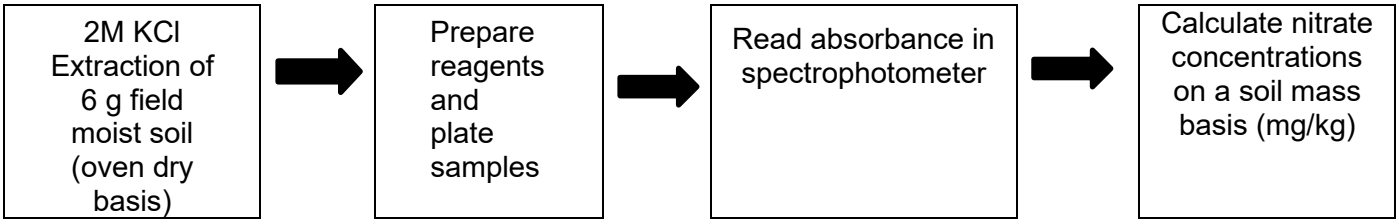
SOP: **Determination of Soil Extractable Nitrate via Vanadium (III) reduction**

Overview:

The following standard operating procedure (SOP) for nitrate is accomplished via **Vanadium (III) reduction**. Vanadium (III) reduces nitrate into nitrite/nitric oxide when combined with a dilute acid solution[1,3]. The reaction of nitrate reduced by Vanadium (III) occurs as followed:

$$NO_3^- + 3V^{3+} + 2H_2O \rightarrow NO + 3VO^{2+} + 2H^+$$

By capturing both nitrite and nitric oxide with **Griess reagents** (sulfanilamide, N-(1-naphthyl)-ethylenediamine), a **pink dye** to each sample is produced, allowing for the colorimetric analysis of nitrate[1,2,4].



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:
Eye Protection: Safety goggles
Body Protection: Lab coat
Hand Protection: Gloves
Particularly hazardous substances:
Vanadium (III) chloride should be handled with care and under a fume hood, as this substance gives off corrosive fumes when exposed to moist air[1]. Once in solution, however, fumes will not occur. Any waste produced while handling Vanadium (III) chloride should be placed either in a labeled waste container or placed in a labeled solid waste bag according to DRS regulations. Specific details on these substances are incorporated in the **Detailed Procedure** below.

Instrumentation & Consumables:

Standards preparation

- 1.5 mL microcentrifuge tubes
- Nitrate standard
    - Location: Refrigerator, S-23
- 2M Potassium Chloride (KCl)
- 1000 µL pipette and tips

Reagent preparation

- Analytical balance (two decimal places sensitivity)
- Solution 1
    - 125 mL Erlenmeyer flask wrapped with tin foil.
    - Vanadium (III) Chloride
        - Location: Refrigerator, S-23 or desiccator, S-27
- Solution 2
    - 500mL Erlenmeyer flask wrapped with tin foil.
    - Sulfanilamide
        - Location: Organic reagents shelf
    - N-(1-naphthyl) ethylenediamine dihydrochloride (NED)
        - Location: Organic reagents shelf

Colorimetry

- 96 well microplates
- Microplate spectrophotometer
- Pipettes and tips (20 µL – 200 µL)

Detailed Procedure:

I. 2M KCl Preparation

1. ***Can use potassium sulfate or potassium chloride, but values cannot be directly compared*****
2. In a 1L volumetric flask, dissolve 150 g KCl into 1L 18.2 MΩ water.
3. Calculate in advance how much 2M KCl reagent you will need for all samples. Each sample will require 30 mL of 2M KCl.
    i. Using the proportion above in step 1, the amount of KCl needed for your desired volume can be calculated.
    ii. A slight excess of KCl should be made to ensure that you don't run low when trying to use the dispensette.
    iii. **Example:** For 100 samples 3 L of 2M KCl is needed. To make up 3.5L 2M KCl—
    ~525 g of KCl will be added into 3.5 L 18.2 MΩ water.
    iv. Extra can be made for future use. Additionally, KCl Extract can be used to determine Ammonium-N (See "SOP: Ammonium-N

Protocol").

II. 2M KCl Extraction

1. Weigh 6 g (+/- 0.05) of field-wet soil on an **oven-dried basis** into a 50 mL centrifuge tube. Record exact weight of soil and centrifuge tube.
    i. To calculate the amount of wet soil needed, see *Example 2* under "Example Calculations" and/or SOP: GWC Standard Operating Procedure (Soil Labs 2023).
2. To the measured field-wet soil, add 30 mL of the 2M KCl extracting solution. Include blank samples, where no soil is added.
3. Shake samples for 1 hour on a reciprocating shaker, on low setting.
4. Remove from reciprocating shaker, and then let rest for at least 30 min for the soil can settle to the bottom.
5. Prepare new 50 mL or 15 mL centrifuge tubes for each extracted sample. Fold and place Whatman #42 filter papers over each new tube.
6. When samples are settled, filter the extracted sample solution into the new centrifuge tubes. Filter at least 1 mL of sample extract.
7. Filtered sample extracts should be stored in the refrigerator if being analyzed immediately, or frozen to preserve samples if immediate analysis is not possible.

III. Standards Preparation

1. Pour a small amount of the nitrate standard into a 15 mL centrifuge tube. This is to avoid any type of contamination on the reagent container.
2. Pipette the required amount of standard reagent (Table 1) to dilute in 2M KCl from 1000 mg/L to 100 mg/L into a 1.5 mL microcentrifuge tube. Proceed to pipette the required amount of 2M KCl and shake the mixture. Serial dilution then continues from the previous diluted standard.
3. Refer to Table 1 for examples of serial dilutions that can be used for creating a standard curve. The amounts will change depending on the required detection range.
4. Once the standards are ready, they should be treated as samples when it comes to plating them on the well microplate. Follow the exact colorimetry procedure for preparing samples for the standards. Make sure to remember their location on the plate, since the absorbance will be needed to create the curve to convert absorbance readings of the samples to concentrations.

Table 1. Standards dilutions

Standard concentration (mg/L)	Amount of 2M KCl (µL)	Serial dilution of standards
		Amount of NO_3^--N standard (µL)
100	900	100 of 1000 mg/L
20	800	200 of 100 mg/L
10	500	500 of 20 mg/L
5	500	500 of 10 mg/L
2.5	500	500 of 5 mg/L
1.25	500	500 of 2.5 mg/L
0	500	-

NOTE: Serial dilution consists of performing the same dilution step repeatedly using the previous diluted solution as the input to the next dilution in each step. To create a serial dilution a concentration factor and a dilution factor are needed. These concepts are explained in the following formulas:

$$Concentration\ factor = \frac{volume_{initial}}{volume_{final}}$$

$$Dilution\ factor = \frac{1}{concentration\ factor}$$

The purpose of using serial dilutions is making the standards with known concentrations to create a standard curve, also known as calibration curve. This tool represents the relationship between two quantities. In this case, it assigns an estimated concentration to the absorbance value of the sample. Without the calibration curve, the absorbance values don't have a meaning. All the absorbance values of the samples must lie between the lowest and the highest absorbance values of the created standards. This means that the concentrations of the standards should be planed around the expected/estimated range of nitrate concentrations of the samples. However, a standard curve involving colorimetry is usually only linear until a certain concentration. Samples outside the range of the standard curve therefore need to be diluted with 2M KCl (e.g., 0.2 mL extract + 0.8 mL 2M KCl to yield 5x dilution) to bring the concentration down to the range of the standard curve, and the sample concentrations will need to be corrected for the corresponding dilution factor.

Note the use of NO3-N standard which is considered an "N basis" in contrast to an ion basis as NO_3^-. Nitrate-N on an N basis refers to only the N for each sample, while the ion basis includes the entire NO_3^- ion. For determining nitrate-N in soil samples, an N basis should be done using nitrate-N standards because of this distinction.

IV. Colorimetry Reagents

1. Solution 1 (**Make FIRST**, and wrap in aluminum foil)
    i. Pour 400 mL 18.2 MΩ water into a 500 mL Erlenmeyer flask. Add:
        1. **0.2 g** sulfanilamide

2. **0.01 g** N-(1-naphthyl)-ethylenediamine

ii. Swirl until completely dissolved.

2. Add Solution 1 to Solution 2
   i. Store reagent in Nalgene bottle, covered with aluminum foil. Reagent can last up to 2-3 weeks in the refrigerator, or indefinitely in a freezer.
3. Solution 2 (**Make LAST**, and wrap in Aluminum foil)
   i. Make 1M HCl by diluting concentrated HCl with 18.2 MΩ water.
   ii. Underneath the fume hood is storage for concentrated HCl.
      1. Transfer a small amount of concentrated HCl from a large container to a smaller container (e.g., 15 mL Falcon tube).
   iii. Pour approximately 25 MΩ water into a 50 mL volumetric flask, then add **4 mL** of concentrated HCl. Dilute up to 50 mL in 18.2 MΩ water.
      1. **NOTE:** ADD WATER BEFORE ACID. AT LEAST 13 mL OF WATER IS NEEDED BEFORE ADDING ACID.
   iv. Pour the 50 mL of 1 M HCl into 150 mL Erlenmeyer flask.
   v. Measure out **0.4 g** (+- 0.2 g) of Vanadium (III) chloride (in fridge, S-23 and desiccator, S-27).
      1. **NOTE:** Always wear gloves, and always measure out in fume hood. A portable balance must be moved into fume hood for measurement. Use thin weighing paper and a spatula. Any waste produced should be placed in a labeled waste container or labeled solid waste bag according to DRS regulations.
   vi. Add Vanadium (III) to HCl. Wrap 150 mL Erlenmeyer flask in aluminum foil. Swirl until completely dissolved.

V. Colorimetric Analysis

1. Preparing samples with reagents (Table 2)
   i. Add Sample
   ii. Add Reagent
   iii. (Invert if using cuvettes)
   iv. Leave samples covered with aluminum overnight (12-16 hours)
   v. Read at 540 nm.

Table 2. Reagent and sample proportions

<1 mg/L (LL 0.005 mg/L)	**1-5 mg/L (LL 0.05 mg/L)**	**1-10 mg/L (LL 0.07 mg/L)**	**1-20 mg/L (LL 0.07 mg/L)**	**1-50 mg/L**
Cuvettes------	**Cuvettes------**	**Cuvettes------**	**Cuvettes------**	**Cuvettes------**
500 µL reagent	1000 µL reagent	1000 µL reagent	1000 µL reagent	3500 µL reagent
500 µL sample	100 µL sample	45 µL sample	20 µL sample	30 µL sample
Microplates-----	**Microplates-----**	**Microplates-----**	**Microplates-----**	**Microplates-----**
150 µL reagent	273 µL reagent	287 µL reagent	294 µL reagent	297 µL reagent
150 µL sample	27 µL sample	13 µL sample	6 µL sample	3 µL sample

I. Clean Up

1. Dial back the pipettes to their corresponding volume.
2. Dispensette: clean IMMEDIATELY after use. Failure to do so will result in KCl crystallization in the dispensette, making it difficult to clean out.
3. Make sure all reagents are back on the shelves.
4. Liquid waste should be kept in a labeled liquid waste container. Any solid waste from handling of Vanadium (III) chloride should be kept in a separate labeled waste bag.
5. Liquid waste from nitrate reagents should be kept in a separate waste container. DO NOT combine or mix with ammonium waste.

II. Calculations

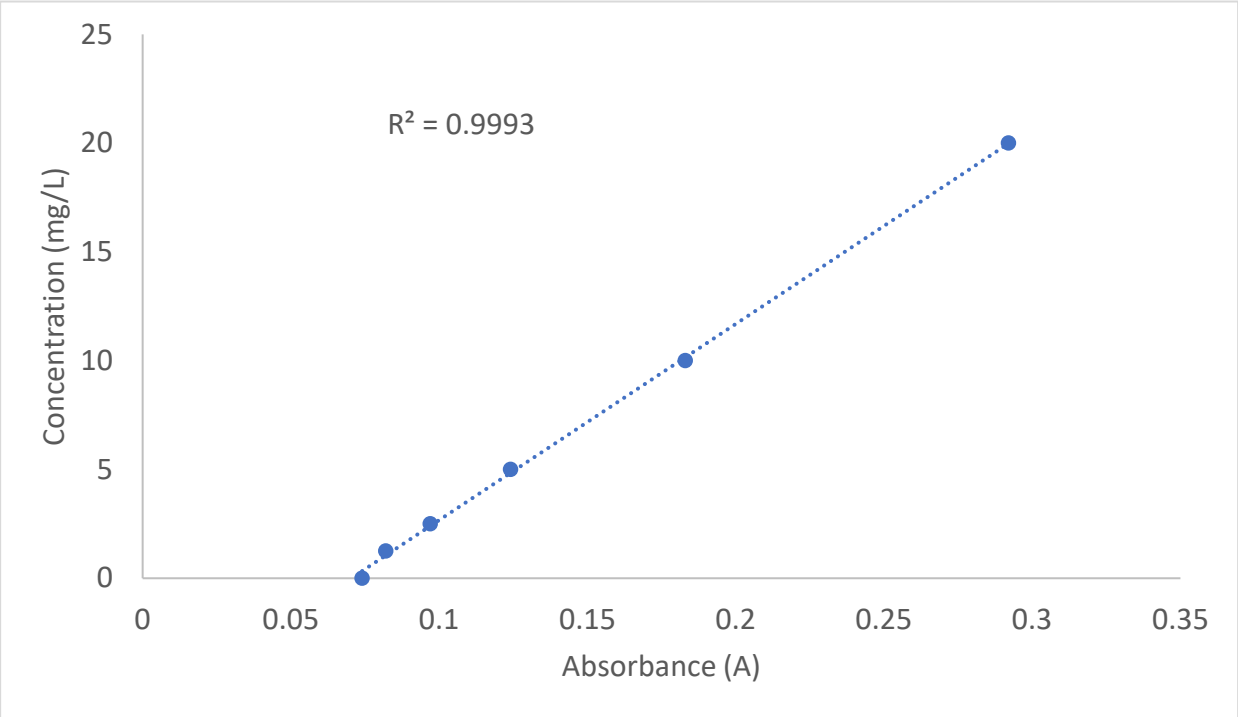
Measurement of nitrate is usually expressed in units of mg/L. To calculate, see Example 1 under “Example Calculations”.

1. Generate a scatter plot graph for the standard curve (Table 1). Add a trend line and check if the R^2 value is acceptable (<0.9). An example of the absorbance values and graph is provided below.
2. Conversions can also be done from mg/L to mg kg-1 soil (see Example 3 under “Example Calculations”.

Table 3. Example of recorded absorbance values from standard samples analyzed through the spectrophotometer.

Absorbance values	0.074	0.082	0.097	0.124	0.183	0.292
Concentration of standards (mg/L)	0	1.25	2.5	5	10	20

Figure 1. Example of nitrate standard curve, with absorbance values recorded from spectrophotometer and concentration of each standard created through serial dilutions.



Example calculations:

Example 1. Sample conversion from absorbance (A) to mg/L (i.e., “ppm”).

	A	B	C	D
1	Absorbance (A)	Concentration (mg/L)	Samples (A)	Samples (mg/L)
2	0.074	0	1.329923274	113.5300274
3	0.082	1.25	1.150895141	97.39359339
4	0.097	2.5	1.355498721	115.8352322
5	0.124	5	1.636828645	141.1924856
6	0.183	10	1.150895141	97.39359339
7	0.292	20	1.023017903	85.86756912
8				

Green (left two) columns are from standard curve. Orange (right two) columns are from sample readings. The standard curve is used to convert the sample absorbance (A) into concentration (mg/L).

=TREND(known_ys, known_xs,new_xs)
=TREND(concentration_values,absorbance_values,sample_value)
=TREND(B$2:B$7,A$2:A$7,C2)
LOCK the "concentration_values" and "absorbance_values"
i.e., add the dollar sign after each letter. DO NOT lock sample value.

Example 2. Calculation to determine fresh weight needed on an oven dried basis.

Sample ID	1
Soil before drying (g)	9.97
Tin Mass	1.76
Soil + Tin after drying (g)	9.85
Soil after drying (g)	8.09
GWC (%)	23.23856613
GWC in 6 g soil	1.394313968
Fresh Weight needed	7.394313968
Actual Weight (g)	7.4
Weight (g) oven dried basis	6.004613842

Sample ID: sample name/number
Soil before drying (g): weigh 10 g (+/- 0.5) of wet soil and record exact weight to 0.00 decimal place.
Tin mass: record weight of tin that soil sample is placed in.

Soil samples should be oven dried @ 105°C for at least 24 h.

Soin and Tin after drying: record weight of soil and tin after samples has been oven dried.

Gravimetric Water content (GWC): = **([soil before drying - soil after drying]) / [soil after drying]) * 100**
= (9.97-8.09)/(8.09)*100 = 23%

GWC in 6 g soil: **=6*(Water content %)** = 6*23% = 1.39 g

Fresh weight needed: **=6+(WC in 6 g soil)** = 6+1.39 = 7.39 g fresh weight needed

Actual weight: Actual fresh weight recorded, +/- 0.05 of the fresh weight calculated above. = 7.40 g

Oven dried basis weight: **=(Actual Weight) / (1+[Water Content %] /100)**
= (7.4) / (1+ 23/100) = 6.005 g oven dried basis used

Example 3. Conversion from extract concentration to soil basis (mg NO3-N/kg soil).

L	M	N	O	P
Samples (mg/L)	Extraction Vol (L)	Actual Weight (g)	Soil Mass (kg)	**mg/ kg soil**
113.5300274	0.03	7.53	0.00753	452.310866
97.39359339	0.03	7.63	0.00763	382.9368023
115.8352322	0.03	7.73	0.00773	449.5545882
141.1924856	0.03	7.44	0.00744	569.3245388
97.39359339	0.03	7.35	0.00735	397.524871
85.86756912	0.03	7.21	0.00721	357.2853084

Orange (left most column) is from sample readings.

Extraction volume: each soil sample was extracted in 30 mL of 2M KCl.

Actual weight (g): recorded wet mass used for soil extraction.

Soil mass (kg): =(actual weight) / 1000

mg/kg soil: **=(concentration * extraction vol) / (soil mass kg)**

References:

1. Doane, T.A., and Horwath W.R., 2005. Nitrate via manual vanadium(III) reduction. *National Environmental Methods Index*. https://www.nemi.gov/methods/method_summary/9171/
2. Doane, T.A., and Horwath W.R., 2003, Spectrophotometric determination of nitrate with a single reagent. Analytical Letters 36(12):2713-2722. Marcel Dekker Publishing.
3. Hendrix, S. A., & Braman, R. S. (1995). Determination of Nitrite and Nitrate by Vanadium(III) Reduction with Chemiluminescence Detection. *Methods*, *7*(1), 91–97. https://doi.org/10.1006/meth.1995.1013
4. Miranda, K. M., Espey, M. G., & Wink, D. A. (2001). A Rapid, Simple Spectrophotometric Method for Simultaneous Detection of Nitrate and Nitrite. *Nitric Oxide*, *5*(1), 62–71. https://doi.org/10.1006/niox.2000.0319

Citation:

SOP: Nitrate Standard Operating Procedure. 2023. 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