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Enzymatic oxalate kit analysis for soil

Background

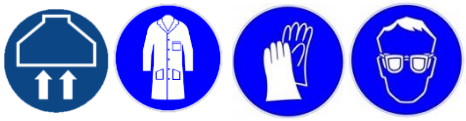
The oxalate concentration of plant and soil samples is ascertained with a commercial Enzymatic Oxalate kit (EOK; Trinity Biotec Plc or LIBIOS), using techniques adapted from Certini et al (2000) and Cailleau et al (2014) to first extract the oxalate. $H_2C_2O_4$ content can be calculated through the relative proportions of indamine dye (590 nm) through the following reaction:

$$H_2C_2O_4 + O_2 \xrightarrow{\text{oxalate oxidase}} 2\,CO_2 + H_2O_2$$

$$H_2O_2 + \text{MBTH} + \text{DMAB} \xrightarrow{\text{peroxidase}} \text{Indamine Dye} + H_2O$$

Enzymatic oxalate kit chemical reaction.

Safety/ Protective equipment



Material

- Samples: soil sieved at 2 mm / ground plant material
- Enzymatic Oxalate kit
- Hydrochloric acid 37 %
- Sodium hydroxide
- Oxalic acid dihydrate salt
- Rotary shaker
- Centrifuge
- Falcon tubes 15 mL
- Spectrophotometer UV-VIS + semi-micro cuvette

Preparation of solutions for soil & plant extraction

- Preparation of a 1 M HCl solution

Half fill a 500 mL volumetric flask half-filled with MilliQ water. Then add 41.5 mL of 37 % hydrochloric acid to the water. Complete to 500 mL with MilliQ water. Put a lid on the flask and then shake well.

- Preparation of a 2 M NaOH solution

Half fill a 100 mL volumetric flask with milliQ water. Dissolve 8.0 g of NaOH in the 100 mL volumetric flask. Wait to cool then complete to 100 mL with MiliQ water. Put a lid on the flask and then shake well.

Preparation of standards

Prepare an oxalic acid standard at 500 mg/L from oxalic acid dihydrate ($C_2H_2O_4 \cdot 2\,H_2O$) in acid washed glassware. Do not to forget to remove the molar

fraction of water when calculating your standards. Dilute the standard to obtain a calibration curve at 0, 10, 50, 100, 200 mg/L oxalic acid.

Soil & Plant Extraction

Perform an initial test with numerous samples that cover a range of your expected concentrations (establish prior to analysis with an optical microscope and carnoy fluid or scanning electron microscope) to ensure that the concentration of the samples is in the range of the standards. If they are outside the range, adjust the dilution of point 5 to ensure correct measurements.

1. Weigh 0.1 g of plant or 2.0 g of soil into a 15 mL Falcon tube. Record the exact value in your laboratory notebook.
2. Add 5 mL of 1 M HCl
3. Place the closed tubes on the orbital shaker at 150 rpm for 16 hours.
4. Centrifuge the tubes at 1080 g (3080 rpm) for 5 minutes.
5. In a new 15 mL tube, remove 1 mL of the supernatant (avoid organic residues) and add 4mL of demineralized water.
6. Adjust the pH to 5-7 with ± 0.4 mL of 2 M NaOH. Check the pH. Record the exact value in your laboratory notebook. Make sure that you adjust appropriately the pH for a wide range of samples. $CaCO_3$ presence in your samples can mean that some samples need less adjustment than others. Once you have a correct quantity of 2 M NaOH for pH correction of a range of your samples you can apply this correction throughout. You will need these dilution figures for re-calculating the exact calcium oxalate (CaOx; CaC_2O_4) content of your samples.

Dosage of oxalic acid *(follow kit instructions)*

When first using the kits, you will need to hydrate the different reagents. Once hydrated the kits have a limited life span in the fridge. Remove the reagents before starting analysis so that they warm to room temperature. Make sure to use a calibrated pipette for all of the hydration steps and different measurements to avoid erroneous measurements. I would suggest calibrating your pipettes for the different measurements during method development and then keeping them separate / not changing the measurements, to ensure that you can rapidly and accurately work with the kits.

7. In a purification tube mix 1 mL of the MiliQ water (blank)/ sample/ standard with 1 mL of the sample diluent provided in the kit.
8. Shake the tubes on the orbital shaker for 5 min at 150 rpm.
9. Centrifuge at 1080 g (3080 rpm) for 5 minutes.
10. Add **500 µL of Reagent 1** into a semi-micro cuvette (optical path 1 cm).
11. Add **500 µL of Reagent 2** to the semi-micro cuvette.
12. Add **100 µL of Reagent 3** to the semi-micro cuvette.
13. Then add **50 µL of MiliQ water/sample/standard** (taken from the purification tube). Be careful when pipetting the sample / blank / standard from the purification tubes to ensure that you do not reagitate the activated charcoal. Remove air from the pipette before placing it into the solution. Then carefully

take the sample / blank / standard from between the suspended charcoal and centrifuged charcoal. Mix the vial with the pipette tip to thoroughly mix the reagents and sample. Dispose of the sample tip between samples.

14. Repose the tubes for *ca.*10 minutes to allow the color to develop. We would suggest passing a middle standard (50 mg/L) in the spectrophotometer and taking continuous measurements, to evaluate when is best to take measurements and how long your specific kit is stable for.
15. Measure the absorbance on the spectrophotometer at 590 nm.
16. Do not forget to measure replicates, blanks, and a middle standard (50 mg/L) throughout your run to check for error, contamination, and machine drift, respectively.

Summary table

Reagents	*Blanc*	*Standard*	*Sample*
Reagent 1	*500 µL*	*500 µL*	*500 µL*
Reagent 2	*500 µL*	*500 µL*	*500 µL*
MiliQ water	*50 µL*	*--*	*--*
Standard	*--*	*50 µL*	*--*
Sample	*--*	*--*	*50 µL*
Reagent 3	*100 µL*	*100 µL*	*100 µL*

Results

The final result must be expressed in mg/Kg of oven dry soil or g/Kg of oven dry soil using the equation below. Take all dilutions into consideration and relate the calcium oxalate concentration to the initially weighed sample mass. Do not forget to account for the change in dilution during pH correction and residual humidity differences between your samples.

$$\text{Observed calcium oxalate content } (\text{mg kg}^{-1}) = \frac{(\text{Spectrophotometer reading } (\text{mg L}^{-1}) \text{x volume(mL) x dilution factor})}{\text{Sample weight x moisture correction ratio}}$$

Equation 1. Calculating the content of calcium oxalate in your samples. You'll need to correct the eventual concentration for the molar mass of the CaC_2O_4 that you have previously seen in your samples (whewellite or weddellite)

Reference

Rowley, M.C., Estrada-Medina, H., Tzec-Gamboa, M. *et al.* Moving carbon between spheres, the potential oxalate-carbonate pathway of *Brosimum alicastrum* Sw.; Moraceae. *Plant Soil* 412**,** 465–479 (2017).