Horn Point Analytical Services Laboratory

SOP Foliar Pigment Analysis

Version 1.2 (Battelle Service Contract); Dec 5, 2018

Plant tissue pigments are passively extracted under low light into 100% methanol. Spectrophotometric methods are used for the quantitative analysis of chlorophylls *a* and b, and bulk carotenoids.

Methodology

Lichtenthaler, H.K. and Buschmann, C. (2001) Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy. *Current protocols in Food and Analytical Chemistry*, F4.3.8-F4.2.4.

Instrumentation

Spectrophotometer: Shimadzu UV-2401PC (UV-VIS) spectrophotometer.
Freezer: -80^{o}C, with 24/7 emergency power and alarmed, for sample storage.

Sample Preparation and Storage

Note: The following procedure is specific for the NEON samples (Battelle service contract). These standard operating procedures are kept on file in the Analytical Services laboratory.

1. Sun-lit foliage samples for pigment analysis will be collected by NEON project field personnel and flash frozen in the field. Subsamples will be stored in an ultra-low (i.e., -80^{o}C) freezer. Within 7 days, frozen subsamples will be packaged with dry ice and shipped overnight to HPL Analytical Services. Shipments will include a hard-copy manifest and be preceded by an email notification of shipment, containing an electronic copy of the manifest and receipt form.

2. Upon receipt of samples, HPL Analytical Services will ensure all samples are in good condition (e.g., dry-ice still present in cooler, samples not damaged, sample identification present and legible, etc.). If samples are received in compromised condition, HPL Analytical Services should notify the Battelle Technical Representative within two business days. An electronic receipt form that will be emailed with the shipping information is also to be completed to document condition of samples upon receipt. Each completed receipt form will be uploaded to the NEON Data Portal.

3. Samples will be stored at -80oC until analysis.

4. To avoid pigment degradation, ***samples should be analyzed within 7 days from receipt*** **so that the holding time of 14 days from collection is not exceeded**. If

this holding time is exceeded, HPL Analytical Services should proceed with analysis, flag data appropriately, and contact the Battelle Technical Representative within 48 hours of the incident.

General Analytical Procedure

Extraction Procedures

1. During all steps, minimize evaporation of extraction solvent and light exposure. Workspace lighting should be the minimum that is necessary to read instructions and operate instrumentation.
2. If samples contain Toxicodendron, samples are to be handled in biosafety hazard level II building (named, BSLII)
3. Extraction solvent is 100% methanol (spectrophotometric grade; > 99%), chilled to 4^{o}C in a refrigerator. Have ready a bottle of 100% methanol with a calibrated bottle-top dispenser for organic solvents chilled in a refrigerator.
4. Immediately prior to extraction, remove subsamples from the ultra-low (-80^{o}C) freezer. If multiple subsamples are removed, the rest are kept in on dry ice and maintained in the dark (i.e., in a cooler).
5. Prepare tubes with 100% methanol: Label tubes and add 10.0 mL chilled 100% methanol, and cap immediately. Store tubes in refrigerator until samples are ready.
6. Remove a single foliar sample from cooler with dry ice. Working quickly to prevent sample warming, cut sample into 1mm wide pieces using a razor blade.
7. Weigh samples using a high accuracy balance (better than 0.1 mg precision);
    - for needles and grasses, target 75 – 100 mg foliar material / 10.0 mL extraction solvent
    - for leaf discs, target 35 - 75 mg foliar material / 10.0 mL extraction solvent
8. Transfer weighed foliar sub-sample to chilled 100% methanol tube; re-cap immediately
9. For each sample, record tube ID and total fresh weight of foliar subsample added to solvent.
10. Transfer vials to the refrigerator, and allow solvent to extract pigments for 18-36 hrs at 4^{o}C. After extraction, samples should be white, indicating pigments have been fully extracted. Samples may require a full 36 hours for this to be complete.
11. Once pigments have been fully extracted, decant extract into clean glass scintillation vials with foil-lined caps, and kept on ice in darkened environment. Remaining extract may be stored in a freezer (-20^{o}C) until all analysis is complete and data has been reported to Battelle.

Spectrophotometric Analysis – QA Standards

12. Turn on UV-Vis spectrophotometer ~ 30 mins prior to use
13. Use quartz cuvettes (1cm pathlength) for all readings
14. For blanks, standards, and samples, absorbance is measured and recorded at wavelengths 750, 665, 652, and 470 nm
15. **Zeroing solution and Blank**: 100% methanol
16. **Quality Assurance (QA) Standard**:
    - Chl *a* standard (Sigma C5753), dissolved in 100% methanol
17. To begin, the spectrophotometer is zeroed, using the zeroing solution.
18. To verify the spectrophotometer is operating within acceptable limits, a blank and the QA standard is analysed at the 4 wavelengths identified. The blank and QA standards must fall within acceptance criteria, as described in Table 1, before analysis of samples may proceed. If the blank or QA standard does not meet the acceptance criteria, then corrective action, as described in Table 1, is undertaken.
19. The QA standard and a blank are run every 30 samples to monitor instrument drift.

Table 1. QA Requirements for Chlorophyll and Carotenoid Analysis

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 (750, 665, 652, 470 nm)	Maintenance and/or recalibration until value meets acceptance criteria	Analyze samples, report data with quality flag as specified by Battelle
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 as specified by Battelle

Spectrophotometric Analysis -- Samples

20. Transfer about 1 mL of extract to the quartz cuvette. Absorbance readings should be between 0.2 and 0.9. Values higher than 0.9 should be diluted and re-analyzed. Values lower than 0.2 should be re-extracted using a smaller volume of methanol, if sufficient material is available. If this is not possible, the data should be reported with a quality flag. If samples have too much sediment (i.e. absorbance reading at 750 nm > 0.05), those samples must be centrifuged and re-analyzed.
21. Raw absorbance data is recorded, and the following pigment concentrations are calculated and reported using the following equations:

Chl *a* (µg/mL) = [16.72*(A665 – A750) – 9.16*(A652-A750)]*DF

Chl *b* (µg/mL) = [34.09*(A652-A750) – 15.28*(A665-A750)]*DF

Bulk Carotenoids (µg/mL) = [(1000*(A470-A750) – 1.63*Chl *a* – 104.96*Chl *b*)/221]*DF

where DF = dilution factor

Data Reporting

The HPL Analytical Services Laboratory will submit results electronically to Battelle using the data ingest sheets provided by Battelle within 45 days.

Sample and batch QA data will be submitted for every sample set. Long-term lab summary and parameter datasheets will be submitted once during each year of the contract, or whenever those change.

HPL Analytical Services Laboratory will continue to hold any remaining sample material in a freezer at -80°C for 30 days after the data is reported. After this time, HPL Analytical Services Laboratory should dispose of the material unless directed otherwise by the Battelle Technical Representative.