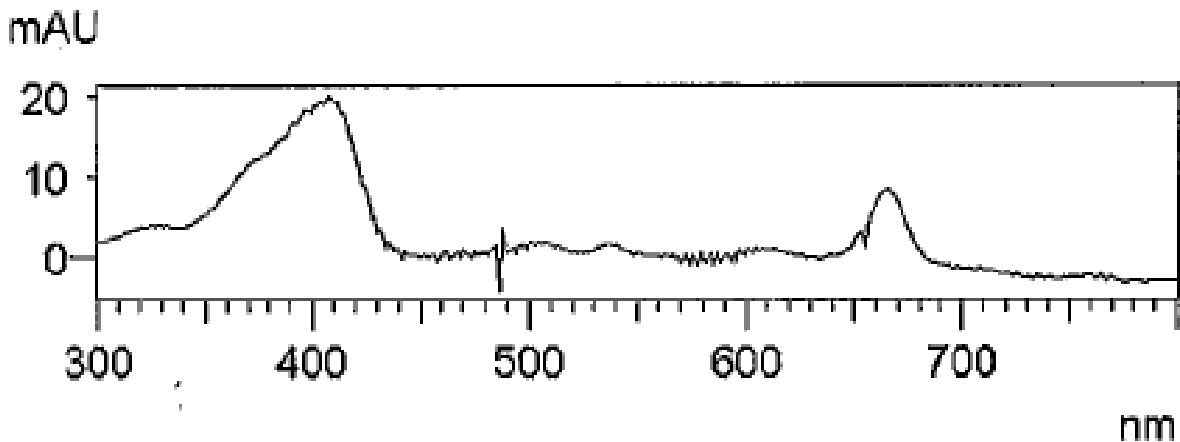
**Compound Name** : Chlorophyll a  
**Lambda max** : 432.00/665.06/337.69/619.48/779.59  
**Lambda min** : 349.40/635.37/473.25/776.18  
**Operation** : None



Compound Name : Chlorophyll b  
Lambda max : 467.23/651.34/314.80/342.73/596.99  
Lambda min : 327.67/405.90/620.72/519.42/738.52  
Operation : None



Compound Name : Pheophytin a  
Lambda max : 407.72/665.67/487.93/329.12/536.95  
Lambda min : 338.29/522.02/473.11/458.41/585.29  
Operation : None



## **25.0 SOP Addendums and Changes**

