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: -80ºC, 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., -80ºC) 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 and enter remarks accordingly in the electronic receipt form. Upon completion of sample custody, the receipt form (that accompanies the electronic shipping manifest) will to be uploaded to the NEON Data Portal within 48 hours of receiving shipment.

3. In general, samples will be stored at -80ºC until analysis. However, if samples can be analyzed within 24 hours of receipt at HPL, they will remain in the coolers in which they were shipped on dry ice.

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. Always work in a dimly lit environment, with no overhead lighting.
2. If samples contain Toxicodendron, samples are to be handled in biosafety hazard level II building (named, BSLII)
3. Prepare extraction area to process samples in an efficient manner: Pre-label tubes, prepare ice bath in cooler, and gather items for slicing and weighing the plant material.
4. Verify calibration of balance. Zero balance.
5. Select the desired number of samples to be analyzed within the given time frame (this is an overnight extraction) and place in a cooler with dry ice.
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 (for needles and grasses) or metal paper cutter (for leaf discs) until the desired weight is reached.
7. Weigh sample using a high accuracy balance (better than 0.1 mg precision);
    - for needles and grasses, target 75 – 100 mg foliar material / 8.0 mL extraction solvent
    - for leaf discs, target 35 - 75 mg foliar material / 8.0 mL extraction solvent
8. Transfer weighed foliar sub-sample to pre-labeled tubes, add desired volume of 100% methanol, cap and parafilm top immediately, and add tube to a rack in an ice bath. Return collection bag with remaining parent sample back to cooler with dry ice.
9. For each sample, record tube ID and total fresh weight of foliar subsample, which was added to solvent.
10. Before pulling the next sample out of the dry ice, use 100% methanol to wipe down all items used with slicing the material. In the same manner, wipe down tweezers and other surfaces the processed material touched, so as to not cross-contaminate the next sample to be processed.
11. Once all samples have been processed for extraction, vortex each tube for 20 seconds and visually verify all subsample material is suspended under the surface of the extraction solvent before returning tube to ice bath.
12. When all tubes have been vortexed, empty cooler of ice bath, and transfer cooler with tubes to a 4^{o}C refrigerator and allow solvent to extract pigments for 18-36 hours.

13. The following day, check each sample tube to verify all foliage lacks color (look white-ish), indicating pigments have been fully extracted. Samples may require a full 36 hours for this process to be complete
    a. If plant material looks like more pigment could be extracted, then return cooler to 4°C refrigerator for another 2-12 hours, not to exceed 36 hours.
14. Once samples are fully extracted, remove tubes from the refrigerator and vortex each for 20 seconds.
15. Centrifuge tubes at 2000rpm for 10 minutes.

Spectrophotometric Analysis -- QA Standards

16. Turn on UV-Vis spectrophotometer ~ 30 mins prior to use and allow the instrument to equilibrate. For blanks, standards, and samples, absorbance is measured and recorded at wavelengths 750, 665, 652, and 470 nm. Use quartz cuvettes (1cm pathlength) for all readings.
17. Auto balance instrument and 'zero' with 100% methanol. **Zeroing solution and Blank**: 100% methanol
18. **Quality Assurance (QA) Standard**:
    - Chl *a* standard (Sigma C5753), dissolved in 100% methanol
19. To verify the spectrophotometer is operating within acceptable limits, a blank and the QA standard are analyzed 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.
20. Include a blank and a QA standard 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

21. After samples have been centrifuged, transfer 1.5 mL of extract from the first sample to the quartz cuvette. Absorbance readings should be between 0.2 and 0.9.
    a. Values higher than 0.9 should be diluted and re-analyzed.
    b. 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.

22. When all samples have been properly analyzed, record and transfer data to an excel spreadsheet for further calculations to determine the concentrations of agreed upon pigments of interest.
23. Compile client's report and submit all data in the electronic format specified by the client, and via the client's data portal.
24. 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 (a whole number, 1 = no dilution, 2 = 50% dilution, etc)

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.