# STANDARD OPERATING PROCEDURES

**for Measurement of Chl *a* and Pheophytin Using 90% Acetone Extraction and Fluorescence Detection**

Prepared by
EcoAnalysts, Inc.
1420 South Blaine Street, Suite 14
Moscow, ID 83843

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LIFE IN WATER

**TITLE AND APPROVAL PAGE**

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Preparer:
**EcoAnalysts, Inc., Moscow, Idaho**

Address and Telephone Number:
**1420 South Blaine Street, Suite 14, Moscow, Idaho 83843 / (208) 882-2588**

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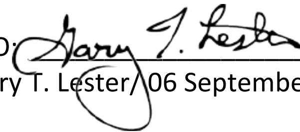
EcoAnalysts, Inc. President/CEO: 
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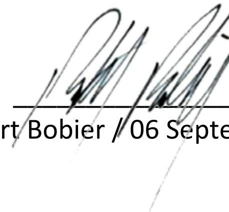
EcoAnalysts, Inc. Quality Assurance Manager: 
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# 1 SCOPE AND APPLICATION

This method is based on EPA Method 445.0 and is used to determine chlorophyll and pheophytin in algae by using fluorescence detection. Chlorophylls and carotenoids are foliar pigments related to photosynthetic efficiency in plants.

# 2 CALIBRATION AND QUALITY CONTROL USING BLANK AND CERTIFIED CHL A STANDARDS

## 2.1 Equipment and Laboratory Setup

a. Volumetric flasks
b. Micropipettes and tips
c. Pasteur pipettes
d. Pipet bulbs
e. Chlorophyll standards
f. Turner Solid Standard Reference
g. Optically matched cuvettes
h. Turner Trilogy Fluorimeter-Model 7200-000
i. Yellow light
j. 90% acetone (Acetone HPLC grade and Nanopure H20)
k. Clay Adams Dynac II Centrifuge and tubes
l. Tissue grinder
m. Aluminum Foil
n. Squirt Bottles
o. 0.1 N HCl

## 2.2 Standard Preparation

Standard calibration runs will be conducted at the beginning of each sample batch run. Standard Preparation: Dissolve standard in 90% acetone to appropriate volume and dilutions to produce the following standard concentrations or similar:

| Final Concentration |
|---|
| 0.5ug/L |
| 1 ug/L |
| 5 ug/L |
| 12 .5 ug/L |
| 25 ug/L |
| 50 ug/L |
| 100 ug/L |
| 250 ug/L |
| 500 ug/L and higher if needed |

### 2.3 LDR and IDR Determinations

a. LDR (Linear Dynamic Range) Determination - Analyze a minimum of 5 calibration standards ranging in concentration from 0.2 to 200 ug/L Chl *a*. Perform linear regression of normalized response vs concentration and obtain slope and y- intercept. Continue measurements for standards of increasing concentration until the fluorescence response indicates a concentration within 10% of known value. This is the upper limit of the instruments linear dynamic range.
b. IDL (Instrumental Detection Limit) Determination -Measure serially diluted standards until known values are no longer determinable and changing concentrations no longer detected.
c. EDL (Estimated Detection Limit) Determination - The EDL will be calculated as the concentration of standard that is equivalent to three times the average response of the blank filters.

### 2.4 Quality Control Checks

For each run, provide measurements for blank and standards of known concentration for the before and after acidification process in addition to measurement of the solid reference secondary standard.

| QA Check | Frequency | Acceptance Criteria | Corrective Action | Procedure if Corrective Action Fails |
|---|---|---|---|---|
| Blank | At least 1 per batch, rerun after 30 samples | Observed value within 5 % of known value | Maintenance and/or recalibration until value meets acceptance criteria | Analyze samples, report data with quality flag |
| QC Reference or Standard | At least 1 per batch, rerun after 30 samples | Observed value within 5 % of known value | Maintenance and/or recalibration until value meets acceptance criteria | Analyze samples, report data with quality flag |

### 2.5 Proficiency Testing

Proficiency Testing for Chlorophyll Pigment Analysis will be completed using Sigma-Aldrich PT Services for any individual analyst performing project pigment analysis.

## 3 ANALYSIS OF UNCORRECTED CHLOROPHYLL *A*, PHEOPHYTIN CORRECTED CHLOROPHYLL *A* AND PHEOPHYTIN *A*

### 3.1 Equipment and Laboratory Setup

a. Volumetric flasks
b. Micropipettes and tips
c. Pasteur pipettes
d. Pipet bulbs
e. Chlorophyll standards

f. Turner Solid Standard Reference
g. Optically matched cuvettes
h. Turner Trilogy Fluorimeter-Model 7200-000
i. Yellow light
j. 90% acetone (Acetone HPLC grade and Nanopure H20)
k. Clay Adams Dynac II Centrifuge and tubes
l. Tissue grinder
m. Aluminum Foil
n. Squirt Bottles
o. 0.1 N HCl

### 3.2 Sample Handling

**a. Sample analysis will be completed within the 14 day holding period.**
**b. All samples will be maintained on ice and/or handled in dark or under yellow light at all times for all of the following steps.**
c. Each sample filter will be placed in glass grinding tube and pushed to bottom with glass rod.
d. Add 4 mL 90% acetone.
e. Grind to slurry and then grind for about 1 minute at 500 rpm taking care not to overheat the sample.
f. Pour slurry into a 15 mL screw-cap centrifuge tube taking care to rinse pestle and grinding tube with no more than 6 mLs 90% acetone.
g. Add this rinse to centrifuge tube containing filter slurry.
h. Cap the tube and shake vigorously.
i. Place in dark before proceeding to next filter extraction.
j. Clean the pestle, grinding tube and glass rod with dH20 and final rinse of 90% acetone between samples.
k. Repeat steps c. through j. for each sample.
l. Last filter to be extracted should be a blank.
m. Shake each tube vigorously again before placing in dark at 4C.
**n.** Steep for 2-24 h. **Steeping is complete when green pigmentation is no longer observed in intact materials upon observation of solution in the extraction centrifuge tube. If unsure, random samples can be measured for Rb at selected time intervals within the 24 time period to determine if Rb's are stable or increasing over time. Once stable, extraction is considered complete.**
o. After steeping is complete, centrifuge samples for 5 min at 1000g.
p. Pour 3 mls supernatant of extracted sample into sample cuvette.
q. Record the fluorescence measurement (Rb (i.e., fluorescence before acidification)).
**r. Dilution with 90% acetone will be used if needed to maintain sample fluorescence readings within the range of the standard curve.**
**s. Pheophytin step: Remove the cuvette from the fluorometer and acidify the extract to a final concentration of 0.003 N HCl using the 0.1 N HCl solution (i.e., 0.09 mL of 0.1 N HCl should be used to acidity 3 mL extract). Mix with pipette while keeping below surface of liquid to prevent aerating the sample.**
t. Wait 90 seconds.
u. Remeasure fluorescence (Ra, i.e., fluorescence after acidification).

### 3.3 Data Analysis and Calculations

Raw Fluorescence Mode: obtain the fluorescence value of a sample in Raw Fluorescence Units (R). Use a standard curve to determine the concentration of the analyte in the samples. A blank sample should be run to determine background fluorescence or scatter. A solid secondary standard may be used to verify instrument stability and function. Standard concentration samples will be used to generate the standard curve and calculation of Favg for each sample batch. Conversion of R's to uncorrected Chl*a* values will be based on regression analysis and results in addition to equations using F provided in EPA Method 445.

<u>**Calculations (See Examples Below)**</u>

**Response Factor (F)**

The Turner Trilogy fluorimeter does not have a sensitivity setting.

F = C/R will be determined for each standard in a calibration curve, the average taken to represent 'Favg' for each particular sample batch.

R = fluorescence reading

C=concentration for chlorophyll a

| | |
|---|---|
| **C_uncorrected (Uncorrected Chl *a*)** | **C_uncorrected=(Rb-Rblank_avg)*Favg** where: C=Chla concentration; Favg=average response factor for multiple standards in a standard run; Rb=fluorescence before acidification; Rblank_avg=average fluorescence of blanks |
| **C_corrected (Corrected Chla)** | **C_corrected=Favg*(r/(r-1))*(Rb-Rblank_avg)-(Ra-Rblank_avg)** where: C=Chla concentration; r=(Rb-Rblank_avg)/(Ra-Rblank_avg); Favg=average response factor for multiple standards in a standard run; Rb=fluorescence before acidification; Ra=fluorescence after acidification; Rblank_avg=average fluorescence of blanks |
| **Pheophytin *a*** | **Pheophytin *a*=Favg*(r/(r-1))*(r(Ra-Rblank_avg)-(Rb-Rblank_avg))** where: C=Chla concentration; r=(Rb-Rblank_avg)/(Ra-Rblank_avg); Favg=average response factor for multiple standards in a standard run; Rb=fluorescence before acidification; Ra=fluorescence after acidification; Rblank_avg=average fluorescence of blanks |

**EXAMPLE CALCULATIONS FOR CHLOROPHYLL a (CORRECTED AND UNCORRECTED) AND PHEOPHYTIN**

| Chla Concentration ug/L (C) | Fluorescence Reading Before Acidification (Rb) | Analysis Date | F (Response Factor)=C/(Rb-Rblank) | Calculated Chla using Favg: C=(Rb-Rblank)*Favg | Calculated Chla using Regression Line Equation of Standard Curve (for quality comparison) |
|---|---|---|---|---|---|
| **Blanks and Standards Using Pure Chlorophyll a from Spinach** | | | Regression Line Equation C=0.000365879317801807*Rb-0.956519247348062 Note: use of Favg is used for calculation of pheophytin - regression analysis is used to check the standard curve and Rb relationship to C. | | |
| **Blank** | 927.35 | 20170831 | Rblank_avg: | 931.55 | |
| **Blank** | 914.74 | 20170831 | | | |
| **Blank** | 941.86 | 20170831 | | | |
| **Blank** | 906.72 | 20170831 | | | |
| **Blank** | 967.1 | 20170831 | | | |
| 0.5 | 3163.24 | 20170831 | 0.000224 | 0.73 | 0.20 |
| 0.5 | 3147.05 | 20170831 | 0.000226 | 0.73 | 0.19 |
| 1 | 3935.63 | 20170831 | 0.000333 | 0.98 | 0.48 |
| 1 | 3836.79 | 20170831 | 0.000344 | 0.95 | 0.45 |
| 5 | 15531.75 | 20170831 | 0.000342 | 4.78 | 4.73 |
| 5 | 15493.73 | 20170831 | 0.000343 | 4.77 | 4.71 |
| 12.5 | 44976.84 | 20170831 | 0.000284 | 14.42 | 15.50 |
| 12.5 | 38179.9 | 20170831 | 0.000336 | 12.20 | 13.01 |
| 12.5 | 38132.41 | 20170831 | 0.000336 | 12.18 | 13.00 |
| 25 | 71849.52 | 20170831 | 0.000353 | 23.22 | 25.33 |
| 25 | 71506.62 | 20170831 | 0.000354 | 23.11 | 25.21 |
| 50 | 143934.81 | 20170831 | 0.000350 | 46.83 | 51.71 |
| 50 | 143154.39 | 20170831 | 0.000352 | 46.57 | 51.42 |
| 100 | 273030.68 | 20170831 | 0.000368 | 89.10 | 98.94 |
| 100 | 272378.15 | 20170831 | 0.000368 | 88.89 | 98.70 |
| | | **Favg:** | **0.000327** | | |
| | | | | | Regression Line Equation C=0.000546235676556346*Rb-0.536409761824721 |
| **Blank** | 521.92 | 20170901 | Rblank_avg: | 520.18 | |
| **Blank** | 518.43 | 20170901 | | | |
| 0.5 | 2090.45 | 20170901 | 0.000318 | 0.74 | 0.61 |
| 1 | 3544.67 | 20170901 | 0.000331 | 1.43 | 1.40 |
| 5 | 10500.57 | 20170901 | 0.000501 | 4.73 | 5.20 |

| | | | | | |
|---|---|---|---|---|---|
| 12.5 | 23587.84 | 20170901 | 0.000542 | 10.93 | 12.35 |
| 25 | 46934.32 | 20170901 | 0.000539 | 21.99 | 25.10 |
| 50 | 92983.49 | 20170901 | 0.000541 | 43.81 | 50.25 |
| 100 | 183772.76 | 20170901 | 0.000546 | 86.84 | 99.85 |
| | | **Favg:** | **0.000474** | | |
| | | **Equations:** | | | |

| | |
|---|---|
| **C_uncorrected (Uncorrected Chl *a*)** | **C_uncorrected=(Rb-Rblank)*Favg** where: C=Chla concentration; Favg=average response factor for multiple standards in a standard run; Rb=fluorescence before acidification |
| **C_corrected (Corrected Chla)** | **C_corrected=Favg*(r/(r-1))*(Rb-Rblank)-(Ra-Rblank)** where: C=Chla concentration; r=Rb/Ra; Favg=average response factor for multiple standards in a standard run; Rb=fluorescence before acidification; Ra=fluorescence after acidification |
| **Pheophytin *a*** | **Pheophytin *a*=Favg*(r/(r-1))*(r(Ra-Rblank)-(Rb-Rblank))** where: C=Chla concentration; r=Rb/Ra; Favg=average response factor for multiple standards in a standard run; Rb=fluorescence before acidification; Ra=fluorescence after acidification |

Rblank_avg: 931.55

| **Analysis Date** | **C Standard Concentration ug/L (Pure Chla Spinach Standard)** | | **F (Response Factor)=C/Rb** | **Rb** | **Ra** | **r (subtraction of blank taken into account)** |
|---|---|---|---|---|---|---|
| 20170831 | 100 | | 0.000367137 | 272378.15 | 189658.06 | 1.44 |
| 20170831 | 50 | | 0.000349273 | 143154.39 | 99759.04 | 1.44 |
| 20170831 | 25 | | 0.000349618 | 71506.62 | 46430.79 | 1.55 |
| 20170831 | 12.5 | | 0.000327805 | 38132.41 | 25295.4 | 1.53 |
| 20170831 | 5 | | 0.000322711 | 15493.73 | 9276.45 | 1.75 |
| 20170831 | 1 | | 0.000260635 | 3836.79 | 2940.15 | 1.45 |
| 20170831 | 0.5 | | 0.000158879 | 3147.05 | 2339.86 | 1.57 |
| | | **Favg:** | **0.000305151** | | | |

| | | C_uncorrected | C_corrected | Pheophytin a |
|---|---|---|---|---|
| 20170831 | 100 | 82.83 | 82.832 | 0.000 |
| 20170831 | 50 | 43.40 | 43.399 | 0.000 |
| 20170831 | 25 | 21.54 | 21.536 | 0.000 |
| 20170831 | 12.5 | 11.35 | 11.352 | 0.000 |
| 20170831 | 5 | 4.44 | 4.444 | 0.000 |
| 20170831 | 1 | 0.89 | 0.887 | 0.000 |
| 20170831 | 0.5 | 0.68 | 0.676 | 0.000 |

Regression Analysis Results for Standard Concentrations Calibration Series for Dates Presented (Standard chl concentration vs fluorescence):

Analysis Date: 20170831

Regression Equation

SUMMARY OUTPUT

Y=0.000365879317801807X-0.956519247348062

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.999461 |
| R Square | 0.998922 |
| Adjusted R Square | 0.998862 |
| Standard Error | 1.059552 |
| Observations | 20 |

**X Variable 1 Line Fit...**

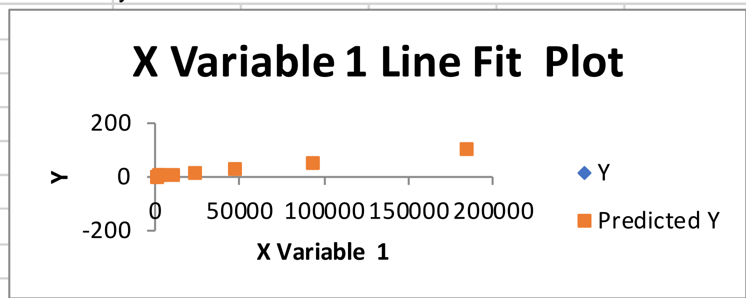


ANOVA

| | *df* | *SS* | *MS* | *F* | *ignificance F* |
|---|---|---|---|---|---|
| Regression | 1 | 18731.03 | 18731.03 | 16684.64 | 3.64E-28 |
| Residual | 18 | 20.20772 | 1.122651 | | |
| Total | 19 | 18751.24 | | | |

| | *Coefficients* | *tandard Err* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | -0.95652 | 0.287259 | -3.32982 | 0.003728 | -1.56003 | -0.35301 | -1.5600274 | -0.35301109 |
| X Variable 1 | 0.000366 | 2.83E-06 | 129.169 | 3.64E-28 | 0.00036 | 0.000372 | 0.00035993 | 0.00037183 |

| Analysis Date: 20170901 | | | **Regression equation** | | | | | |
|---|---|---|---|---|---|---|---|---|
| SUMMARY OUTPUT | | | y=0.000546235676556346x-0.536409761824721 | | | | | |




| *Regression Statistics* | |
|---|---|
| Multiple R | 0.99997265 |
| R Square | 0.99994529 |
| Adjusted R Square | 0.99993845 |
| Standard Error | 0.25547366 |
| Observations | 10 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *ignificance F* |
|---|---|---|---|---|---|
| Regression | 1 | 9543.377866 | 9543.378 | 146221 | 2.45E-18 |
| Residual | 8 | 0.52213431 | 0.065267 | | |
| Total | 9 | 9543.9 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | -0.5364098 | 0.096150378 | -5.57886 | 0.000523 | -0.75813 | -0.31469 | -0.758132932 | -0.31468659 |
| X Variable 1 | 0.00054624 | 1.42848E-06 | 382.3886 | 2.45E-18 | 0.000543 | 0.00055 | 0.000542942 | 0.00054953 |