



# STANDARD OPERATING PROCEDURES

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## CHLOROPHYLL DETERMINATION

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SUPERSEDES: SOP #2030; Revision 1.0; 01/24/92; U.S. EPA Contract EP-W-09-031.



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### 1.0 SCOPE AND APPLICATION

The objective of this standard operating procedure (SOP) is to describe procedures for determining the concentration of chlorophyll in photosynthetic tissue of plants. This analysis, in conjunction with in vivo chlorophyll fluorescence measurement and other plant physiological and toxicological techniques, will be used to assess the impact of contaminants on plants. Chlorophylls are light-harvesting pigments integral to the photosynthetic process. Chlorophyll concentration data will provide information on a plant's photosynthetic potential. The method will be used during the growing season for field applications, or at anytime during the year in laboratory applications.

These are standard (i.e., typically applicable) operating procedures which may be varied or changed as required, dependent upon site conditions, equipment limitations, or limitations imposed by the procedure. In all instances, the ultimate procedures employed should be documented and associated with the final report.

Mention of trade names or commercial products does not constitute U.S. Environmental Protection Agency (U.S. EPA) endorsement or recommendation for use.

### 2.0 METHOD SUMMARY

Plant photosynthetic tissue will be collected from plots established according to the sampling plan of the individual site or the design of the experiment.

Photosynthetic tissue of a known (measured) area will be ground in a solution of 1 part 0.1 Normal (N) ammonium hydroxide solution to 9 parts acetone [volume to volume (v:v)]. The slurry is centrifuged and the supernatant is diluted to a concentration that gives an absorbance reading between 0.2 and 0.8 at wavelengths of 663 nanometers (nm) and 645 nm. The absorbance of each solution is recorded at these wavelengths and chlorophyll a and b concentrations are calculated.

### 3.0 SAMPLE PRESERVATION, CONTAINERS, HANDLING AND STORAGE

Tissue samples will be placed in aluminum foil immediately following collection. The foil-wrapped sample will then be placed in a resealable plastic bag and held in darkness. Tissue samples must be frozen at approximately -56.6 °C to prevent chlorophyll breakdown. Dry ice may be used to achieve such low temperatures in storage, especially while out in the field. Frozen samples can be held no longer than seven days before analysis. If dry ice or another mode of freezing the tissue is not available, samples can be held on wet ice (4 °C) for no longer than three days prior to analysis. Darkness must be maintained during the storage period and analyses must be carried out in subdued light to avoid chlorophyll degradation. Also, to avoid degradation, analytical procedures must be performed with opaque containers or containers covered with aluminum foil when it is possible. If necessary, the undiluted extracts can be held at -10 °C or less for seven days in total darkness in rubber-stoppered borosilicate glass centrifuge tubes wrapped with parafilm to maintain an airtight seal.

### 4.0 INTERFERENCES AND POTENTIAL PROBLEMS

If the handling and storage precautions detailed above are not carried out, results will be inaccurately low, and valid comparisons cannot be made. Cross-contamination between samples can occur during laboratory analysis. All glassware must be cleaned between use and the following rinses (in order) applied: acetone,



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distilled water, acetone, distilled water, and a final acetone rinse. Inaccurate pipetting in sample dilution will cause magnification of errors. Additionally, personnel expected to perform this analysis must have experience in basic laboratory procedures and training in this analytical procedure.

### 5.0 EQUIPMENT/APPARATUS

Equipment required in the field includes shears or a knife, aluminum foil, appropriate protective gloves, resealable plastic bags, large black garbage bags (or other means of providing a darkened environment), wet ice, dry ice, coolers, sample labels, markers, logbook, data sheets, Chain of Custody records, and custody seals.

Equipment required for the laboratory analysis will include a scale, a small pair of scissors, a tissue homogenizer including at least ten glass homogenization tubes with Teflon pestal with 10 mL capacities, tabletop centrifuge, 12 mL borosilicate glass centrifuge tubes, test tube rack, 5 mL and 10 mL pipettes and bulb, spatulas, 10 mL graduated cylinders (at least 10), a visible light spectrophotometer with an optically matched set of cuvettes (1-cm cell size), pasteur pipettes and bulbs, squeeze bottles with acetone and distilled water for rinsing, a freezer, markers, and a logbook.

### 6.0 REAGENTS

The following reagents are needed for chlorophyll extraction and analysis procedures:

- ammonium hydroxide (NH<sub>4</sub>OH) solution
- 80% aqueous acetone
- distilled water

Preparation of the ammonium hydroxide solution is as follows:

1. Dissolve 0.35 g NH<sub>4</sub>OH in 100 mL distilled water to form 0.1 N NH<sub>4</sub>OH solution
2. Mix 0.1 N NH<sub>4</sub>OH solution with the reagent-grade acetone in a ratio of 1:9 (v:v) (NH<sub>4</sub>OH:acetone)

Preparation of the 80% aqueous acetone solution is as follows:

1. Mix distilled water and reagent-grade acetone in a ratio of 2:8 (v:v) (distilled water:acetone)

Additionally, technical-grade acetone should be available for use in the cleaning of glassware.

### 7.0 PROCEDURES

#### 7.1 Site Preparation

##### 7.1.1 Plant Population Survey

First, the site will be characterized and species of interest will be chosen according to the ERT/SERAS SOP #2037, Terrestrial Plant Community Sampling. Then plant sample collection areas and specific plants to be sampled will be chosen according to the site-sampling plan. Once chosen, these areas/plants will be identified or marked using stakes,



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flagging tape, or bouys.

Samples will be collected from reference areas in addition to contaminated areas and the results compared statistically.

### 7.1.2 Sample Collection

Tissue samples larger than the tissue needed for analysis (refer to section 7.2.2) will be cut from the plant and immediately placed in aluminum foil. The sample is then labelled, placed in a resealable plastic bag, and frozen on dry ice in a cooler. Samples may be held on dry ice for seven days. If dry ice is not available, wet ice can be used with holding time not to exceed three days. Samples must be kept in a darkened environment to prevent chlorophyll degradation. Appropriate sample documentation is made in accordance with ERT/SERAS SOP #2002, Sample Documentation.

## 7.2 Laboratory Analysis

### 7.2.1 Equipment Preparation

Homogenization tubes and pestals, solutions, and glassware (except cuvettes) will be thoroughly chilled in a freezer prior to starting the analyses. The laboratory will be darkened (dim light) and the spectrophotometer turned on and allowed to stabilize. The instrument will be calibrated and operated according to the manufacturer's operating manual.

### 7.2.2 Tissue Processing

An area of at least 1000 square millimeters ( $\text{mm}^2$ ) will be cut from each tissue sample using a template (example: 36 mm diameter circle =  $1018 \text{ mm}^2$ ; or 20 x 50 mm rectangle =  $1000 \text{ mm}^2$ , etc.) The tissue sample will be cut into small pieces using scissors and placed in the homogenization tube and 2-mL of extraction solution will be added using a 5-mL pipette. The material will be carefully ground with a pestal while being kept chilled over ice. After grinding for approximately 30 seconds (until the tissue is a fine slurry), extraction solution will be used to wash any sample material adhering to the pestal. This will be done by pouring 3 mL of extraction solution over the pestal into the homogenization tube. The tube is then placed on ice

or refrigerated in the dark for two hours and reground to extract any remaining chlorophyll. The extract will then be carefully poured into a centrifuge tube. An additional 5 mL of 80% aqueous acetone solution will be pipetted over the pestal into the homogenization tube and the remaining contents poured into the centrifuge tube.

When a tissue homogenizer is not available, samples may be ground either by hand, using glass mortars and pestals and the addition of acid-washed quartz sand, or mechanically, using a high-speed blender. In either case it must be insured that the chlorophyll is extracted completely from the tissue, (i.e., the tissue is devoid of green color).



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Sample extracts, now in centrifuge tubes, are centrifuged for twenty minutes at high speed (approximately 500 x gravity). The supernatant solution is decanted into a 10 mL graduated cylinder and the volume brought to 10 mL with 80% aqueous acetone.

At this point, the supernatant may be stored at -10°C if necessary, according to the method described in Section 3.0.

### 7.2.3 Dilution and Spectrophotometry

Dilution is required if the initial reading is out of the linear range of instrument detection. The supernatant is diluted by adding 80% aqueous acetone, as necessary, to give a reading in the range of 0.2 to 0.8 absorbance units at wavelengths of 645 nm and 663 nm. A solution which has an absorbance of 1.00 should, when diluted in half, have an absorbance of 0.50. Final volume of the diluted sample extract will be recorded. The 80% aqueous acetone is used as the blank to zero the instrument initially and after every wavelength resetting. Zero checks, and any deviations from zero, must be recorded in a logbook or laboratory notebook. All samples being analyzed will be read at 645 nm, then read again after resetting the wavelength to 663 nm.<sup>(1)</sup> Dilutions must be accurately made and recorded (in a logbook or laboratory notebook) in order to calculate the chlorophyll concentration in the original tissue sample. Calculations for chlorophyll concentrations are made after the absorbances are read at both wavelengths.

## 8.0 CALCULATIONS

The following calculations will be made to ascertain sample chlorophyll concentrations. Concentrations will be expressed on an area basis.

$$\text{Chlorophyll a [milligrams/milliliter (mg/mL)]} = 12.7 A_{663} - 2.69 A_{645}$$

$$\text{Chlorophyll b (mg/mL)} = 22.9 A_{645} - 4.68 A_{663}$$

where:

$A_{645}$  = absorbance at a wavelength of 645 nm;

$A_{663}$  = absorbance at a wavelength of 663 nm.

$$\text{Total Chlorophyll (mg/mL)} = \text{Chlorophyll a} + \text{Chlorophyll b.}$$

$$\text{Total Chlorophyll (mg) in original tissue sample} = \text{Total Chlorophyll (mg/mL)} \times \text{final volume (mL).}$$

$$\text{Total Chlorophyll a (mg) in original tissue sample} = \text{Chlorophyll a (mg/mL)} \times \text{final volume (mL).}$$

$$\text{Total Chlorophyll b (mg) in original tissue sample} = \text{Chlorophyll b (mg/mL)} \times \text{final volume (mL).}$$

To express on the basis of area, divide the amount of chlorophyll by the area ( $\text{mm}^2$ ) in the tissue sample analyzed.

$$\begin{aligned} \text{Area of a circle} &= \pi(\text{radius}^2) \\ &= 3.1416(r^2) \end{aligned}$$



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### 9.0 QUALITY ASSURANCE/QUALITY CONTROL

A sampling plan, which includes appropriate sample size estimates, will be created prior to sampling and followed during sample collection.

All data must be documented on field data sheets and in logbooks. Samples will also be duplicated at a minimum rate of ten percent. The order of sample analyses will be randomized between the contaminated and reference area samples.

All instrumentation must be operated in accordance with the operating instructions supplied by the manufacturer, unless otherwise specified in the work plan. The spectrophotometer will be properly calibrated according to the manufacturer's operating manual. The instrument's zero adjustment will be checked with the solvent blank between readings. Equipment checkouts and calibration activities must occur prior to sampling/operation and they must be documented. Additionally, calculations will be rechecked at a rate of ten percent by an additional person.

### 10.0 DATA VALIDATION

The data generated will be reviewed according to the quality assurance/quality control considerations listed in Section 9.0. Multiple analyses will be carried out on each plant and data will be statistically analyzed.

### 11.0 HEALTH AND SAFETY

When working with potential hazardous materials, follow U.S. EPA, OSHA, and corporate health and safety procedures.

Appropriate protective gloves will be worn when collecting plant samples and when working in the laboratory.

### 12.0 REFERENCE

- (1) Arnon, Daniel I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant Physiology. Volume 24, pp. 1-15.