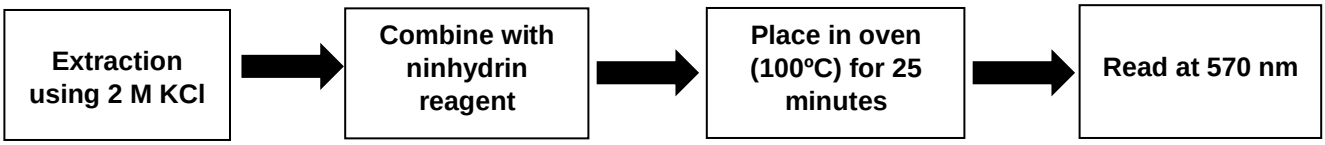
SOP: **Free Amino Acids (ninhydrin)**

Overview:

This standard operating procedure (SOP) describes a protocol for estimating free amino acids in soil. The method was adapted from Jones et al. (2002). Key instruments are a shaker table, oven, and ultra-violet spectrophotometer and key consumables are ninhydrin and sodium acetate. Key safety considerations are the use of nitrile gloves. Soils in field-moist condition are typically used.



Safety:

All standard safety protocols and online safety training via UIUC Division of Research Safety (DRS) are required.
Personal protection (PPE) for this procedure include:
Hand Protection: Nitrile gloves
Particularly hazardous substances: …
Specific details on these substances are incorporated in the **Detailed Procedure** below.

Instrumentation & Consumables:

Sample preparation

- Analytical balance capable of weighing to two decimal places
- 50 mL disposable polypropylene centrifuge tubes (Falcon tubes)
- Centrifuge tube racks

Standard preparation

- Pure glycine
  - Optional: may use other amino acids for standard solution
- 1000 ppm NH_4^+ standard solution
  - **Important:** The estimated concentration of NH_4^+ in each sample is needed. Please use the colorimetric method (Found on Margenot Lab website: https://margenot.cropsciences.illinois.edu/methods-sops/) on the same soil samples within 3 days of this experiment.

Extraction

- 50 mL disposable polypropylene centrifuge tubes (Falcon tubes)

    - A second set of tubes are needed for the filtered extractions. It is recommended to have these organized and labeled before the time of extraction.
- 50 mL dispensette
- 2.0 M KCl (or 0.5 M K_2SO_4)
    - If made in batches, combine before use
    - Store in a closed container indefinitely at room temperature
- Shaker table
- Whatman 70 mm filter paper, grade 42, pore size 2.5 µm
- Centrifuge

Ninhydrin Reaction

- Sodium acetate buffer
    - Sodium acetate
    - DI water
    - Glacial acetic acid
- Ninhydrin reagent
    - Hydradintantin dihydrate (location: organics cabinet)
    - Ninhydrin (location: fridge S-13)
    - 2-Methoxyethanol (also called methyl cellosolve, location: underneath fume hood)
- 1.5-2 mL microcentrifuge tubes
- Ethanol (50% v/v)

Colorimetry

- 50 µL-1 mL pipettor and tips
- Ultra-violet spectrophotometer
- 96-well microplates

Detailed Procedure:

I. **Sample preparation**
    1. Measure 6 ± .05 g oven-dry equivalent of field-moist soil into Falcon tubes. Each soil sample requires at least 1 replicate but at least 2 replicates are ideal.
        i. The mass of the soil is based on the 1:5 soil (g) to KCl (mL) ratio; 5 g oven-dry equivalent may be used with 25 mL KCl.

II. Reagent preparation
    1. Sodium acetate buffer (200 mL, store at 2-8ºC up to 2 months)
        i. Add 108.8 g of sodium acetate to 80 mL of DI water.
        ii. Bring pH to 5.2 using ~20 mL of glacial acetic acid.
        iii. Bring volume to 200 mL using DI water.

2. Ninhydrin reagent (10 mL, prepare fresh prior to each experiment):
    i. Add 0.03 g of hydradintantin dihydrate and 0.2 g ninhydrin to 7.5 mL of pure 2-Methoxyethanol.
    ii. **Just prior to analysis**, add 2.5 mL sodium acetate buffer.

Note: make enough ninhydrin reagent to add 50 µL to each sample.

III. Standard preparation

1. Glycine standard (0.1 mM = 100 µM):
    i. Add 0.0375 g of glycine to 50 mL of 2 M KCl solution. This will make 10 mM glycine solution.
    ii. Dilute the 10 mM glycine solution to a 0.1 mM solution:

Volume standard (µL)	Volume KCl (µL)	Final concentration (mM)
100 µL of 10 mM	900 µL	1 mM
100 µL of 1 mM	900 µL	0.1 mM (e.g. 100 µM)

Note: make enough 100 µM glycine solution to use 100-300 µL for the experiment. If K_2SO_4 is being used to extract samples instead of KCl, then dilute standards in K_2SO_4.

2. NH_4^+ interference (25 ppm):
    i. Dilute 1000 ppm NH_4^+ to 25 ppm using 2 M KCl solution:

Volume standard (µL)	Volume KCl (µL)	Final concentration (mM)
100 µL of 1000 ppm	900 µL	100 ppm
250 µL of 100 ppm	750 µL	25 ppm

Note: make enough 25 ppm NH_4^+ solution to use 100-300 µL for the experiment. If K_2SO_4 is being used to extract samples instead of KCl, then dilute standards in K_2SO_4.

    ii. **Important:** The estimated concentration of NH_4^+ in each sample is needed. Please use the colorimetric method (Found on Margenot Lab website: https://margenot.cropsciences.illinois.edu/methods-sops/) on the same soil samples within 3 days of this experiment. *Remember to use units of µM for calculations.*

IV. Extraction

1. Using a dispensette, add 30 mL of 2.0 M KCl to the soil samples.
2. Recap tubes and place on the shaker table (low setting – 120 rpm) for 1 hour.

3. While extractants are shaking, fold filter paper into funnels on each of the labeled filtration tubes. This can be done by folding the circular filter paper into quarter-circles, then opening from the top and pressing into tube.
4. After 1 hour, remove tubes from the table and pour the extractants through the filters until 5-10 mL of clear solution has accumulated. If extractants are not clear after filtration, repeat the process with a new tube and filter paper. Centrifugation prior to filtration is not necessary but highly recommended for time efficiency.
5. Extractants may be stored at -20ºC for 2-4 weeks prior to analysis.

V. Ninhydrin reaction

1. Add 100 µL of sample extractant (e.g. supernatant) to a microcentrifuge tube followed by 50 µL of ninhydrin reagent. Do this for standards as well.
2. In a second set of microcentrifuge tubes, add 100 µL of sample extractant and 50 µL sodium acetate. These will serve as blanks.
3. Cap and invert the tubes, then place in the oven at 100ºC for 25 minutes.
4. Once the tubes are removed from the oven, allow to cool for ~5 minutes.
5. Add 950 µL ethanol to each tube.

VI. Colorimetry

1. Transfer 200 µL of each sample and standard to a microplate well.
2. Read at 570 nm using a UV spectrophotometer.

VII. Clean up

1. Dispensette should be cleaned **immediately** after use to prevent crystal formation. Return to maximum volume, then pump deionized water 5-10x. Empty and allow to dry before storing.
2. If the centrifuge was used, remove Falcon tube holders and wipe away any liquid at the bottom. If KCl crystalizes around the tube holders, they can be extremely difficult to take apart and change tube sizes.
3. Dispose of remaining reagent in a capped and labeled waste bottle.
4. Any remaining 2.0 M KCl solution may be drained in the sink after diluted 20x and flushing the sink with 1-2 L of tap water after.
5. Falcon tubes and filters may be thrown away in regular trash bins.

VIII. Calculations

Measurement of amino acids is usually expressed in units of µM. To calculate, see equation below:

$$Amino\ acids\ (\mu M) = \frac{O - B - A}{(\frac{S}{100})}$$

O = sample absorbance value
B = blank absorbance value
S = 100 µM glycine standard absorbance value

A = interference of NH_4^+ = $\frac{Ac}{As} \times Ar$

- *Ac* = NH_4^+ background concentration, as determined by the colorimetric method (µM)
- *As* = NH_4^+ standard concentration (µM)
- *Ar* = the absorbance value of the NH_4^+ standard using the ninhydrin colorimetric procedure

NOTE: to convert NH_4^+ background concentration from ppm to µM use the following formula.

$$NH_4^+ \ concentration \ (\mu M) = \frac{NH_4^+ \ concentration \ (ppm) \times 1000}{18.039}$$

Example calculation:

$$\text{Amino acids } (\mu\text{M}) = \frac{0.111 - 0.048 - \left(2.21 \ x \ 10^{-5}\right)}{\left(\frac{0.116}{100}\right)} = 54.29 \ \mu\text{M}$$

0.111 = sample absorbance value
0.048 = blank absorbance value
0.116 = 100 µM glycine standard absorbance value

2.21 x 10^{-5} = interference of NH_4^+ = $\frac{0.053}{1427.83} \times 0.596$

- 0.053 = NH_4^+ background concentration, as determined by the colorimetric method (µM)
- 1427.83 = NH_4^+ standard concentration (µM)
- 0.596 = the absorbance value of the NH_4^+ standard using the ninhydrin colorimetric procedure

References:

Jones et al. 2002. Simple method to enable the high resolution determination of total free amino acids in soil solutions and soil extracts. *Soil Biology and Biochemistry* 34: 1893-1902. https://doi.org/10.1016/S0038-0717(02)00203-1

Citation:

SOP: Free Amino Acids (ninhydrin). 2024. Soils Lab, University of Illinois Urbana-Champaign. Urbana, IL. Accessed at: https://margenot.cropsciences.illinois.edu/methods-sops/

Questions can be directed to Andrew Margenot at margenot@illinois.edu