STANDARD OPERATING PROCEDURES

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

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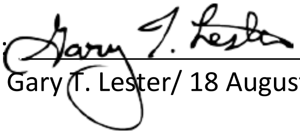
EcoAnalysts, Inc. President/CEO: 
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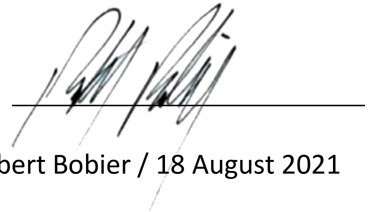
EcoAnalysts, Inc. Quality Assurance Manager: 
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Table of Revisions

Revision Number	Revision Date	Revisor	Reason for Revision
2	20200512	L. Underwood	More clarification on procedures for NEON
3	20210818	S. Hengen	Revised section 2.1 to describe secondary standard created from the primary standard; 2.2 adding in secondary standard values. Updated glassware language throughout the document. Updated language in 3.3 for corrective action for high absorbance reading for 750nm and how diluent volume is measured.

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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 capacity in plants. In this method, methanol solvent is used to separate pigments for analysis.

1.1 Equipment and Laboratory Setup

- Pasteur pipets
- Pipet bulbs
- 4-10 mL volumetric flasks
- 20 mL scintillation vials
- Graduated pipettes
- Chlorophyll from Spinach standards (when Spinach unavailable, Chlorophyll from *Anacystis nidulans* algae will be used)
- 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 towels
- Tin foil
- Distilled water
- Freezer
- Refrigerator
- Balance
- Forceps
- 100 mL amber glass containers
- Single-channel manual pipette and tips
- Traceable thermometers

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 as ampules of a predetermined weight. These ampules are stored in the dark in a -20 C freezer. Each ampule of standard material is dissolved in 100% methanol (spectrophotometric grade, > 99%) in the dark conditions or yellow light. If using 1mg (1000 ug) of standard material, it will be dissolved in 100% methanol solution using a 100 mL volumetric

flask for an initial concentration of 10 ug/ml pigment. This is the primary standard. Once created, the primary standard is again stored in the dark in a -20 C freezer. A log will be kept for the standard to determine longevity and degradation based on observed and known values. When the observed value of the standard no longer meets the QA acceptance criteria (Section 2.2) a new primary standard will be prepared. Additional standard ampules will be on hand in case of degradation as distributor availability allows. Standards will be tested prior to running samples.

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 µg/ml (undiluted portion of the primary standard), 6 µg/ml (primary standard aliquot diluted 3:2 using a single channel manual pipette with 100% methanol), and 4 µg/ml (primary standard aliquot diluted 2:3 using a single channel manual pipette with 100% methanol). Secondary standards are prepared in this manner for both chl *a* and chl *b* prior to each sample run to avoid degradation of lower chlorophyll concentrations over multiple sample runs. A log will be maintained to monitor standard concentrations.

Standard Concentration (µg/ml)	Volume of Standard (ml)	Volume of Methanol (ml)	Acceptance Range (µg/ml)
10 (primary concentration	n/a	n/a	9.5 to 10.5
6	3	2	5.7 to 6.3
4	2	3	3.8 to 4.2

2.2 NEON Quality Control 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 (10, 6 and 4 µg/ml for each) will be run at the onset of each batch. Chl *a* and chl *b* standards (6 µg/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. When standard observed concentration is more than 5% from the known value a new standard will be prepared. New standard will be on hand prior to values not meeting criteria. Standards will be tested prior to running samples.	Analyze samples, report data with quality flag . Report quality flag of '1' in qaQF field of the Batch QA ingest file. Include details in remarks field. In the Sample Data table, 'relativeAccuracyScale', select the corresponding letter designation as defined by NEON field descriptions

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. Documentation of Starna standard analysis and results will be maintained. Should a 'fail to pass' result occur, steps will be taken to troubleshoot the issue (check chambers, lamps, connections) and the test rerun. Should a problem persist, a Perkin-Elmer representative will be contacted for immediate remote assistance. The Client will be notified if the problem cannot be rectified and/or impacts analytical workflow.

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 -20 C 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 -14C, and where the allowable tolerance is between -30 to -15C. A temperature log is maintained with the use of calibration fluid in the freezer. The dry ice will be checked daily and replenished as 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 48 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 in the Sample Data Table by marking the 'handling QF' as '1', and contact the client within 48 hours of the incident.

3.3 Sample Analysis

1. Sample material will be prepared for extraction within 48 hours of receipt.
2. Sample material will be handled to achieve random relative representation of each enclosed sample by cutting portions of the sample with a razor blade, homogenizing, and then subsampling to desired mass range. 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.

3. Using a graduated pipette 20 ml aliquots of 100% methanol will be prechilled in sealed screw top vials in a freezer at -20C 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.
4. Extraction in the dark at 4C for 48 hours has been observed to work effectively if sample material is prepared as described above.
5. 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.
6. A 100% methanol blank and 10 µg/ml, 6 µg/ml, and 4 µg/ml standard will be run at onset of each batch prior to samples. Additional 100% methanol blank, 6 µg/ml standards will be run for every 10 samples.
7. 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.
8. Absorbance readings for 665 nm should be between 0.2 and 0.9 ug/ml. 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. Diluent fluid used will be measured with a volumetric pipette to ensure accurate dilution factors. Should absorbance readings at 750 nm be > 0.05 ug/ml, cuvette will be cleaned and checked for particulates and the sample will be re-run.
9. Blanks and 6 µg/ml standard for chl a and chl b will be analyzed every 10 samples to assess instrument performance and stability.
10. 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 instrument drifting is detected based on baseline reading, 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 backup spectrophotometer will be utilized, and results compared.

4.0 Data Reporting

The Spectrophotometer software records and outputs data electronically. This dataset is then uploaded electronically to a spreadsheet for further analysis. 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

Note that for undiluted samples, the dilution factor = 1, for a 50% dilution the dilution factor = 2, etc.

4.1 Review and Submission

1. The Primary technician will generate data transferred from The Spectrophotometer software electronically. The dataset is then uploaded to spreadsheets with final calculated values reported. An initial review of standard measurement deviation and sample data is performed by the primary technician. Data that have been reviewed will be highlighted to indicate the review is complete. The Principal Scientist, Lisa Underwood, will review standard readings, absorbance values, and corrected chlorophyll prior to submission. Results will be submitted using the client's requested data sheets. Data ingest sheets will be shared or uploaded as directed.
2. If issues with data are detected, EcoAnalysts will correct errors and re-submit. This process will continue until the data file is accepted.

5.0 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.

6.0 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.