# Chlorophyll-a Double-Extraction with Ethanol

This protocol originates from the Aridlands Ecology Lab at University of Colorado at Boulder.

## Materials

- Mortar and pestle
- 15 mL screw-cap vials
- Polystyrene cuvettes, Fisher # 14-377-010
- 5000µL and 1000 µL pipettes and pipette tips
- Spectrophotometric grade Ethanol
- Magnesium Carbonate ($MgCO_3$) solid form
- Plastic funnel
- Filter paper
- Orbital shaker
- Water bath setup
- 0.1 M HCl

## Solutions

Neutralized Ethanol Solution:
Add 0.3g of $MgCO_3$ to 1L Ethanol and stir to neutralize. Then filter using funnel and filter paper. Consider using the vacuum option on the gas chromatograph.

## Procedure

**Extracting the Chlorophyll-a**

*Note: All processing should be done in minimal light to help prevent degradation of chlorophylls.*

1. Grind soil sample in mortar and pestle until homogeneous and place 3 g soil into labeled 15 mL screw-cap vial.
2. Add 6 mL of Ethanol solution to each vial and shake gently by hand.
3. Boil samples in water bath for 5 minutes. Begin time when samples actually begin to boil.
    - Make sure to loosen caps to allow heat to escape but not enough to allow evaporation. (If water is too hot, samples may boil over.)
4. Remove vials from water bath and allow to cool for 10 minutes. Make sure the samples are in the dark.

5. Tighten caps and place all vials horizontally in shaker and let them shake for 20 minutes.
6. After shaking, centrifuge samples at 4000 rpm for 10 minutes.
7. Carefully pour supernatant into separate labeled vial. This sample is ready for analysis on the spectrophotometer.
8. Repeat steps 2-7 for all following extractions.

**Measuring the Chlorophyll-a**

- **Note:** The spectrophotometer setup (warm up and calibration) is specific to the Aridland Ecosystems Lab's spectrophotometer. Please use the appropriate setup protocol for your specific spectrophotometer. The measurement steps are universal.

Preparing the Spectrophotometer

1. Warm up the spec first by plugging it in and turning it on.
2. Let the spec warm up for 10-15 minutes. During this time it will run through a self test.
3. Turn on the lamp. Press the “Vis” button until you see “Vis” appear in the digital screen.
4. When the spec is ready the screen will read a wavelength (λ) of 486. Align the cuvette tray using a new cuvette with a sticky note rolled up inside of it (green sticky notes work best). If the door is propped slightly open you can see the blue light hitting the cuvette holder. The holder should be positioned so that the light hits the center of the cuvette.
5. The instrument is now ready to calibrate.
6. Calibration should be done using the same solution that the samples were extracted with. This means extra solution should be made during the sample extraction process. **Cuvettes should be handled with care. Dirty cuvettes will give bad readings. Only touch the foggy sides of the cuvette.**
7. Place a cuvette with blank solution into the cuvette holder. Close door.
8. Type in the wavelength, push the “λ” button and then push “calibrate.” Calibrate for all wavelengths that you will be measuring at. Calibration can be done for up to 10 wavelengths. The absorbance reading should be zero for all calibrated points. In this situation, use wavelengths 665 nm and 750 nm.

Measuring Samples

1. When measuring samples the door must be completely closed and the cuvette holder aligned.
2. Only use new and clean cuvettes for running samples. They are not that expensive and not worth cleaning.
3. Make sure that the extraction solution is compatible with the cuvette material. Acetone etches polystyrene, so certain precautions should be taken when using acetone.
4. Using the 5000 µL pipette, transfer 3 mL of extract to the cuvette.

5. Promptly measure the absorbance at 665 nm and 750 nm by typing in the wavelength and pressing “λ.”
6. Using the 1000 µL pipette, transfer 100 µL of 0.1 N HCl into the same cuvette, then tap or stir the vial after the acid addition and wait 90 seconds for the reaction.
7. Measure the absorbance again at 665 nm and 750 nm.
8. When finished, turn off the spectrophotometer.

## Calculations

Calculating Chlorophyll-a concentrations: Use the following equations to determine concentration of chlorophyll-a in each soil sample:

for analysis done with acidification: (29.6 * (6650 - 665a) * V) / (g soil-1) * L
V = volume of solvent (mL), g soil = gram dry soil, L = path length

for analysis done without acidification: (11.9035 * (6650) * V) / (g soil-1) * L
V = volume of solvent (mL), g soil = gram dry soil, L = path length