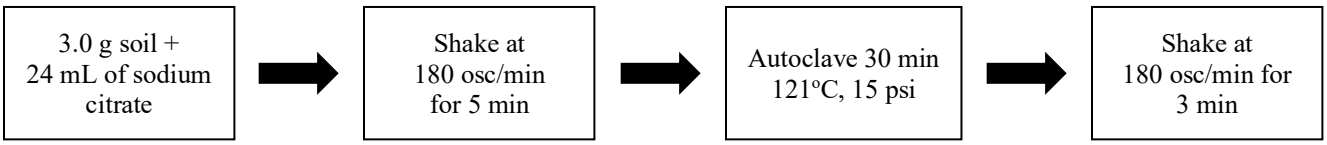
Procedure for Autoclaved-Citrate Extractable (ACE) Soil Protein

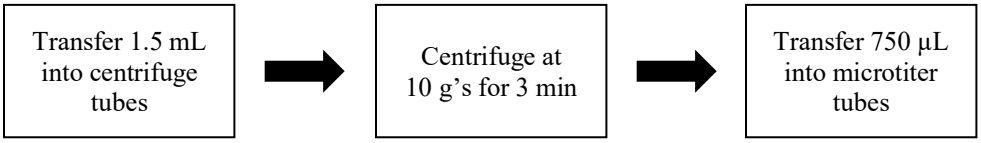
Procedure Overview

This document describes a procedure for the determination of autoclaved-citrate extractable soil protein as outlined by Hurisso et al. (2018). This procedure is a modification of an approach used to extract proteins from soil and fungi (Keen & Legrand 1980, Wright & Upadhyaya 1996). Soils that are air-dried and ground to <2 mm are typically used.

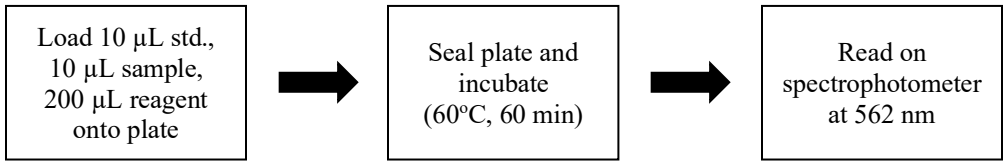
Extraction



Clarification



Quantification



Instrumentation and Materials:

Sodium Citrate ($Na_3C_6H_5O_7$) Stock Solution Preparation

- Reagent grade Tribasic sodium citrate dehydrate ($C_6H_5Na_3O_7$; FW=294.10 g mol^{-1})
- Reagent grade Citric acid ($C_6H_8O_7$; FW=192.12 g mol^{-1})
- Stir bar and stir plate
- 20 L carboy, 250 mL beaker, 1 L Erlenmeyer flask
- pH meter

Sample Extraction and Clarification

- Glass extraction tubes with caps
- Metal storage racks for extraction tubes
- Analytical balance capable of weighing to two decimal places
- Soil checks (pulverized, homogenous soil as internal lab reference samples)
- Adjustable bottle-top dispenser fitted to a bottle of sodium citrate (20 mM, pH 7.0) and calibrated to deliver 24 mL
- Horizontal shaker

- Autoclave-safe plastic tubs
- Adjustable 100-1000 µL pipettor and tips
- 2 mL microcentrifuge tubes
- Microcentrifuge
- Microtiter tubes (1.1 mL open top tubes in strips of 8) and caps

Reagent and Standard Preparation
- Pipetting reservoir for multichannel pipette
- 50 mL disposable polypropylene centrifuge tube with cap (Falcon tube)
- Adjustable 100-1000 µL pipettor and tips
- 25 mL graduated cylinder
- Bicinchoninic acid (BCA) reagents A and B (Fisher Sci. # PI23225)
- ThermoScientific™ Pierce Bovine Serum Albumin (BSA) Protein Assay Standards (Fisher Sci. # PI23208)

Sample Quantification
- Adjustable 1-10 µL and 30-300 µL multichannel pipettor and tips
- Clear polystyrene flat-bottom cell culture 96-well plate
- Plate sealing tape and roller
- Plate incubator (heating plate)
- Centrifuge with microtiter plate inserts
- Spectrophotometer capable of reading 562nm

Detailed Procedure:

I. 20 *mM* Sodium Citrate ($Na_3C_6H_5O_7$) Stock Solution Preparation (makes 20 L):

1. Prepare 100 mL of 1 *M* citric acid solution for pH adjustment (19.2 g citric acid and bring to 100 mL with DI water).
2. Weigh 117.64 g of tribasic sodium citrate dihydrate and place in a 1 L Erlenmeyer flask. Add a stir bar and ~500 mL of DI water.
3. Stir at a moderate speed until completely dissolved.
4. Pour into the clean 20 L carboy. Transfer stir bar to carboy.
5. Rinse the flask with DI water and pour into the carboy.
6. Fill carboy with DI water to 20 L, place on large stir plate, and stir at moderate speed.
7. Adjust pH of sodium citrate solution to target pH of 7.0 with citric acid. Solution is weakly buffered; adding 1 mL increments of citric acid solution is recommended.

II. Sample Extraction

1. Label one glass extraction tube for each sample. Weigh 3.00 g (± 0.05 g) of air-dried soil into the tube (may be done in advance).
2. Soil checks should be prepared in the same manner as the unknown soils and serve as laboratory reference samples. It is recommended to pulverize and homogenize a large batch of air-dried soil for long-term use. The soil checks allow for a quality control check across protein analyses performed on different batches, over multiple days, and with different reagents.

3. Using the bottle-top dispenser, add 24 mL of the prepared 20 *mM* sodium citrate stock solution to each tube containing soil.
4. Cap tubes tightly.
5. Transfer tubes to separate rack configured to protect glass tubes from breaking while on the shaker.
6. Place rack horizontally on a shaker at 180 oscillations per minute for 5 minutes ("low" setting on Eberbach reciprocal shaker).
7. After 5 minutes, remove samples from shaker and invert the tubes a couple times to ensure that there is no soil clinging to the sides of the tube.
8. Loosen caps so they are not airtight but still placed on the tube to protect contents.
9. Place tubes in metal rack in an autoclave-safe plastic tub as a precaution to keep autoclave clean. In the event that a sample boils over, the sample will need to be rerun.
10. Autoclave (121^{o}C, 15 psi, slow/liquid setting) for 30 minutes.
11. Remove samples from autoclave. Wear heat protective gloves. Autoclave surfaces are hot.
12. Set aside to equilibrate to room temperature before clarification.

III. Sample Clarification

1. Label a set of 2.0 mL microcentrifuge tubes that correspond with extraction tubes.
2. Tighten caps on extraction tubes again and place on shaker for 3 minutes at 180 oscillations per minute. Ensure that there is no soil clinging to the sides of the tube.
3. Transfer 1.50 mL (2 x 750 µL) of mixture, using an adjustable 100-1000 µL pipette, to corresponding 2 mL microcentrifuge tube (use a new, clean tip for each sample). Close microcentrifuge tube cap.
4. Place and evenly distribute tubes in microcentrifuge.
5. Centrifuge at 10,000 g for 3 min. On occasion, samples with high clay content will not separate completely. If this happens, simply repeat this step until complete separation occurs.
6. Transfer 750 µL of the cleared extract liquid layer to a microtiter tube in a 96-well format rack, using a 1000 µL pipettor with a new, clean, tip. Avoid dislodging the pellet of solids at the bottom of the tube. Note this step isn't necessary but facilitates using a multi-channel pipette for loading the plate.
7. If the samples will not be quantified on the same day, they can be stored for 24 hours in the refrigerator. Transfer to microtiter tube and cap to store. Before sample quantification steps below, allow samples to equilibrate to room temperature.

IV. Reagent and Standard Preparation

1. Prepare the BCA working reagent in a 50 mL Falcon tube. The working reagent is a 50:1 mixture of two parts: Reagent A and Reagent B.
2. To make enough for one 96 well plate, use 25 mL of A and 500 µl of B.
3. Prepare BSA standards for multi-channel use, by pipetting aliquots (~750 µL) from each of the vials into corresponding and labeled microtiter tubes. The standards should include the following concentrations: 0, 125, 250, 500, 750, 1000, 1500, and 2000 µg per mL BSA. The 0 µg mL^{-1} standard is a blank and consists of only the sodium citrate buffer. All absorbance values should be adjusted by subtracting the average blank absorbance from each sample absorbance.

V. Sample Quantification

1. Ready a 96-well plate, ensuring bottom is free from scratches or dust.
2. Preheat the heating plate to 60°C.
3. Carefully remove strip caps from tubes with standards (0, 125, 250, 500, 750, 1000, 1500, and 2000 micrograms per milliliter BSA).
4. Using the adjustable 1-10 µL multichannel pipette and tips, pipette 10 µL of the standards into the first and last column of the reaction plate.
5. Pipette 10 µL of each sample into wells of the reaction plate.
6. Retrieve the pre-mixed working reagent and transfer it to a clean, dry pipette reservoir.
7. Using the adjustable 30-300 µL multichannel pipette and tips, add 200 µL of working reagent to each well. The colorimetric reaction with proteins begins at this step.
8. When the reaction plate is filled, cover with plate sealing tape and seal completely with roller.
9. Place plate in the heating plate and cover with lid. Start a timer set for 60 minutes.
10. After 60 minutes, remove the reaction plate from the heating plate and place on benchtop to cool for 5 minutes undisturbed.
11. Turn on plate reader, start the plate reader program (Gen5), and select the appropriate protocol from the Gen5 menu to read the plate at 562 nm.
12. When plate has cooled, centrifuge for at 2,000 g for 1 minute to consolidate any condensate that might have formed on tape surface into sample.
13. Carefully remove the tape.
14. Place plate on tray of plate reader and click "OK" to read.
15. Save file and export to Excel.

VI. Calculating Mass of Protein for Unknown Soil Samples

1. The amount of protein is a function of the amount extracted from soil. Note that this assay does not extract all soil protein, but rather an operationally-defined pool that serves as a functional indicator in soil.
2. Use the following equation to determine protein content:

Protein (g kg^{-1} soil) =

$$[(a(Abs_u - Abs_b)^2 + b(Abs_u - Abs_b) + c) \times 24\ mL] / (Wt \times 1000)$$

Where:
- a = coefficient (a) of standard curve
- b = coefficient (b) of standard curve
- c = intercept (c) of standard curve
- Abs_u = absorbance of unknown soil sample
- Abs_b = average absorbance of blank
- Wt = weight of air-dried soil sample in grams

This equation provides a total mass of extracted protein (µg) per unit of soil (g). The equation technically reports µg mg^{-1} soil, which is equivalent to and reported as g ACE-protein kg^{-1} soil.

Example Calculation

Construct standard curve with the following values:

Y-axis BSA Standard Concentrations (µg/mL)	125	250	500	750	1000	1500	2000
X-axis Average Adjusted Absorbance (562 nm)	0.195	0.379	0.743	1.123	1.380	1.849	2.251

This produces the quadratic equation: $y = 199.15x^2 + 414.53x + 53.364$; $R^2 = 0.9994$
Unknown sample absorbance: 0.920
Average absorbance of Blank: 0.105
Unknown sample weight: 3.00 g

Protein (g kg^{-1} soil) =

$$[(199.15(0.920 - 0.105)^2 + 414.53(0.920 - 0.105) + 53.364) \times 24] / (3.00 \times 1000)$$

= 4.19 g Protein kg^{-1} soil

Clean-up and Disposal

1. Discard all microcentrifuge tubes and microtiter tubes into a trash bin.
2. Place reaction plate and reagent reservoir in hood or let dry on counter. These can be thrown away once completely evaporated.
3. Using a phosphate free detergent, clean all surfaces of tubes and caps including tube that was used to prepare the reagent. Final rinses should be done with DI water.

References

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Wright S.F. and Upadhyaya A. 1996. Extraction of an abundant and unusual protein from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi. Soil Science 161: 575-586.

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Questions can be directed to Steve Culman at culman.2@osu.edu