STANDARD OPERATING PROCEDURES

for Spectrophotometric Measurement
Chl a, Chl b, and Bulk Carotenoids using Methanol Solvent

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LIFE IN WATER

TITLE AND APPROVAL PAGE

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1 SCOPE AND APPLICATION

This method is based on EPA Method 446.0 and is used to determine chlorophyll and carotenoid concentrations in plant foliage by visible spectrophotometry. Chlorophylls and carotenoids are foliar pigments related to photosynthetic efficiency in plants. In this method, methanol solvent is used to separate pigments for analysis.

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

2.1 Equipment and Laboratory Setup

a. Volumetric flasks
b. Pasteur pipets
c. Pipet bulbs
d. 4-10 mL volumetric flasks
e. Chlorophyll standards
f. Optically matched quartz cuvettes (3.5 ml; 1 cm)
g. PerkinElmer Lambda 25 Spectrophotometer with 1 cm light path and Software
h. Red (or yellow if acceptable) light
i. 100% methanol (spectrophotometric grade, > 99%)

2.2 Standard Preparation

Dissolve standard in 100% methanol (spectrophotometric grade, > 99%) to appropriate volume and dilutions to produce the following standard concentrations:

Final Concentration
1 ug/ml and lower depending on lower limit at which absorbance at a selected wavelength falls between 0.005 and 0.008 (for IDL).
3 ug/ml
6 ug/ml
9 ug/ml
12 ug/ml
15 ug/ml
18 ug/ml and higher depending on the upper limit where measured absorbance no longer yields calculated concentrations ± 10% of the known concentration (for LDR).

2.3 LDR and IDR Determinations

2.3.1 LDR (Linear Dynamic Range) Determination

1. Record absorbances at 665 nm, 652 nm, 470 nm and 750 nm for each standard concentration;
2. Plot a graph of absorbance vs concentration for each wavelength;

3. Perform a linear regression of absorbance (at pigment's wavelength maximum) vs. concentration and obtain slope and y-intercept (m and b, respectively);
4. Incrementally analyze standards of higher concentration until the measured absorbance response of a standard no longer yields a calculated concentration that is ±10% of the known concentration.

2.3.2 IDL (Instrumental Detection Limit) Determination

1. Zero the spectrophotometer using 100% methanol;
2. A standard solution of pigment is serially diluted until absorbance units for a selected wavelength (the wavelength of maximum absorption for pigment of interest) falls between
   0.005 and 0.008.

2.4 EDL (Estimated Detection Limit) Determination

1. The EDL will be calculated as the concentration equivalent of three times the standard deviation of seven replicate samples similar in type to sample material to be analyzed for the project of interest.
2. Pigment concentration should be between 2 and 5 times the IDL – dilution or spiking may be required.

2.5 Requirements for Chlorophyll and Carotenoid Analysis

2.5.1 Quality Assurance Requirements for Proceeding to Chlorophyll and Carotenoid Sample Analysis

2.5.2

QA Check	Frequency	Acceptance Criteria	Corrective Action	Procedure if Corrective Action Fails
Blank	At least 1 per batch, rerun after 30 samples	Absorbance < 0.05 at all wavelengths	Maintenance and/or recalibration until value meets acceptance criteria	Analyze samples, report data with quality flag
QA 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

2.5.3 Analyst Proficiency Testing

Proficiency Testing for Chlorophyll Pigment Analysis will be completed using Sigma-Aldrich PT Services. See Appendix 1 for current certification.

3 ANALYSIS OF CHLOROPHYLL AND CAROTENOIDS IN PLANT FOLIAGE

3.1 Equipment and Laboratory Setup

a. Volumetric flasks
b. Pasteur pipets
c. Pipet bulbs
d. 4-10 mL volumetric flasks
e. Chlorophyll standards
f. Optically matched quartz cuvettes (3.5 ml; 1 cm)
g. PerkinElmer Lambda 25 Spectrophotometer with 1 cm light path and Software
h. Red (or yellow if acceptable) light
i. 100% methanol (spectrophotometric grade, >99%)
j. Dry ice
k. Balance with 0.0001 g accuracy
l. Clay Adams Dynac II Centrifuge and tubes

3.2 Sample Handling

Immediately upon receipt of plant foliage samples, all containers are inspected for damage and sample condition and results are recorded on the Chain of Custody (COC) form. Sample labels are checked against chain of custody forms and/or packing slips and any discrepancies are noted. Receipt records are reported to the client within two business days of sample receipt. Chain of custody logs are reported, throughout the project, according to timelines and methods requested by the client.

Samples are logged into the EcoAnalysts, Inc. and given a EcoAnalyst Sample ID for tracking.

Samples will be received on dry ice and will be kept on dry ice and maintained in the dark during all transport to and from the ultra-low temperature freezer.

3.3 Sample Storage and Holding Time

1. EcoAnalysts does not have an in-house ultra-low temperature freezer and will be using space in a nearby laboratory at University of Idaho as needed. During transport between EcoAnalysts' lab where sample prep, extraction and analysis will take place and the freezer, all samples will be kept on dry ice and maintained in the dark.
2. Samples will be stored in a freezer at -80°C until analysis. Freezer is equipped with a backup generator.
3. Sample analysis will begin within 24 hours to the extent possible after receipt with pigment extraction.
4. All samples will be maintained on dry ice and/or handled in dark under yellow or red light.
5. To avoid pigment degradation, all samples will be analyzed within 7 days from receipt. If this holding time is exceeded, EcoAnalysts will proceed with analysis, flag the data, and contact the client within 48 hours of the incident.

3.4 Sample Analysis

1. Sample material will be handled to achieve maximum possible relative representation of all enclosed within any particular packet. Sample material preparation to facilitate complete extraction within 48 hr is as follows: conifer needles and elongate grasses will be cut into 1 mm lengths (desired mass: 0.15-0.2 g foliage/20 mls methanol); leaf discs will be cut into 1 mm strips (desired mass: 0.07-0.15 g foliage/20 mls methanol)
2. Sample material will be weighed with a balance with at least 0.0001 g accuracy and transferred to screw cap glass vials with 20 mls of 100% methanol chilled to 4C for extraction.
3. Extracts will be incubated in the dark at 4C for 48 hours (observed to work if sample material is

prepared as described above).

4. All sample extracts, blank, and standards will be brought to room temperature immediately prior to absorbance measurement unless otherwise specified by the client.
5. Absorbances at 665 nm, 652 nm, 470 nm and 750 nm for each sample extract will be measured in optically matched quartz cuvettes.
6. A 100% Methanol blank and selected standard will be run at onset and for every 10 samples
7. Absorbance readings will be between 0.2 and 0.9. Extract material will be adjusted by dilution or re-extraction if needed to attain acceptable absorbance values in this range taking care to adjust calculations accordingly. Should absorbance readings at 750 nm be > 0.05, extract material will be centrifuged to eliminate possible sediment interference.

3.5 Data Reporting

The following equations will be used to report pigment concentrations in addition to absorbance measurements for 665 nm, 652 nm, 470 nm and 750 nm, fresh foliage mass for which pigments were extracted and volume of extraction solvent:

a. Chl a (µg/mL) = [16.72*(A665 – A750) – 9.16*(A652-A750)]*dilution factor
b. Chl b (µg/mL) = [34.09*(A652-A750) – 15.28*(A665-A750)]*dilution factor
c. Bulk Carotenoids (µg/mL) = [(1000*(A470-A750) – 1.63* Chl a – 104.96*Chl b)/221]*dilution factor

1. Results will be submitted using the client's requested data sheets. Data ingest sheets will be shared or uploaded as directed.
2. Data will be generated by hand using spreadsheets developed with the assistance of Battelle. QC and validation capabilities will be put in place and tested to ensure function for future correctness in data submissions.
3. If issues with data are detected, EcoAnalysts will correct errors and re-submit. This process will continue until the data file is accepted.

4 SPECIAL HANDLING OF METHANOL

Methanol is flammable and poses health risks. EcoAnalysts will distribute MSDS/Chemical Safety information to all laboratory personnel and will use a hazardous waste pickup service to dispose of methanol. EcoAnalysts regularly handles hazardous materials and is familiar with proper storage and disposal.