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Chlorophyll-*a* Double-Extraction with Ethanol

Materials:

Mortar and pestle
15mL 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
.1M 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.

Processing and Extraction:

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

1. Grind soil sample in mortar and pestle until homogenous, and place 3g soil into labeled 15mL screw-cap vial.
2. Add 6mL of Ethanol solution to each vial and shake gently by hand.
3. Boil samples in water bath for 5 minutes, begin timing once 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 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 4000rpm for 10 minutes.
7. Carefully pour supernatant into separate labeled vial. This sample is ready for analysis on the spectrophotometer.

*If samples or extracts need be held overnight, they must be kept in the fridge at 4°C.

8. Repeat steps 2-7 for second extraction.

Determining Chlorophyll-*a* Concentration on the Spectrophotometer:

Warm up the Spec:

1. Plug in the Spec.
2. The spectrophotometer takes approximately 10-15minutes to warm up. 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.

Calibrating the Spec:

1. 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 side of the cuvette.**
2. Place a cuvette with blank solution into the cuvette holder. Close door.
3. 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 665nm and 750nm.

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 are 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 5000µL pipette, transfer 3mL of extract to cuvette.
5. Promptly measure the absorbance at 665nm and 750nm by typing in the wavelength and pressing "λ".
6. Using the 1000 µL pipette, transfer 100µL of .1M HCl into the same cuvette, then tap or stir the vial after the acid addition and wait 90sec for the reaction.
7. Measure the absorbance again at 665 nm and 750 nm.

When finished with readings turn Spec off by unplugging the instrument.

Calculating Chla concentrations:

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

for analysis done with acidification: $(29.6 * (665_0 - 665_a) * V) / (g\ soil^{-1}) * L$

V = volume of solvent (mL), g soil = gram dry soil, L = path length

for analysis done without acidification: $(11.9035 * (665_0) * V) / (g\ soil^{-1}) * L$

V = volume of solvent (mL), g soil = gram dry soil, L = path length