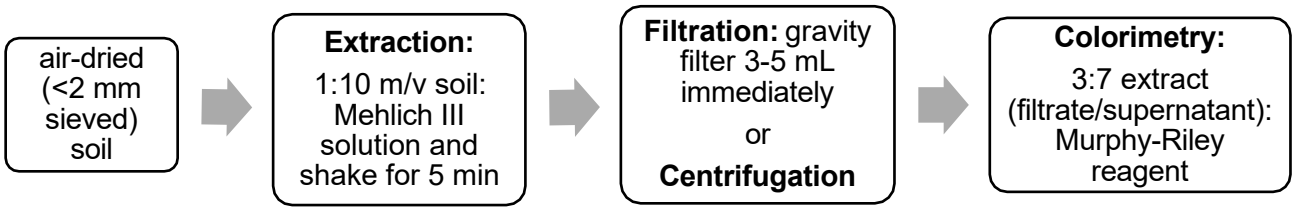
SOP: **Mehlich III-extractable P**

Overview:

This standard operating procedure (SOP) describes a protocol for weak organic acid soil extraction followed by molybdate-colorimetry to estimate the "plant-available," or more operationally, the "test" phosphorus (P) pool in soil. Although other labile organic and inorganic P compounds are extracted from soil, colorimetric P measurements will not detect these *or* may over-estimate orthophosphate if these P forms are hydrolyzed during the extraction and determination processes (Cade-Menun et al 2018). The Mehlich III extractant combines acetic acid (CH_3COOH), ammonium nitrate (NH_4NO_3), ammonium fluoride (NH_4F), nitric acid (HNO_3) and ethylenediaminetetraacetic acid (EDTA) at pH 2.5 to determine soil test P and can be used over a wider range of soil pH than other soil tests. Critically, this method estimates orthophosphate in the extract as molybdate-reactive P (MRP), in contrast to ICP-based quantification.

The method was developed by Mehlich (1984) to modify the original Mehlich-1 method in 1981 to be applicable to a wider range of soils. This protocol is based on Tran and Simmard (1993). Air-dried soil that are ground to pass a 2 mm sieve is 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:
Eye Protection: Safety goggles
Body Protection: Lab coat
Hand Protection: Gloves
Particularly hazardous substances: Concentrated sulfuric acid and nitric acid and glacial acetic acid should be handled in the fume hood. Ammonium fluoride is rat poison and hazardous. Make sure to check Material Safety Data Sheet (MSDS) if unsure about how to handle these chemicals. Specific details on these substances are incorporated in the **Detailed Procedure** below.

Instrumentation & Consumables:
Sample preparation

- Analytical balance (two decimal places sensitivity)
- 50 mL centrifuge tube
- <2 mm sieved soil samples
- 1 L, 2 L, 100 mL class A volumetric flasks
- Nalgene beaker
- 1L plastic storage bottle
- 100 mL graduated cylinder

Extraction

- Horizontal shaker (low setting – 120 rpm required)
- Macro-centrifuge (optional, see notes)
- Stir plate and stir bar
- Dispensette
- 50 mL centrifuge tubes
- Pipette and tips (40-1000 µL)

Filtration/centrifugation

- 15 mL centrifuge tubes (*only if filtration is done*)
- Filter funnels (*only if filtration is done*)
- Whatman 42 filter paper (42.5 mm diameter, 2.5 µ pore size) (*only if filtration is done*)
- Microcentrifuge
- Microcentrifuge tubes and racks
- Cold storage of filtrates (4°C for short-term storage)

Colorimetry

- Cuvettes (or 96 well microplates)
- Beckman Spectrophotometer capable of reading at 882 nm (or microplate spectrophotometer)
- Pipette and tips (100-1000 µL)
- Commercial P standard (1000 mg P/L)

Extracting Solution Preparation

- Ammonium fluoride (NH_4F)
- Ethylene diamine tetra acetic acid (EDTA)

Working Solution Preparation

- Ammonium nitrate (NH_4NO_3)

- Acetic acid (glacial) (≥ 99.7% ACS grade)
- Concentrated Nitric acid (HNO_3 72% ACS grade)

Murphey-Riley Reagent Preparation
- Concentrated Sulfuric acid (H_2SO_4 95-98% ACS grade)
- Ammonium molybdate tetrahydrate (CAS number: 12054-85-2)
- Antimony potassium tartrate
- Ascorbic acid

Detailed Procedure:

I. **Sample Preparation**
   1. Measure **3.00 g** of air-dried soil into 50 mL centrifuge tube. Falcon tube is recommended for avoiding leak during extraction. Record exact weight of soil to at least 1/100th of a gram (3 ± .xx g)

II. **Reagent Preparation**
   1. Extracting solution preparation (*5 L of working solution can be used for 150 sample extractions*): This is a weak organic acid soil extraction procedure that has the advantage of being applicable for several elements. The extract is composed of 0.2 M CH_3COOH, 0.25 M NH_4NO_3, 0.015 M NH_4F, 0.013 M HNO_3, and 0.001 M ethylene diamine tetra acetic acid (EDTA).
      i. Stock Solution
         a. Fill a 1.000 L volumetric flask to about **500-600 mL** with 18.2 mΩ water
         b. Weigh out **55.56 g** of NH_4F into a plastic Nalgene beaker using the analytical balance.
         c. Transfer NH_4F to volumetric flask using a funnel and squirt bottle of 18.2 mΩ water. *Caution: ammonium fluoride is rat poison and hazardous. Read MSDS and follow proper PPE.*
         d. Weigh out **29.23 g** of EDTA into plastic Nalgene beaker using top loading balance.
         e. Transfer EDTA to volumetric using a funnel and squirt bottle of 18.2 mΩ water
         f. Let dissolve and dilute to volume.
         g. Store in a plastic bottle in a refrigerator. The stock remains good for **4 weeks.**

      ii. Working solution
         a. Fill a clean 2.000 L volumetric flask to about 700 mL with 18.2 mΩ water.
         b. Add **100.05 g** NH_4NO_3 (weighed out on top loading balance)
         c. Add **50 mL** of stock solution (measured out using a clean 50ml volumetric)
         d. Add **57.5 mL** of glacial acetic acid (measured out in a graduated `cylinder under the hood!)
         e. Add **41 mL** of 10% v/v nitric acid

a) Fill a clean **100 mL** volumetric with about 80 mL 18.2 mΩ water.
b) Add **10 mL** of nitric acid into the volumetric and dilute to volume.

f. Dissolve ingredients and bring to volume.
g. Transfer to a 5.000 L carboy.
h. Add an additional ~3 L 18.2 mΩ water to carboy to make up to 5 L volume, and a stir bar. Stir on stir plate until well mixed. For many samples, make 10-20 L of the working solution and store in 5-gallon carboy. *Working solution can be stored and used at room temperature (25 °C) for 2 weeks.*
i. Transfer workable volumes of the working solution into 1 gallon glass jugs (dispensette fits nicely on these) for extraction.

2. Standards
   i. j. Calibration standards (ranging from 0 – 20 mg P/L) need to be made in the same extracting solution used for samples. Dilute commercial standard (1000 mg P/L) in Mehlich III extracting solution and sequentially dilute them to make calibration standards that are within the linear range and cover the concentration you are expecting for your soil samples. It is essential to use the same extracting solution for standards as for samples because molybdate colorimetry of P is sensitive to pH (color development and intensity, precipitation).

3. Colorimetry reagents
   i. Murphy-Riley Solution A
      a. Dissolve **4.3 g** ammonium molybdate in **400 mL** of deionized water in a 1-litre beaker.
      b. Dissolve **0.40 g** antimony potassium tartrate in **400 mL** deionized water, then add to the ammonium molybdate solution in the beaker.
      c. Slowly and carefully, while stirring, add **54 mL** conc. H_2SO_4.
      d. Allow to cool and make to **1000 mL** with deionized water. Mix well and store in a dark bottle in a refrigerator. *The reagent is stable for up to 6 months at 4°C
   ii. Murphy-Riley Solution B
      a. Ascorbic acid, 1%: Dissolve **1.00 g** of ascorbic acid in 100 mL deionized water. Make a fresh solution daily as needed.
   iii. Final Murphy-Riley (MR) reagent: combine **56 mL** of solution B + **44 mL** of solution A, and mix (should turn to light yellow color).

III. Extraction

1. Extract the pre-weighed soil in the 50 mL centrifuge tube by adding 30 mL of Mehlich III extracting solution (**soil to solution ratio of 1:10**).
   i. **Note:** soil mass can be increased to reduce variability if soils are not <2 mm sieved; this is also strongly recommended for non-air-dried

soils).
   ii. **Note:** break up extraction of samples into smaller groups, as extracting too many at once will lead to overextraction.
2. Shake soil on low speed (120 RPM) for 5 mins.

IV. Filtration or centrifugation

1. Filtration
   i. Remove samples from shaker and filter through Whatman 42 filters into clean 15- or 50-mL centrifuge tubes. Labels on flasks can be transferred to the tubes as the samples are poured.
      a. *Note: centrifugation (macro centrifuge - 5000 rpm, 3 minutes at 25 °C) followed by filtration can also be used, but since extraction time of Mehlich is 5 min, centrifugation will lead to appreciable continued extraction of P (compare with 30 min for Olsen, 16 h for Colwell or 16-18 h for Hedley fractionations). Thus, this step should only be included if suspended soil particles are interfering with the filtration process.
   ii. Gravity filter at least 3-5 mL and proceed to colorimetry immediately.
2. Centrifugation *(another equally valid option to use microcentrifuge for smaller quantities of extract, without prolonged contact with soil)*
   i. Immediately after shaking, quickly uncap and pipette out 1 mL of soil + extract suspension into labelled micro-centrifuge tubes (hold up to 1.5 mL) and centrifuge at 15000 rpm (17,968 g for a typical Beckman-Coulter 24-place microcentrifuge) for 1.45 min at 25 °C in a microcentrifuge.
   ii. Immediately post centrifugation, pipette out 60 µL of the clear supernatant into a 96-well plate (wells can hold 400 µL volume, but a usual recommended working volume is 350 µL), to avoid any further contact with soil.

V. P – Colorimetry

1. Note: run colorimetry within the same day to avoid hydrolysis of organic P in the (acidic) Mehlich III extract over longer storage periods
2. Colorimetry is performed directly in the well plates (standard disposable polyacrylamide or another cheap polymer). 96 well plates hold up to 350 µL. Or cuvettes (standard disposable [polyacrylamide or another cheap polymer]) may be used. Cuvettes marked as 1.5 mL cuvettes can hold up to 2.5-3 mL.
3. Conduct colorimetry using a 3:7 ratio of Mehlich III extracts (and standards!) and Murphy-Riley reagent. The reaction generally takes >20

4. minutes and should be no longer than 60 minutes.
    i. Table 1. Example volumes that have worked in the past:

Method	Mehlich III Extract (µL)	Murphy-Riley Reagent (µL)
Cuvette	300	700
96-well plate	60	140

5. Absorbance is measured at A882 in a spectrophotometer (or microplate spectrophotometer). Use 0 mg/L standard as blank reading in the Beckman spectrophotometer (abs = 0.000).
    1. To prepare standards from a 1000 mg/L P stock solution: Create a 100-mg/L (can also pick a lower concentration like 20 mg/L) aqueous stock solution by combining 1 ml 1000 mg/L stock with 9 ml nanopore water. Then, combine the new 100 mg/L stock with the extracting solution (Mehlich III working solution) in 15 ml centrifuge tubes to produce a range of standards. An example with 100 mg/L stock solution:

Standard concentration (mg/L)	100 mg/L stock (mL)	Mehlich III working solution (mL)
0.00	0.000	10.000
0.25	0.025	9.975
0.50	0.050	9.950
1.00	0.100	9.900
2.00	0.200	9.800
2.50	0.250	9.750
5.00	0.500	9.500
10.00	1.000	9.000
15.00	1.500	8.500
20.00	2.000	8.000

VI. Clean up

1. Make sure to clean up dispensette (with nanopure water – fill up to 50 mL twice and dispense) and bring to the original maximum volume of 50 mL, shaker (especially if tubes leak), and centrifuge (especially if tubes leak). Clean analytical balance with brush and kimwipe after each use in case of chemical spillage.
2. Collect solutions (except those that can go into drain with copious amount of water; check DRS for further information) into chemical waste bottle, clearly labelled with contents and their concentrations. Request pick up when the bottle is almost full (≥75%). Clean up any spills with absorptive tissues and soap water immediately as needed. For spilled dilute acids, immediately neutralize with sodium bicarbonate, and then clean up.

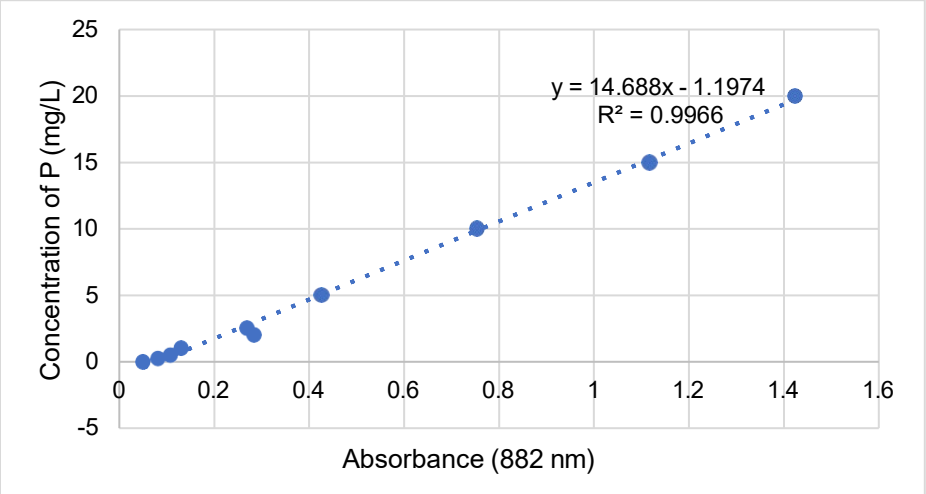
VII. Calculations

1. Measurement of available P or Mehlich III-extractable P is usually expressed in units of mg P kg^{-1} soil (See "Example Calculation"). To calculate:
   a. Convert raw absorbance to concentration (mg P L^{-1}) using calibration curve (as noted previously, calibration standards should be treated the same as samples. Absorbance can be directly converted to concentrations in the extract before dilution if samples were separately diluted). Multiply the concentration by dilution factor if diluted.
   b. Multiply the concentration by the extract volume (e.g., 30 mL = 0.03 L) and divide by soil mass (3.00 g = 0.003 kg) to yield concentration in mg P kg^{-1} soil.

Example Calculation:

Absorbance (X) = 0.292
Dilution = 1
Calibration curve: y = 14.688x - 1.1974
Extraction volume = 0.03 L
Soil mass = 0.003 kg

Concentration in extract (Y) = [14.688*0.292 - 1.1974] * 1 = 3.09 mg L^{-1}
Concentration in soil basis = 3.09 mg L^{-1} * 0.03 L / 0.003 kg = 30.91 mg P kg^{-1} soil



References:

1. Cade-Menun, B. J., Elkin, K. R., Liu, C. W., Bryant, R. B., Kleinman, P. J. A., & Moore, P. A. (2018). Characterizing the phosphorus forms extracted from soil by the Mehlich III soil test. *Geochemical Transactions*, *19*(1), 7. https://doi.org/10.1186/s12932-018-0052-9
2. Mehlich, A. (1984). Mehlich 3 soil test extractant: A modification of Mehlich 2 extractant. *Communications in Soil Science and Plant Analysis*, *15*(12), 1409–1416. https://doi.org/10.1080/00103628409367568
3. Tran, T. S., & Simard, R. R. (1993). Mehlich III-extractable elements. In *Soil sampling and methods of Analysis* (2nd ed.). https://www-taylorfrancis-com.proxy2.library.illinois.edu/chapters/mono/10.1201/9781420005271-14/mehlich-3-extractable-elements-carter-gregorich

Suggested reading:

J.B. Jr. 1998. Soil test methods: past, present, and future use of soil extractants. Commun. Soil Sci. Plant Anal. 29: 1543–1552.

Mallarino, A.P. 2003. Field calibration for corn of the Mehlich-3 soil phosphorus test with colorimetric and inductively coupled plasma emission spectroscopy determination methods. Soil Sci. Soc. Am. J. 67: 1928–1934.

Mallarino, A.P., and Sawyer. 2013. Interpretation of soil test results. Publ. PM 1310 (Rev.). Iowa State Univ. Extension.

Sharpley, A., Daniel, T.C., Sims, J.T., and Pote, D.H. 1996. Determining environmentally sound soil phosphorus levels. J. Soil Water Conserv. 51: 160–166.

Sims, J.T., Maguire, R.O., Leytem, A.B., Gartley, K.L., and Pautler, M.C. 2002. Evaluation of Mehlich 3 as an agri-environmental soil phosphorus test for the mid-Atlantic United States of America. Soil Sci. Soc. Am. J. 66: 2016–2032.

Citation:

SOP: Mehlich III-P. 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