STANDARD OPERATING PROCEDURE FOR THE DETERMINATION OF FOLIAR CHLOROPHYLL *a*, *b*, & CAROTENOIDS BY THE SPECTROPHOTOMETRIC METHOD FOR THE NEON PROGRAM, ABBREVIATED

CHM 2052A

Adapted from:
Lichtenthaler, H. K. and C. Buschmann. 2001

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Great Lakes Environmental Center, Inc.
(GLEC)

Michelle Moore — 6/3/2025
Michelle Moore — Date
Technical Author

Dennis McCauley — 6/4/2025
Dennis McCauley — Date
President/Senior Environmental Scientist

Jennifer Hansen — 6/3/2025
Jennifer Hansen — Date
GLEC Quality Assurance Officer

Changes made in this version:

- Added use of grinding mill.
- Added control charting calculation for RPD.
- Added new sections for corrective action, contingencies and criteria.
- Updated to Version 2.0.

Changes made in version August 15, 2024, Version 1.1:

- Changing from Version 1.0 to 1.1.
- Changed the word strips to pieces, Section 2.1.
- Added that methanol should be chilled to 4°C.
- Added sample codes for Toxicodendron species.
- Added direction to start extractions within 14 days of collection date.
- Deleted 3 Result Codes in Table 1.
- Added distinction between extraction times for leaf and needle type samples.
- Added language to ensure that samples have turned white.
- Removed reference to a glass cutting board.
- Added that pieces should be 1 mm or smaller with smaller being preferred for needles.
- Added that needles should be at high end of the weight range.
- Added that samples should be analyzed within 1 to 2 hours of target extraction time.

TABLE OF CONTENTS

I. SCOPE AND APPLICATION

1.1 This Standard Operating Procedure (SOP) describes the procedure for the passive extraction of plant tissue pigments, under low light using 100% methanol and the subsequent spectrophotometric determination of chlorophylls *a* and *b*, and carotenoids. In plants, chlorophylls and carotenoids are pigments found in the foliage that regulate photosynthesis.

1.2 Foliar plant tissue is ground in a mill or cut into 1 mm pieces (if needed) and extracted for spectrophotometric analysis at wavelengths of 470, 652, 665 and 750 nm. Chlorophyll *a*, *b* and carotenoid concentrations will be determined by inserting the absorbance at each wavelength into calculations.

II. EQUIPMENT AND SUPPLIES

2.1 Aluminum foil – used to protect plant material and extracts from light.

2.2 Analytical balance, capable of weighing to the nearest 0.0001 g.

2.3 Disposable weigh boats.

2.4 Insulated cooler.

2.5 Cryofreezer, -80℃

2.6 Cutting board.

2.7 Cuvettes – for the spectrophotometer, optically matched, with 1 cm path length.

2.8 Dry ice.

2.9 Eppendorf pipette.

2.10 Forceps – for handling plant tissue.

2.11 Freezer, -6℃.

2.12 Graduated cylinder – 25 mL.

2.13 Kimwipes, or equivalent.

2.14 Lamp, red or yellow filtered.

2.15 Manicure scissors.

2.16 Pasteur pipettes, disposable glass. Pasteur pipette bulb.

2.17 Pipettes, volumetric – glass, variety of sizes.

2.18 Razor blade or similar tool.

2.19 Refrigerator, 4°C.

2.20 Solvent-safe Eppendorf pipette tips.

2.21 GENESYS 50 UV-Vis spectrophotometer.

2.22 Volumetric flask – 200 mL.

2.23 Glass stirring rod.

2.24 Parafilm.

2.25 Glass funnel.

2.26 Starna filters set RM-1N2N3N.

2.27 IKA A 10 Basic Mill - IKA™ 25002269, or equivalent.

III. REAGENTS AND STANDARDS

3.1 Reagents –

3.1.1 100% methanol. Spectrophotometric grade, ≥ 99%, chilled to 4ºC.

3.2 Standard Solutions –

3.2.1 QAR solution – Use purchased solid chlorophyll *a* to prepare approximately 200 mL of standard in 100% methanol under yellow or red filtered light.

IV. SAMPLE RECEIPT, STORAGE AND HOLDING TIME

4.1 Sample Receipt

4.1.1 Upon receipt, ensure that samples are in good condition (e.g. dry ice is still present in cooler, samples are stored within, not on top of it, sample

packaging is not damaged, and sample identification is present and legible). If receipt criteria are not met, immediately alert Battelle and Domain Support Facility (DSF) staff.

4.1.2 Ensure that all samples containing a *Toxicodendron* species are obviously labeled as such. This includes samples with the word 'CLIP' in the identifier and marked as 'sampleType = toxicodendron possible' in the shipping manifest, or samples with these taxon codes in their identifier: TODI, TORA2, TOVE.

4.1.3 Document the anomalous temperature or sample package conditions on the data receipt form that was emailed to GLEC staff.

4.2 Sample storage – Store the plant material at -80ºC in a freezer with an auto-notification system in case of temperature or electrical failure.

4.3 Sample holding time – Start extractions within 7 days from receipt date to ensure the holding time of 14 days (from collection date - day 0). If this holding time is exceeded, proceed with extraction and analysis, flag data, and contact the Battelle Technical Representative within 48 hours of the incident.

4.4 On the extraction date, when the foil packet is removed from the bag, unwrapped and the samples inspected, add any additional anomalous sample conditions to the receipt form prior to uploading the completed form to the NEON Data Portal.

V. QUALITY CONTROL

5.1 Solvent blank (SB) – Analyze an SB at the beginning of each analytical batch, and after the last sample has been analyzed, to ensure that there has been no instrument drift. Transfer approximately 3 mL of 100 % methanol to a cuvette. The absorbance reading must be < 0.05 at all target wavelengths. See Section 11.1 for corrective action, if it is not.

5.2 NEON QA Reference Standard (QAR) – At the beginning of each analytical batch, analyze a 5 µg/mL standard. Calculate the chlorophyll *a* concentration using Equation 3. Calculate the percent recovery (R) using Equation 1, where D is the result for the QAR sample; i.e., Chl a result in Equation 1; and C is the known fortified concentration:

$$R = \frac{D}{C} \times 100 \qquad \textbf{Equation 1}$$

The mean QAR recovery for a batch must be within 15% of the known value. If it is not, see Section 11.2 for corrective action.

5.3 Laboratory Duplicate (LD) - Analyze one sample in duplicate for each batch of 30 or fewer field samples to measure precision. Calculate the RPD using the following equation:

Equation 2 $$\text{RPD} = \left[\frac{|x_1 - x_2|}{(x_1 + x_2)/2}\right] \times 100$$

Where:

x_1 = The concentration observed in the original sample; and
x_2 = The concentration observed in the duplicate sample.

If the results are not within the laboratory-generated control limits, see Section 12.3 for contingency. Control limits are calculated (see Section 8.2) using up to the past two years of previously determined RPDs per Standard Methods (APHA, 2023).

5.4 Data Qualifier Codes - Use the codes listed in Table 1 to flag results with associated QC problems.

5.5 Use Starna filters to confirm the calibration of the spectrophotometer daily.

VI. CALIBRATION

6.1 Allow the spectrophotometer to warm up for 15 minutes and perform the calibration check procedures just prior to sample analysis.

6.2 Swipe to page two on the touch screen of the spectrophotometer and select multi-wavelength mode. Enter the calibration ID. Enter each of the desired wavelengths; 652, 665, 470 and 750 nm. Click ok to go onto the analysis page.

6.3 Zero the instrument. Pour ~ 3 mL of 100 % methanol into a cuvette. Wipe the sides of the cuvette with a Kimwipe and place it in the instrument. Click Blank in the bottom left corner to zero the instrument at 652, 665, 470 and 750 nm.

6.4 Read the 100% methanol at each wavelength to ensure each still reads <0.05 ug/mL by entering the sample name; and clicking Analyze.

VII. PROCEDURE

7.1 Extraction

7.1.1 Begin extraction either the morning after receipt or early the following morning at 7 or 8 AM. After confirmation that samples have turned

white (no green pigment is remaining), analysis will be performed, for leaf samples, 30 to 32 hours after extraction (maximum 36 hours) and, for needle type samples, at approximately 48 hours after extraction.

7.1.2 Weigh each aluminum weigh pan with accompanying tin foil cover and record the weight.

7.1.3 Prepare work area in the hood such that there is a cutting surface that has been cleaned with methanol. Clean razor blades and manicure scissors with methanol.

7.1.4 Prepare enough solvent-washed 20 mL test tubes so there is one for each sample. Store the rack of tubes on ice in a cooler.

7.1.5 Remove subsamples from the cryofreezer and place them in a cooler on dry ice. Begin processing the first sample immediately.

7.2 Under yellow or red light, visually inspect each sample prior to processing and update the sampleCondition field as necessary.

7.2.1 Grind the entire sample in the grinding mill. Use extra precautions when working with samples that contain a *Toxicodendron* species.

7.2.1.1 If it is not too large, place the entire sample in the grinding mill. If a NEON sample is particularly large, it may be necessary to grind the sample in portions to avoid overloading the mill. The entire sample must be ground and homogenized before subsampling for extraction.

7.2.1.2 Needle type samples should be ground for a standard 10 seconds. If not fully ground after 10 seconds, the grinding time can be increased to 20 seconds. Broadleaf samples should be ground for a standard 20 seconds. Grinding time should be limited to avoid heat generated by the griding mill which can contribute to chlorophyll degradation, therefore, samples should not be milled for more than 20 seconds.

7.2.1.3 If there are particularly wet or sticky samples that are not sufficiently ground after the maximum time in the mill, hand cut the material, using a razor blade, then dice as small as possible. Pieces smaller than 1mm are preferred.

7.2.1.4 If the sample is milled but not hand chopped, enter *grinding mill* in the samplePrepMethod field of the bench sheet. If the sample is milled, then hand chopped, enter *grinding mill plus hand chop* in this field.

7.2.2 Subsample a portion of the homogenized sample into the correspondingly labeled weigh boat. Use another portion of the sample for a batch replicate, if applicable. Place the remainder of the sample back into the foil, return to the bag and place on dry ice until it can be returned to the cryofreezer.

7.2.2.1 For needles and grasses, place an estimated subsample of between 0.11 to 0.18 g of sample in each weigh boat. Needles should be on the high end of this range.

7.2.2.2 For leaves from broadleaf plants, place an estimated subsample of between 0.05 to 0.14 g of sample in each weigh boat.

7.2.3 Cover weigh boat with tin foil to transport it to the balance. Record weight. The fresh mass will be automatically calculated.

7.2.4 Return to the dark room with the weighed subsample and the tube from the freezer. Transfer the subsample to the tube, add 15 mL of methanol chilled to 4ºC cap it, ensure that all plant material is in the methanol and not stuck to the sides of the tube.

7.2.5 Cover the tube with foil to block light, and place it in a rack in the cooler on ice.

7.2.6 Re-wrap remaining parent sample in foil and place in whirlpak bag. Return the sample to the cryofreezer.

7.2.7 Decontaminate surfaces, the mill compartment, and tools with methanol, repeat steps 7.2.1 to 7.2.6.

7.2.8 When all samples have been transferred to extraction tubes, transport the rack(s) of samples to a dark 4ºC refrigerator. See Section 7.1.1 for extraction lengths. At the end of the extraction, verify visually that all green color is gone from the tissue by examining it apart from the solution. If broadleaf samples retain green color; i.e., are not fully extracted within 32 hours, the sample can be extracted for an additional 4 hours. Consult with Battelle if green color still remains; the extraction

may need to be repeated. On the afternoon of the first day of extraction and in the morning of the second day, invert the extraction tubes to disperse chlorophyll.

7.3 Analytical Procedure

7.3.1 Prepare to analyze samples within 1 to 2 hours of the target extraction time (Section 7.1.1).

7.3.2 Turn on the spectrophotometer and allow to warm up for 15 minutes and allow the foil-covered extracted samples to come to room temperature (approximately 20 minutes).

7.3.3 Zero the instrument by filling the cuvette with 100% methanol, inserting into the cuvette holder and pressing Blank.

7.3.4 Before proceeding with analysis, read and record each of the 3 Starna filters at 440 nm, 465 nm, 546.1 nm, 590 nm and 635 nm.

7.3.5 On the datasheet record the sampleCode, analyst, date, time, handlingQF (see Table 1 for result codes) and remarks, if the handlingQF is other than zero, the qaReferenceIDs for blanks and QC samples and the standardChlAConc for each QC sample. Absorbance readings for each wavelength will be stored on the instrument. After the analysis is complete, data will be transferred to the datasheet. Data is also stored on the spec until it is manually deleted. It will not be deleted until the report has been submitted.

7.3.6 In a dark room with red or yellow light, observe each extract by peaking under the foil. Centrifuge cloudy samples for 5 minutes at 2000 rpm. If a sample is centrifuged, check the centrifuged box on the bench sheet.

7.3.7 Before each analytical batch of 30 or fewer samples, read a 100% methanol SB at each wavelength; 665, 652, 470 and 750 nanometers (nm). The absorbance for each wavelength will be shown on the screen when a sample is inserted. If an absorbance of 0.05 or greater is observed, see Section 11.1 for corrective action.

7.3.8 Analyze a QAR sample. Pour ~ 3 mL of 5 µg/mL chlorophyll *a* QAR standard in a cuvette. Record readings in nanometers (nm) for each wavelength.

7.3.9 Use Equation 3 for chlorophyll *a* determination based on absorbance at 652, 665 and 750 nm. If the target meets criteria (Section 5.2), proceed with analysis. The spreadsheet calculation for the chlorophyll *a* recovery is shown in Equation 1.

7.3.9.1 If recovery is not acceptable, see Section 11.2 for corrective action.

7.3.9.2 If the corrective action fails, see Section 12.2.

7.3.10 Invert the tube several times to ensure that chlorophyll is well dispersed in the solvent, let the foliar material settle, then use a Pasteur pipette to transfer ~ 3 mL of extract from the first extracted sample in the analytical batch to a cuvette.

7.3.10.1 Ensure that each sample marked as a needle sample on the bench sheet, aligns with a visual inspection of the sample. If it does not, the sample must be reextracted.

7.3.10.2 Visually inspect the sample to ensure that it is white (no green pigment remaining). It may be necessary to turn on the overhead light, very briefly, to be sure. Keep in mind that some sample types may maintain a yellowish color when fully extracted. If green pigment remains, evaluate whether or not the sample was adequately homogenized into small enough pieces.

7.3.10.2.1 If not adequately homogenized: Schedule a re-extraction.

7.3.10.2.2 If adequately homogenized: If it's a leaf sample, try to extend the extraction time until green pigment disappears. If it's a needle sample, contact Battelle for guidance. Cover it again with foil and place it back on ice or in the refrigerator.

7.3.11 Wipe cuvette with a Kimwipe and place in spectrophotometer.

7.3.11.1 If absorbance is greater than 0.9 spectrophotometric units at 665 nm, dilute the sample with 100 % methanol and re-analyze.

7.3.11.2 If absorbance is less than 0.2 spectrophotometric units at 665 nm, re-extract the sample, using a smaller volume of methanol, if sufficient material is available. If there isn't enough sample remaining, and the original result will be reported, add a data quality flag.

7.3.11.3 If absorbance is higher than 0.05 spectrophotometric units at 750 nm, there is too much turbidity in the extract. Centrifuge the sample for 5 minutes at 2000 rpm and re-analyze.

7.3.12 Repeat steps in Sections 7.3.10 to 7.3.11 for each sample in the analytical batch.

7.3.13 If more than one analytical batch (more than 30 samples) will be analyzed, begin with reading a new SB and QAR, prior to analyzing the next 30 or fewer samples. For each additional SB and QAR sample, the analyticalRepNumber should be increased by one.

7.3.14 Analyze a final SB and QAR after the last sample has been analyzed.

7.3.15 For each sample, populate the handlingQF field, using codes in Table 1, indicating whether the samples were analyzed within target holding times and extraction time was within the target range.

7.3.16 For each sample, populate the measurementQF field, using codes in Table 1, indicating whether the values were within the acceptable absorbance range, and, if not, enter an explanation in the remarks field.

7.3.17 For each sample, populate the chlQAMaterialQF field, using the codes in Table 1, indicating whether or not the mean observed value of the opening and closing QAR for the sample set was within 15% of the known value.

7.3.18 For each sample, populate the absorbanceStandardQF field, using the codes from Table 1, indicating whether or not quality criteria were met for the Starna filter check. See Table 1 for flags.

VIII. DATA ANALYSIS AND CALCULATIONS

8.1 The equations for chlorophylls *a* and *b* and bulk carotenoids are below. The dilution factor is 1 for undiluted samples, 2 for 1 part sample + 1 part methanol, 4 for 1 part sample + 3 parts methanol, etc.

$$\textbf{Chl a } \left(\frac{\textbf{ug}}{\textbf{mL}}\right) = [\mathbf{16.72} * (\mathbf{A665} - \mathbf{A750}) - \mathbf{9.16} * (\mathbf{A652} - \mathbf{A750})] * \mathbf{DF}$$

Equation 3

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

Equation 4

Where: A### represents the absorbance at the specified wavelength
DF = dilution factor

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

Equation 5

Where: A### represents the absorbance at the specified wavelength
DF = dilution factor

8.2 Control limit for laboratory duplicate RPD

8.2.1 Control limits are determined for the acceptability of the RPDs between laboratory duplicate samples.

8.2.1.1 The Control Limit is the average of all RPDs for up to the past two year period, plus three times the standard deviation.

Control Limit $= \bar{x} + 3s$

Where: $\bar{x}$ = the mean RPD of all laboratory duplicates
And s= the standard deviation of RPD for the laboratory duplicates.

8.3 Generate reports

8.3.1 Verify the upload of the data. Save to the GLEC LIMS.

8.3.2 Use NEON Foliar chlorophyll-specific macros to populate the sample result and QC result tables with the new data, and update fields derived from the log in report. These tables follow the field and data criteria specifications required by NEON for the foliar data ingest upload.

8.3.3 Export the two tables created in Section 8.2.2 to Excel, and format them for data ingest upload. Save as a comma delimited (csv) file.

8.3.4 Upload the final lab ingest files created in Section 8.2.3 to the NEON Data Portal.

IX. QUALITY ASSURANCE

9.1 Data reports are reviewed by a qualified GLEC staff member before submission to the client. This QA Review is an independent review; it is performed by

someone not associated with the data generation. This review evaluates the computations performed, and the accuracy and traceability of the data. It is the responsibility of the person who generated the report to satisfactorily address any of the QC reviewer's comments and concerns and to generate the final report.

9.2 Hard copies of original documents are maintained in secure files at GLEC. Electronic data or other information is filed and stored by the project name on GLEC's server which is backed-up daily.

X. WASTE MANAGEMENT/POLLUTION PREVENTION

10.1 Methanol waste must be collected into a designated container, labeled with a GHS compliant label, and stored in the Nutrient Chemistry Laboratory or the chemical shed pending disposal. Dispose of hazardous material by appointment with RecycleSmart.

10.2 This method should be conducted with active pollution prevention as an objective, by: modifying processes to reduce waste at the source.

XI. CORRECTIVE ACTIONS FOR OUT OF CONTROL DATA

11.1 If the solvent blank fails criteria (Section 5.1), reanalyze a new aliquot of the SB. If the result is again ≥ 0.05 spectrophotometric units, re-zero the instrument, re-read the SB and the samples analyzed since the previous acceptable SB. If the corrective action fails, see Section 12.1 for contingency.

11.2 If the recovery for a QAR sample is not within 15% (Section 5.2), re-prepare and reanalyze the standard and recalculate the recovery. If recovery is still unacceptable, see Section 12.2 for contingency.

XII. CONTINGENCIES FOR HANDLING OUT OF CONTROL OR UNACCEPTABLE DATA

12.1 If the corrective action for a solvent blank (Section 11.1) fails, analyze samples and report the data with a measurementQF flag of 3 and explain in remarks.

12.2 If recovery of the QAR is still unacceptable upon reanalysis (Section 11.2), proceed with analysis. If the average of QAR recoveries is outside 15%, report low accuracy using the chlQAMaterialQF field in Table 1, below. If the QAR has become compromised, enter a 1 in the qaQF field.

12.3 If the recovery and/or RPD results for a sample and laboratory duplicate are not within the acceptable range (see Section 5.3), enter a "3" for the measurementQF

and explain in the remarks field. The standard remark for RPD failure between duplicates should be; “The RPD between duplicates was not within control limits”.

12.4 Use the result flags in Table 1 for other issues, as applicable.

XIII. DATA ASSESSMENT AND QUALIFICATION CRITERIA FOR QUALITY CONTROL MEASURES

Table 1. Data Qualifier Codes

sampleCondition	
ok	
arrived wilted	
arrived brown	
arrived thawed	
other	
handlingQF	
0	No issues to report
1	exceeded standard holding time, 14 days from sample collection
2	exceeded standard extraction time, 48 hours or less
3	exceed both standard holding time and standard extraction time
5	other handling issue, explained in remarks
measurementQF	
0	No issues to report
1	absorbance value below the optimal range, 0.2
2	absorbance value above the optimal range, 0.9
3	other measurement issue, explained in remarks
chlQAMaterialQF	
OK	Average observed relative accuracy of chlorophyll QA material within 15% of known value
criteria not met	Average observed relative accuracy of chlorophyll QA material > 15% from known value
not available	chlorophyll QA material not run with this set of samples
absorbanceStandardQF	
OK	Starna filter absorbance meets specifications at all wavelengths

criteria not met	Starna filter absorbance did not meet specifications at all wavelengths
not available	Starna filters not measured with this set of samples
qaQF	
0	No issues to report
1	Known issue with a QAR

XIV. REFERENCES

14.1 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.

14.2 GENESYS 50 UV-VIS spectrophotometer Manual.

14.3 SM Method 1020 B Section 13. Quality Control. In Standard Methods for the Examination of Water and Wastewater, 24th Edition, 2023. American Public Health Association (APHA), American Water Works Association, and Water Environment Federation, Washington, DC.