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Standard Operating Procedure for Spectrophotometric Determination of Chlorophyll α in waters and sediments of Fresh/Estuarine/Coastal Areas.

(References: EPA 446.0, SM10200H)

Document #: NASLDoc-034

Revision 2024-1
Replaces Revision 2023-1
Effective May 1, 2024

I attest that I have reviewed this standard operating procedure and agree to comply with all procedures outlined within this document.

Employee (Print)	Employee (Signature)	Date
Employee (Print)	Employee (Signature)	Date
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Revised by: _____ Date: _____

Reviewed by: _____ Date: _____

Laboratory Supervisor: _____ Date: _____

Revisions affecting 2024-1

No changes were made.

1. SCOPE and APPLICATION

1.1 This is an acetone extraction method to determine chlorophyll α in fresh, estuarine waters, and coastal waters.
1.2 A Method Detection Limit (MDL) of 0.62 µg/L active chlα and 0.74 µg/L phaeophytin was determined using the Student's *t* value times the standard deviation of a minimum of 7 replicates. If more than seven replicates are used to determine the MDL, refer to the Student's *t* test table for the appropriate n-1 value.
1.3 The quantitation limit for chlα is dependent upon the volume of sample filtered. The reporting limit is equal to the MDL.
1.4 This procedure should be used by analysts experienced in the theory and application of chlorophyll analysis. A three-month training period with an analyst experienced in the analysis using the spectrophotometer is required.
1.5 This method can be used for all programs that require spectrophotometric analysis of chlorophyll α.
1.6 This procedure is based on EPA Method 446.0 and Standard Methods 10200H, 22nd Edition.

2. SUMMARY

2.1 Chlorophyll α is extracted from phytoplankton cells using a 90% solution of acetone. The cells are physically disrupted by mechanical grinding or sonication. The samples are refrigerated in the dark from 2 to 24 hours (overnight is preferable). After the appropriate time, the samples are centrifuged to separate the sample material from the extract. Because the waters of the Maryland portion of the Chesapeake Bay are relatively turbid, the sample extract is filtered through a 0.45 um PTFE or nylon syringe filter before analysis. For later analysis, the extract may be transferred into a clean tube, stored frozen, and centrifuged again for 20 minutes. The extract is analyzed on a spectrophotometer. To determine phaeophytin and active chlα, the extract is then acidified using 1N HCl, and reread. The concentrations are then calculated using Lorenzen's modified monochromatic equation. Uncorrected chlorophyll may be determined using the Jeffrey and Humphrey trichromatic equation.

3. DEFINITIONS

3.1 Absorbance – A measure of the amount of light at a specific wavelength absorbed by a liquid.
3.2 Acceptance Criteria – Specified limits placed on characteristics of an item, process, or service defined in a requirement document. (ASQC)
3.3 Accuracy – The degree of agreement between an observed value and an accepted reference value. Accuracy includes a combination of random error

(precision) and systematic error (bias) components which are due to sampling and analytical operations; a data quality indicator. (QAMS)

3.4 Aliquot – A discrete, measured, representative portion of a sample taken for analysis. (EPA QAD Glossary)

3.5 Analytical Range – The analytical range is dependent on the volume of water filtered and the volume of acetone used in the extraction.

3.6 Batch – Environmental samples, which are prepared and /or analyzed together with the same process and personnel, using the same lot(s) of reagents. An **analytical batch** is composed of prepared environmental samples (extracts, digestates, or concentrates) and/or those samples not requiring preparation, which are analyzed together as a group using the same calibration curve or factor. An analytical batch can include samples originating from various environmental matrices and can exceed 20 samples. (NELAC/EPA)

3.7 Blank- A sample that has not been exposed to the analyzed sample stream in order to monitor contamination during sampling, transport, storage or analysis. The blank is subjected to the usual analytical and measurement process to establish a zero baseline or background value and is sometimes used to adjust or correct routine analytical results. (ASQC)

3.8 Calibrate- To determine, by measurement or comparison with a standard, the correct value of each scale reading on a meter or other device, or the correct value for each setting of a control knob. The levels of the applied calibration standard should bracket the range of planned or expected sample measurements. (NELAC)

3.9 Calibration – The set of operations that establish, under specified conditions, the relationship between values indicated by a measuring device. The levels of the applied calibration standard should bracket the range of planned or expected sample measurements. (NELAC)

3.10 Calibration Curve – The graphical relationship between known values, such as concentrations, or a series of calibration standards and their analytical response. (NELAC)

3.11 Calibration Method – A defined technical procedure for performing a calibration. (NELAC)

3.12 Calibration Standard – A substance or reference material used to calibrate an instrument. (QAMS)

- 3.12.1 Initial Calibration Standard (STD) – A series of standard solutions used to initially establish instrument calibration responses and develop calibration curves for individual target analytes.
- 3.12.2 Initial Calibration Verification (ICV) – An individual standard, analyzed initially, prior to any sample analysis, which verifies the acceptability of the calibration curve or previously established calibration curve.
- 3.12.3 Continuing Calibration Verification (CCV) – An individual standard that is analyzed after every 10-15 field sample analysis.

3.13 Certified Reference Material (CRM) – A reference material one or more of whose property values are certified by a technically valid procedure, accompanied by or traceable to a certificate or other documentation which is issued by a certifying body. (ISO 17025)

3.14 Corrective Action – Action is taken to eliminate the causes of an existing nonconformity, defect, or other undesirable situation in order to prevent a recurrence. (ISO 8402)

3.15 Deficiency – An unauthorized deviation from acceptable procedures or practices. (ASQC)

3.16 Demonstration of Capability – A procedure to establish the ability of the analyst to generate acceptable accuracy. (NELAC)

3.17 Detection Limit – The lowest concentration or amount of the target analyte that can be determined to be different from zero by a single measurement at a stated degree of confidence.

3.18 Duplicate Analysis – The analyses of measurements of the variable of interest were performed identically on two subsamples (aliquots) of the same sample. The results from duplicate analyses are used to evaluate analytical or measurement precision but not the precision of sampling, preservation, or storage internal to the laboratory. (EPA-QAD)

3.19 External Standard (ES) – A pure analyte (anacystis nidulans algae, or equivalent) that is measured in an experiment separate from the experiment used to measure the analyte(s) in the sample. The signal observed for a known quantity of the pure external standard is used to calibrate the instrument response for the corresponding analyte(s). The instrument response is used to calculate the concentrations of the analyte(s) in the unknown sample.

3.20 Field Duplicates (FD1 and FD2) – Two separate samples collected at the same time and place under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of FD1 and FD2 provide a measure of the precision associated with sample collection, preservation, and storage, as well as with laboratory procedures.

3.21 Field Reagent Blank (FRB) – An aliquot of reagent water or other blank matrix that is placed in a sample container in the laboratory and treated as a sample in all respects, including shipment to the sampling site, exposure to the sampling site conditions, storage, preservation, and all analytical procedures. The purpose of the FRB is to determine if method analytes or other interferences are present in the field environment.

3.22 Holding time – The maximum time that samples may be held prior to analysis and still be considered valid. (40 CFR Part 136) The time elapsed from the time of sampling to the time of extraction or analysis, as appropriate.

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

3.24 Laboratory Duplicates (LD1 and LD2) – Two aliquots of the same sample were taken in the laboratory and analyzed separately with identical procedures. Analyses of LD1 and LD2 indicate precision associated with laboratory procedures, but not with sample collection, preservation, or storage procedures.

3.25 Laboratory Reagent Blank (LRB) and Reagent Blank (RB) – The Reagent Blank is a matrix blank (i.e., 90% acetone) that is treated exactly like a sample including exposure to all glassware, equipment, solvents, and reagents that are used with other samples. The RB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the instrument. The LRB consists of a blank filter pad extracted in 90% acetone at the end of the loading process. It is analyzed at the end of each run. LRB data are used to assess contamination from the laboratory environment.

3.26 Laboratory Control Sample (LCS) – A sample matrix, free from the analytes of interest, spiked with verified known amounts of analytes from a source independent of the calibration standard or a material containing known and verified amounts of analytes. The LCS is generally used to establish intra-laboratory or analyst-specific precision and bias or to assess the performance of all or a portion of the measurement system. (NELAC)

3.27 Limit of Detection (LOD) – The lowest concentration level that can be determined by a single analysis and with a defined level of confidence to be statistically different from a blank (ACS), also known as MDL.

3.28 Limit of Quantitation (LOQ) – The minimum levels, concentrations, or quantities of a target variable (target analyte) that can be reported with a specified degree of confidence. The LOQ is set at 3 to 10 times the LOD, depending on the degree of confidence desired. Also known as Quantitation Limit.

3.29 Linear Dynamic Range (LDR) – The absolute quantity over which the instrument response to an analyte is linear. This specification is also referred to as the Linear Calibration Range (LCR).

3.30 May – Denotes permitted action, but not required action. (NELAC)

3.31 Method Detection Limit (MDL) – The minimum concentration of an analyte that can be identified, measured, and reported with 98% confidence that the analyte concentration is greater than zero.

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

3.33 Must – Denotes a requirement that must be met. (Random House College Dictionary)

3.34 Path Length – The path length is the width of the cuvette cell (length between optical non-frosted sides). For this method, 5 and 1 cm path length cuvettes are used.

3.35 Precision – The degree to which a set of observations or measurements of the same property, obtained under similar conditions, conform to themselves; a data quality indicator. Precision is usually expressed as standard deviation, variance, or range, in either absolute or relative terms. (NELAC)

3.36 Preservation – Refrigeration, freezing, and/or reagents added at the time of sample collection (or later) to maintain the chemical and or biological integrity of the sample.

3.37 Quality Control Sample (QCS) – A sample of analytes of known and certified concentrations. The QCS is obtained from a source external to the laboratory and is different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials and is also known as the CRM.

3.38 Run – One sample analysis from start to finish, including printout.

3.39 Run Cycle – Typically a day of operation – the entire analytical sequence of runs from the first run to the last run.

3.40 Safety Data Sheets (SDS) – Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.41 Sample Volume – Volume of water filtered.

3.42 Sensitivity – The capability of a test method or instrument to discriminate between measurement responses representing different levels (concentrations) of a variable of interest.

3.43 Shall – Denotes a requirement that is mandatory whenever the criterion for conformance with the specification requires that there be no deviation. (ANSI)

3.44 Should – Denotes a guideline or recommendation whenever noncompliance with the specification is permissible. (ANSI)

3.45 Standard Reference Material (SRM) – Material that has been certified for specific analytes by a variety of analytical techniques and/or by numerous laboratories using similar analytical techniques. These may consist of pure chemicals, buffers, or compositional standards. The materials are used as an indication of the accuracy of a specific analytical technique. Also known as CRM.

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

4. INTERFERENCES

4.1 Light and heat cause the chlorophyll molecule to break down. Therefore, the samples should be kept cold in the dark and care should be taken during cell disruption so as not to overheat the sample. When ready to analyze, the extract

must be at room temperature, and the analysis performed under reduced lighting.

4.2 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 absorbances (665, 664, 647, and 630 nm) to account for the turbidity of the clarified sample. If the absorbance at 750 nm is above 0.007 absorbance units (AU), the sample may be filtered one more time.

4.3 The spectral overlap of chlorophyll α, *b*, and *c* and phaeophytin can cause over or under-estimation of chlorophyll and/or phaeophytin. 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 α may be overestimated in the presence of phaeophytin. In the monochromatic equation, chlorophyll α may be slightly overestimated in the presence of chlorophyll *b* and phaeophytin may be overestimated in the presence of carotenoids.

5. SAFETY

5.1 Safety precautions must be taken when handling reagents, samples, and equipment in the laboratory. Protective clothing including lab coats, safety glasses, and enclosed shoes should be worn. In certain situations, it will be necessary to also use gloves and/or a face shield. If solutions come in contact with eyes, flush with water continuously for 15 minutes. If solutions come in contact with skin, wash thoroughly with soap and water. Contact Solomons Rescue Squad (911) if emergency treatment is needed and also inform the CBL Associate Director of Administration and Facilities of the incident. Contact the CBL Associate Director of Administration and Facilities if additional treatment is required.

5.2 The toxicity or carcinogenicity of each reagent used in this procedure may not have been fully established. Each chemical should be regarded as a potential health hazard and exposure should be as low as reasonably achievable. Cautions are included for known hazardous materials and procedures.

5.3 Do not wear jewelry when troubleshooting electrical components. Even low voltage points are dangerous and can injure if allowed to short circuit.

5.4 The following hazard classifications are listed for the chemicals used in this procedure. Detailed information is provided on Safety Data Sheets (SDS).

Table 1:

Chemical	Health Hazard	Fire Hazard	Instability Hazard	Specific Hazard	
Hydrochloric Acid	3	0	2	ACID, COR	

Acetone	1	3	0		

On a scale of 0 to 4 the substance is rated on four hazard categories: health, flammability, reactivity, and contact. (0 is non-hazardous and 4 is extremely hazardous)

HAZARD RATING
Health Hazard - Blue: 4 – deadly, 3 – extreme danger, 2 – hazardous, 1 – slightly hazardous, 0 – normal material

Fire Hazard - Red: Flash Points: 4 – below 73° F, 3 – below 100° F, 2 – below 200° F, 1 – above 200° F, 0 – will not burn

Instability Hazard - Yellow: 4 – may detonate, 3 – Shock and heat may detonate, 2 – violent chemical change, 1 – unstable if heated, 0 - stable

Specific Hazard - White: Acid = ACID, Alkali = ALK, Corrosive = COR, Oxidizer = OXY

6 EQUIPMENT AND SUPPLIES

6.1 A scanning spectrophotometer capable of measuring wavelengths within the visible range. This laboratory uses Shimadzu UV2401PC and UV2450PC spectrophotometers.
6.2 Freezer, capable of maintaining -20° ± 5° C and refrigerator, capable of maintaining 4° ± 2° C.
6.3 Lab ware – All reusable lab ware (glass, Teflon, plastic, etc) should be sufficiently clean for the task objectives.
6.4 A centrifuge.
6.5 A Teflon pestle for grinding, either by hand or power, and/or a sonicator.
6.6 5-cm path length and 1-cm path length cuvettes of either special optical glass or quartz.

7 REAGENTS AND STANDARDS

7.1 Purity of Water – Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to ASTM Specification D 1193, Type I. Freshly prepared water should be used for making the standards intended for calibration. The detection limits of this method will be limited by the purity of the water and reagents used to make the standards.
7.2 Purity of Reagents – Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is

first ascertained that the reagent is of sufficiently high purity to permit its use without compromising the accuracy of the determination.

7.3 Acetone ($H_2C=O=CH_2$), 90% v/v

Acetone, reagent grade	900 ml
Reagent water	100 ml

Using a graduated cylinder, add 100 ml reagent water to 900 ml acetone.

7.4 Hydrochloric Acid, 1N –

Hydrochloric acid (HCl), concentrated,	8.6 ml
Reagent water, q.s.	100 ml

In a 100 ml volumetric flask, add 8.6 ml of concentrated hydrochloric acid to ~60 ml of reagent water. Dilute to 100 ml with reagent water.
Premade solutions are also available through VWR, Fisher, and Thomas Scientific.

7.5 Blanks – A reagent blank of 90% acetone is used.

7.6 Standards – Standards used are one of the following:

7.6.1 Turner Designs Spectrophotometer standard, PN 10-950. This includes one 20 ml ampoule of known concentration in 90% acetone, accompanied by a certification from Turner Designs.

7.6.2 Chlorophyll α from Anacystis nidulans algae, PN C6144-1MG, ordered from Sigma/Aldrich. If chlorophyll from algae is not available, chlorophyll α from spinach, PN C5753-1MG, may be substituted.

7.7 Quality Control Sample (QCS) – For this procedure, the QCS can be any certified sample that is obtained from an external source. If a certified sample is not available, then use the standard material. This laboratory uses a solid filter Didymium reference cell.

8 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

8.1 Water collected for chlα should be filtered through a Whatman GF/F glass fiber filter (nominal pore size 0.7 μm), or equivalent.

8.2 Water collected for chlα should be filtered as soon as possible. If immediate filtration is not possible, the water samples should be kept on ice in the dark and filtered within 24 hours.

8.3 The filtered sample is kept frozen at -20° C or lower. Filter pads should be folded in half and may be stored in folded aluminum foil pouches.

8.4 Frozen chlα filters should be extracted within 28 days. Once the sample is extracted, the clarified extract is ready to be analyzed or may be stored at -20° C or lower and should be analyzed within the original holding time.

9 QUALITY CONTROL

9.1 The laboratory 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 instrument blanks and calibration standard material, analyzed as samples, as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of data generated.

9.2 Initial Demonstration of Capability

9.2.1 The initial demonstration of capability (iDOC) – is used to characterize instrument performance (MDLs) and laboratory performance (analysis of QC samples) prior to the analyses conducted by this procedure.

9.2.2 Method Detection Limits (MDLs) – MDLs should be established for chlα using a low-level ambient water sample. To determine the MDL values, analyze a minimum of seven replicate filtered aliquots of water. Perform all calculations defined in the procedure (Section 11) and report the concentration values in the appropriate units. Calculate the MDL as follows:

$$MDL = St_{(n-1,1-\alpha=0.99)}$$

Where,

t(n-1,1-α=0.99) = the Student's t-value appropriate for a single-tailed 99th percentile
t statistic and a standard deviation estimate with n-1 degrees of freedom.
n = number of replicates
S = Standard Deviation of the replicate analyses.

9.2.3 MDLs should be determined yearly. If more than 7 replicates are analyzed, use the appropriate n-1 value obtained from the table for the Student's *t* test.

9.3 Assessing Laboratory Performance

9.3.1 Laboratory Reagent Blank (LRB) and Reagent Blank (RB) – The laboratory must analyze at least one RB at the beginning, after every 10 samples, and at the end with each batch of samples. The RB consists of 90% acetone. The LRB is a blank filter loaded and extracted with 90% Acetone at the end of the loading process. It is analyzed at the end of each run. LRB data are used to assess contamination from the laboratory environment.

9.4 Data Assessment and Acceptance Criteria for Quality Control Measures

9.4.1 The instrument's optical performance is checked quarterly using a didymium reference standard which presents a wide range of crisply resolvable peaks which are easily used to correlate the wavelength

indicator on the spectrophotometer to the known peak. Each peak reading should fall within the manufacturer's tolerance of the wavelength readout. If the criteria are not met, the instrument must be seen by a service technician.

9.5 Corrective Actions for Out of Control Data

9.5.1 The sample is first analyzed using the 5 cm path length cuvette. If the 665 nm reading is above 1.000 absorbance units, the sample should be reread using the 1 cm cuvette. If the 665 nm reading is above 1.000 absorbance units using the 1 cm cuvette, the sample should be diluted appropriately and reanalyzed.

9.5.2 If the absorbance of the RB (reagent blank) shows an upward trend, AUTOZERO, and re-BASELINE, then reread that RB.

10 CALIBRATION AND STANDARDIZATION

10.1 Calibration – Quarterly optical performance checks are performed using certified reference material such as Didymium or Holmium Oxide cell or filter used to check wavelength accuracy.

11 PROCEDURE

11.1 Sample Preparation – water column

11.1.1 Filter a known volume of water through a Whatman GF/F filter pad (nominal pore size 0.7 μm) or equivalent. Good color is needed on the pad. Do not rinse the pad.

11.1.2 Fold pad in half, sample inside, wrap in aluminum foil, label, and freeze for analysis within 28 days.

11.1.3 Before analysis, briefly thaw pads and then place them in a 15 ml centrifuge tube. Using a repipettor, add 10 ml of 90% acetone. Include the drip released at the upward priming of the repipettor. Work under subdued lighting.

11.1.4 Write all information on the lab bench sheet.

11.1.5 Acceptable methods of cell disruption are either mechanical grinding or sonication. This laboratory uses mechanical grinding by hand. Using a Teflon pestle, grind the filter against the side of the tube until the filter is well ground. When hand grinding, 10-15 seconds is all that is necessary. Sonication and power grinding require vigilance because excess heat will degrade the chlorophyll. Shake the sample after cell disruption. Allow the sample to extract for a minimum of 2 and not to exceed 24 hours in the dark under refrigeration at 4° ± 2° C. Overnight is recommended.

11.1.6 Remove tubes from the refrigerator.

11.1.7 Shake tubes, and then centrifuge at 500-675g for 30 minutes. After centrifuging, the extract is filtered through 0.45 um PTFE or nylon syringe filters. If analyzing immediately, using a syringe, withdraw the sample and filter into the cuvette, using a few drops to sample rinse the cuvette. If the samples are not analyzed that day the extract must be transferred to a clean tube. Pull the extract from the first tube and filter while transferring it to a second numbered centrifuge tube. The transferred samples should be stored in the freezer, but must still be analyzed within 28 days of collection. When ready to analyze the samples, centrifuge again for 20 minutes at 500-675g.
The present centrifuge in this laboratory is set to 1700 rpm. To calculate rpm, use this formula:

$RCF = 1.12r(rpm/1000)^2$,

Where: RCF = relative centrifugal force
r = radius of the rotor in millimeters (usually found on the manufacturer's website)
rpm = speed of rotation

11.2 Pollution Prevention and Waste Management

11.2.1 This method generates hazardous waste.

11.2.2 Acetone waste is stored in 4-liter jugs in the cabinet under the hood and transferred to the hazardous waste area of the Storage Facility on campus.

11.2.3 Do not pour acetone down the sink.

11.2.4 Decant the waste acetone into the waste jugs, and then allow the remaining ground filter pad or sediment to dry in the hood.

11.2.5 The dried waste may then be put in the trash.

11.3 Using the Shimadzu UVProbe software:

11.3.1 Turn on the spectrophotometer (either the UV2401 or the UV2450) and the computer. Open the UVProbe software. Select photometric mode and connect to the instrument to turn on the lamps. Allow the instrument to run the lamp check and click OK. Allow the lamps to warm up for a minimum of 45 minutes before beginning sample analysis. Press GO TO WL and change the wavelength to 750 nm. Open the Method.

11.3.2 Using the 5 cm path length cuvettes, fill both the reference and sample cuvettes with 90% acetone. Wipe the windows of the cuvettes carefully with lens paper to dry. Allow the acetone blanks to sit for several minutes to reduce schlieren effects. Click on AUTOZERO, and then run a BASELINE. When the baseline is

complete, label the first line of the sample table as blk1. Click on READ UNK (unknown) or press F9 to begin scanning. All wavelengths should be very close to zero. If not, AUTOZERO again, and rerun the BASELINE. Run blk2 if needed.

11.3.3 The reference cuvette is filled with 90% acetone and is left in place. Periodically check the liquid level to be sure the meniscus does not interfere with the light path, adding more 90% acetone as needed.

11.3.4 Begin analyzing samples. Enter the sample name in the sample table twice, once with a "b" designation for before acid, and again with an "a" designation for after acid.

11.3.5 Rinse the sample cuvette with acetone after each sample. Then rinse with a small amount of sample before filling.

11.3.6 If analyzing after extraction, filter approximately 0.5 ml of the sample in to the cuvette as a sample rinse, then filter approximately 6.0 ml of sample into the sample cuvette. If analyzing after transferring/storage, sample rinse with a small amount of sample and dispense the sample into the sample cuvette using a polyethylene transfer pipette. Wipe the windows of the cuvette carefully with lens paper and place in the cell holder.

11.3.7 Check the absorbance at 750 nm. If it is at 0.007 or below, press F9 to start the scan. If it is above 0.007, wait a short time to see if the absorbance drops. If not, the sample should be filtered one more time through a 0.45 um PTFE syringe filter. If the 750 nm absorbance is still not below 0.007, proceed with the scan. It may be necessary to recheck the zero if several samples in a row start above 0.007 at the 750 nm reading. Any sample with the before acid 750 nm absorbance above 0.007 shall have a qualifier added to the report.

11.3.8 After the first scan is read, add enough 1N HCl to the sample to achieve a concentration of 0.003 N HCl within the sample. One drop of acid is used in the 1-cm path length cuvettes and 100 ul in the 5-cm cuvettes. Gently stir the sample for 30 seconds and wait another 30 seconds before starting the scan. A total of 90 seconds is needed to complete the reaction before reading. A 30-second wait is built into the method.

11.3.9 Repeat steps 11.3.4 through 11.3.8 for all samples, adding a blank after every 10 samples.

11.3.10 Run a blank at the end

11.3.11 Save the file. Right-click on Properties.

11.3.12 Hide columns TYPE, EX, and CONC. Print file.

11.3.13 Save the file again as an ASCII file to be imported into a spreadsheet for calculation.

12. Calculations:

Chlorophyll corrected for phaeophytin (µg/L):

$$\text{Chlorophyll } \alpha \text{ corrected (ug/L)} = \frac{26.7(664_B - 665_A) \times V_1}{V_2 \times L}$$

Phaeophytin (µg/L):

$$\text{Phaeophytin } \alpha \text{ (ug/L)} = \frac{26.7\,[1.7(665_A) - 664_B] \times V_1}{V_2 \times L}$$

Uncorrected chlorophyll (µg/L):

$$\text{Chlorophyll } \alpha \text{ uncorrected (ug/L)} = \frac{[11.85(664_B) - 1.54(647_B) - 0.8(630_B)] \times V_1}{V_2 \times L}$$

Chlorophyll/Phaeophytin 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 extract (mL)
- V_2 = volume of sample filtered (L)
- L = path length (cm)

13 References:

13.1 EPA Method 446.0.

13.2 APHA, Standard Methods for the Examination of Water and Wastewater, Method #10200H, 22nd Edition.