STANDARD OPERATING PROCEDURES

for Spectrophotometric Measurement of Chlorophyll a, Chlorophyll b, and Bulk Carotenoids Using Methanol Solvent

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

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TITLE AND APPROVAL PAGE

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Prepared by:
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: Gary T. Lester

Gary T. Lester/ 12 May 2020

EcoAnalysts, Inc. Quality Assurance Manager: _____

Robert Bobier / 12 May 2020

Table of Revisions

Revision Number	Revision Date	Revisor	Reason for Revision
2	20200512	L. Underwood	More clarification on procedures for NEON

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1.0 SCOPE AND APPLICATION

This method is based on that described by Lichtenthaler and Buschmann (2001) 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.

1.1 Equipment and Laboratory Setup

- Volumetric flasks
- Pasteur pipets
- Pipet bulbs
- 4-10 mL volumetric flasks
- Chlorophyll standards
- Optically matched quartz cuvettes (3.5 ml; 1 cm)
- PerkinElmer Lambda 25 Spectrophotometer with 1 cm light path and Software
- Red (or yellow if acceptable) light
- 100% methanol (spectrophotometric grade, > 99%)
- PPE includes: Lab coat, gloves, and protective eye wear
- Starna Glass Reference Filters
- Razor blades
- Aluminum dishes
- Spatulas
- Kim wipes
- Paper toweling
- Distilled H2O

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

2.1 Standard Preparation

The standards are purified chl *a* and chl *b* ordered from Sigma in 1 mg quantities. Each 1 mg of standard material is dissolved in 100 ml 100% methanol (spectrophotometric grade, > 99%) for an initial concentration of 10 ug/ml pigment. This is the primary standard.

An aliquot of primary standard is taken to prepare the secondary standards to be used for calibration checks at the onset and during each analytical batch run. Secondary standard concentrations include: 10 ug/ml (undiluted portion of the primary standard) and 5 ug/ml (primary standard aliquot diluted 1:1 with 100% methanol). Secondary standards are prepared in this manner for each chl *a* and chl *b*.

2.2 Requirements for Chlorophyll and Carotenoid Analysis

Quality Assurance Requirements for Proceeding to Chlorophyll and Carotenoid Sample Analysis

QA Check	Frequency	Acceptance Criteria	Corrective Action	Procedure if Corrective Action Fails
Blank	At onset and every 10 samples per batch.	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	Chl *a* and chl *b* standards (8 and 5 ug/ml for each) will be run at the onset of each batch. Chl *a* and chl *b* standards (5 ug/ml for each) will be run every 10 samples thereafter within the batch.	Observed value within 5 % of known value	Maintenance and/or recalibration until value meets acceptance criteria	Analyze samples, report data with quality flag

2.3 Instrument Performance Verification

Instrument performance verification will be conducted at the beginning of any day on which sample analysis is to be conducted using certified Starna density filters. Should a 'fail to pass' result, steps will be taken to troubleshoot the issue (check chambers, lamps, connections) and the test rerun. Should a problem persist, a Perkin-Elmer representative can be contacted for immediate remote assistance.

3.0 ANALYSIS OF CHLOROPHYLL AND CAROTENOIDS IN PLANT FOLIAGE

3.1 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. The Client will be notified at this time of any issues with the condition of the samples. NEON POC's will determine if samples should be analyzed if they have been flagged for issues. 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. LIMs (AEGIS) and given an EcoAnalysts' Sample ID for tracking.

Samples will be received on dry ice and maintained on dry ice (approximately -80 C) in a Styrofoam cooler in the dark in a -6C freezer to help preserve dry ice integrity. The freezer itself is equipped with thermometer in which an alarm will sound should the freezer temperature rise above -6C. A temperature log is maintained with the use of calibration fluid in the freezer. The dry ice will be checked daily and replenished as the needed to maintain the samples within the

required holding conditions. Personal protective equipment outlined above should always be worn while handling dry ice.

3.2 Sample Storage and Holding Time

1. Samples will be stored on dry ice until analysis.
2. Sample analysis will begin within 24 hours to the extent possible after receipt with pigment extraction.
3. All samples will be maintained on dry ice and/or handled in dark under yellow or red light.
4. 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.

3.3 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 pieces (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). Razors, dishes, etc. are cleaned between samples.
2. 20 ml aliquots of 100% methanol will be prechilled in sealed screw top vials in a freezer at -6C for at least several hours in preparation for sample extraction. Sample material will be weighed with a balance with at least 0.0001 g accuracy and transferred to the prechilled methanol. The vials will be resealed and returned to the refrigerator to be maintained in dark and at 4C for 48-hour extraction process.
3. Extraction in the dark at 4C for 48 hours has been observed to work effectively 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. The Spectrophotometer is turned on and allowed to warm up for 45 minutes prior to taking any measurements.
5. Sample material will be transferred to optically matched quartz cuvettes and then absorbances measured at 665 nm, 652 nm, 470 nm and 750 nm for each sample extract.
6. A 100% methanol blank and selected standard will be run at onset and for every 10 samples
7. Absorbance readings for 665 nm will be between 0.2 and 0.9. Extract solution 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.
8. Blanks and 5 ug/ml standards for chl a and chl b will be analyzed every 10 samples to assess instrument performance and stability.
9. The ability to detect abnormalities such as drifting from sample to sample and over time is possible for each absorbance scan as each new scanline can be viewed on a cumulative graph. There are areas for each scan for which all absorbance readings should be same as those of the blank. Any shifting from one scan to another would be indicative of drift. If drifting is detected, we will investigate to see if we can determine the cause of drift. The Spectrophotometer may be too warm and need to be shut off to cool down for 1-2 hours. If there is not a correctable action to take to stop drifting Perkin Elmer will be contacted for machine support to ensure the software is not causing the drift. If these solutions do not solve the issue, the back up spectrophotometer will be utilized, and results compared.

3.4 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:

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

Review and Submission

1. The Primary technician will generate data by hand using spreadsheets developed with the assistance of Battelle. An Initial review is performed by the primary technician. Data that has been reviewed will be highlighted to indicate the review is complete. A secondary Chlorophyll technician will also review the data for errors as an additional QC check.
2. Results will be submitted using the client's requested data sheets. Data ingest sheets will be shared or uploaded as directed.
3. If issues with data are detected, EcoAnalysts will correct errors and re-submit. This process will continue until the data file is accepted.

4.0 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.

5.0 REFERENCES

Lichtenthaler, H. K. And C. Buschmann. 2001. Chlorophylls and carotenoids: measurement and characterization by UV-VIS spectroscopy. Curr. Protoc. food Anal. Chem. 1(1), F4.3.1-F4.3.8.