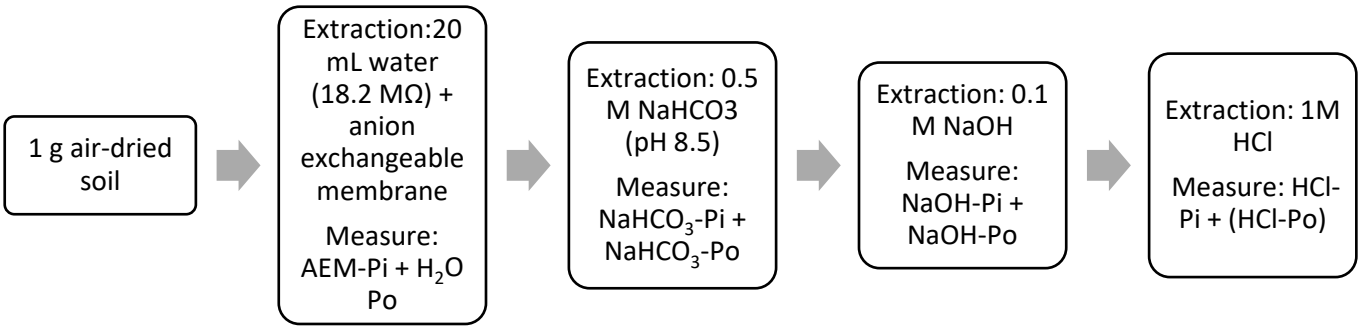
SOP: **Hedley fractionation**

Overview:

This standard operating procedure (SOP) describes a protocol for commonly used sequential extraction of soils to estimate pools of soil phosphorus (P) hypothesized to vary by availability and forms. The method was originally reported by Hedley et al. (1982) and modified by Tiessen and Moir (2007). Soils that are ground to pass a <2 mm sieve are typically used.

Note: Interpretations of extracted P fractions as certain P pools based on hypothesized difference in availability and speciation vary and potential artifacts of the extractions are reported (Cross and Schlesinger, 1995; Klotzbücher et al., 2019; Gu et al., 2020).



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: Nitrile Gloves

Particularly hazardous substances: Concentrated sulfuric acid and hydrochloric acid should be handled in the fume hood. Persulfate is an oxidizer, which can cause fire when in contact with combustibles, and exposure to high temperature should also be avoided. Make sure to check Safety Data Sheet 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 (Falcon brand is preferred for avoiding leak)

Reagent preparation

- pH meter

- Analytical balance (at least two decimal places sensitivity)
- Anion exchangeable membrane (AEM, also called resin) strip (1x4 cm, VWR International, West Chester, PA)
- $NaHCO_3$ (0.5 M; 0.5 M at pH 8.5)
- NaOH (0.1 M; 5 %; 10 %)
- HCl (1 M)
- H_2SO_4 (0.2 M; 1.2 M)
- Sodium persulfate
- Commercial P standard (1000 mg P/L)
- Ammonium molybdate
- Antimony potassium tartrate
- Ascorbic acid

Sequential Extraction

- Horizontal shaker
- Centrifuge
- Microcentrifuge
- Dispensette
- 15 mL centrifuge tubes
- Pipette and tips (100-1000 uL)

Digestion

- 15 mL centrifuge tubes
  - use Falcon brand (material: PP) for digestion to avoid leak
- 10% persulfate in 1.2 M H2SO4
- Oven (able to maintain temperature at 90 °C overnight)

Colorimetry

- Cuvettes (or 96 well microplates)
- Spectrophotometer capable of reading at 882 nm (or microplate spectrophotometer)
  - Note: this SOP's colorimetry procedures are based on cuvettes, but can be scaled down for microplate while keeping same ratio of reagents and extracts
- Pipette and tips (100-1000 uL)

Detailed Procedure:

I. **Sample Preparation**

1. Measure 1.00 ± 0.02 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 one gram (1.XX g)
2. Prepare empty 50 mL centrifuge tube for blanks (no soil, but treated the same way as samples to account for background P throughout the fractionation)

II. **Reagent preparation**

1. Extractants and digestant
    i. AEM strip
        a. Shake AEM strip in 0.5 M $NaHCO_3$ for > 3 hours, and then decant the $NaHCO_3$ (careful not to drop any strip) and then shake the AEM strip in water (18.2 MΩ). Repeat this procedure three times and this will ensure that AEM is pre-loaded with bicarbonate.
        b. AEM strip can be reused until they deform, but make sure to pre-load them before every use.
    ii. 0.5 M $NaHCO_3$ at pH 8.5
        a. Adding 2 chips of NaOH per 1 L of 0.5 M $NaHCO_3$ should get pH closer to 8.5. Adjust pH further with NaOH (e.g., 10% NaOH)
    iii. 0.1 M NaOH
    iv. 1 M HCl (Molarity Calculator by Sigma-Aldrich can be helpful)
    v. 1.2 M and 0.2 M H_2SO_4 (Molarity Calculator by Sigma-Aldrich can be helpful)
    vi. 10% persulfate (sodium persulfate) in 1.2 M H_2SO_4
2. Standards
    i. Calibration standards (ranging from 0 – 20 mg P/L) need to be made in each extracting solution. Dilute commercial standard (1000 mg P/L) in each 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.
        a. It is essential to use the same extracting solution 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, with stirring and while cooling in an ice bath (may be unnecessary but the beaker does heat up when you add sulfuric acid), 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 4 weeks 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)

iv. 5% NaOH (for adjusting pH for colorimetry)

III. **Sequential Extraction**

1. Extract the pre-weighed soil in the 50 mL centrifuge sequentially in the order of AEM strip (in 18.2 MΩ water), 0.5 M $NaHCO_3$ (pH 8.5), 0.1 M NaOH, and 1 M HCl.
   Note: Do not recommend doing 1M HCl extraction for very calcareous soil as vigorous effervescence occurs when acid contacts carbonate in soil.
   i. Extraction volumes are 20 mL for water/AEM and 40 mL for subsequent extractions
   ii. All extractions are carried out on horizontal shaker (120 rpm; "low" for Eberbach E6010.00) for 16 hrs. Wrapping parafilm around cap can help preventing spills while shaking
   iii. After each extraction (except AEM/water, which requires fishing out of AEM strip before centrifugation, described more in detail below), centrifuge each tube at 4000 rpm for 10-45 min until clear supernatant. For example, 20 min for AEM + water, 10-15 min for rest of the extractions.
   iv. After centrifugation, pipette out 10-15 mL into 15 mL centrifuge tube and decant the rest of the extract while avoiding loss of soil samples

IV. **Desorption, Digestion, and P colorimetry**

1. Extraction #1: Water/AEM
   i. AEM-Pi
      a. Desorption: unlike other extractions, Pi in this extraction is quantified after desorption from the anion-exchange membrane (AEM) strip. Residual extract (water) after removal of AEM strip is used for quantifying H_2O-Pt, as described in ii) below.
         1) (Before centrifuging extraction tubes) With tweezers, fish out AEM strip, and attempt to remove as much sediment by shaking strip in the extraction tube
         2) Using a squeeze bottle with double distilled or nano-pure water, use a thin but powerful stream of water to wash sediment off AEM strip (do not wash into extraction tube, as this will dilute the H_2O-Po)
         3) Immerse washed AEM strip in 12 mL of 0.2 M H_2SO_4 (20-25 mL for soils with high P availability) and shake 3 h
            Note: Concentration of H_2SO_4 used for elution is different from 0.25 M used in Microbial biomass phosphorus (hexanol) protocol. Concentration may be changed as long as full elution can be achieved
      b. Colorimetry: 100 µL 5% NaOH + 600 µL eluent + 700 µL MR
         Notes: This colorimetry reaction is the slowest of them, due to low pH of reaction. The addition of NaOH helps. Reaction

is complete when standards have stopped increasing. 45-60 min.

ii. H_2O-Pt
   a. Digestion: 3 mL of H_2O extract + 2 mL 10% persulfate (in 1.2 M H_2SO_4), digest at 90 °C for 16 h, or autoclave for 30 min (as true for all other digestion, make sure standards for Pt fractions are also digested the same way as samples)
   b. Colorimetry: 250 µL 5% NaOH + 300 µL digestate µL + 700 µL MR. Notes: add in this order, as it ensures mixing of base and acidic digestate.

2. Extraction #2: $NaHCO_3$
   i. $NaHCO_3$-Pi
      a. Colorimetry: 300 µL $NaHCO_3$ extract + 700 µL MR.
      Notes: Add MR to extract slowly, as effervescence results from acid being added to base. If there is substantial organic P in extracts, mineralization of organic P into Pi will result in slow upward creep of absorbance
      In rare cases may need + 1000 µL ddH_2O to bring absorbance into linear range.
   ii. $NaHCO_3$-Pt
      a. Digestion: 3 mL of digestate + 3 mL 10% persulfate (in 1.2 M H_2SO_4), digest at 90 °C for 16 h, or autoclave for 30 min
      Notes: Adding acid to $NaHCO_3$ can cause strong effervescence, thus add slowly to avoid spills. Wait approximately 10 min before capping the tube to allow effervescence to go away
      b. Colorimetry: 250 µL 5% NaOH + 300 µL digestate µL + 700 µL MR
      Notes: add in this order, as it ensures mixing of base and acidic digestate

3. Extraction #3: NaOH
   i. NaOH-Pi
      a. Precipitation of SOM
         1) 1000 µL NaOH extract + 120 µL 1.2 M H_2SO_4 in a microcentrifuge tube
         2) Spin down at 15,000 rpm for 3 min (should see dark pellet of precipitated SOM)
         3) Use clear aliquot for colorimetry
         Notes: remember to perform this step for P standards (made up in NaOH extraction solution)
      b. Colorimetry: 104 µL 5% NaOH + 125 µL acidified NaOH eluent µL + 500 µL MR
      Notes: + 1000-1500 µL ddH_2O often needed to bring absorbance into linear range
   ii. NaOH-Pt

a. Digestion: 3 mL of NaOH extract + 3 mL 15% persulfate (in 1.2 M H_2SO_4), digest at 90 °C for 16 h, or autoclave for 30 min
   Notes: for high SOM soils, may need to increase volume of acidic persulfate solution
b. Colorimetry: 208 µL 5% NaOH + 250 µL digestate + 700 µL MR
   Notes: + 1000-1500 µL ddH2O often needed to bring absorbance into linear range

4. Extraction #4: HCl
   i. HCl-Pi
      a. Colorimetry: 250 µL 5% NaOH + 300 µL HCl extract + 700 µL MR
         Notes: Add MR to extract slowly, as effervescence results from acid being added to base. If there is substantial organic P in extracts, mineralization of organic P into Pi will result in slow upward creep of absorbance
   ii. HCl-Pt*
      a. Digestion: 3 mL of HCl extract + 2 mL 10% persulfate (in 1.2 M H_2SO_4), digest at 90 °C for 16 h, or autoclave for 30 min
      b. Colorimetry: 450 µL 5% NaOH + 300 µL digestate µL + 700 µL MR
         Notes: add in this order, as it ensures mixing of base and acidic digestate

   **Not included in standard Hedley, but evidence suggests it may be a small portion of soil organic P*
5. Colorimetry is performed directly in the cuvette (standard disposable [polyacrylamide or other cheap polymer]). Cuvettes marked as 1.5 mL cuvettes can hold up to 2.5-3 mL.
6. Absorbance is measured at A882. Use 0 ppm standard as blank reading (abs = 0.000).
7. For a given extraction, I prefer to start the Pi colorimetry, and then set up the digestion for Pt of that extract.
8. Digestions are performed to convert Po to Pi, and Pt is quantified in the digestate as Pi. Then Po is calculated as difference between Pt and Pi
9. Golden Rule: Treat standards the same as samples. This goes for the working standards being made up in the same solution as extracts. For example, working standards for $NaHCO_3$ extraction should be made in 0.5 M $NaHCO_3$ (pH 8.5) and used as such for $NaHCO_3$-Pi. For P standards for $NaHCO_3$-Pt, digestate the working standards made up in 0.5 M $NaHCO_3$ (pH 8.5) in the same manner as samples. If colorimetry reactions are diluted to bring absorbance into linear range, do the same for standards. Etc.

V. **Clean up**

1. Make sure to clean up dispensette (rinse with dilute sulfuric or hydrochloric acid, followed by water, especially after pumping NaHCO3 after use), shaker (especially if tubes leak), and centrifuge (especially if tubes leak).
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. 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.

VI. **Calculations**

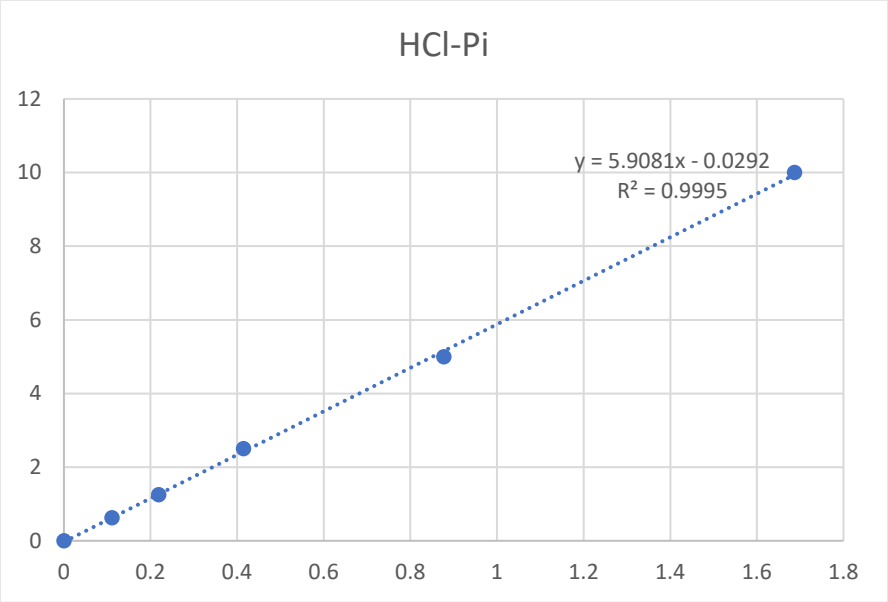
Measurement of P fractions is usually expressed in units of mg P kg^{-1} soil. Pi and Pt are converted to the final unit separately and then Po is calculated by difference at the end.

1. Convert raw absorbance to concentration (mg P L^{-1}) using calibration curve constructed specifically for each fraction (as noted previously, calibration standards should be treated exactly the same way as samples, so the 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.
2. Multiply the concentration by the extract volume (e.g., 0.02 L for H2O-Pt and 0.04 L for HCl-Pi) and divide by soil mass (1 g = 0.001 kg) to yield concentration in mg P kg^{-1} soil

Example calculation:

Given, for HCl-Pi
Absorbance = 0.760
Dilution = 1
Calibration curve: y = 5.9081x - 0.0292
Extraction volume = 0.04 L
Soil mass = 0.001 kg

Concentration in extract = (5.9081*0.760 – 0.0292) * 1 = 4.46 mg L^{-1}
Concentration in soil basis = 4.46 mg L^{-1} * 0.04 L / 0.001 kg = 178 mg P kg^{-1} soil



References:

Cross, A.F., Schlesinger, W.H., 1995. A literature review and evaluation of the. Hedley fractionation: Applications to the biogeochemical cycle of soil phosphorus in natural ecosystems. Geoderma 64, 197-214.

Gu, C., Dam, T., Hart, S.C., Turner, B.L., Chadwick, O.A., Berhe, A.A., Hu, Y., Zhu, M., 2020. Quantifying Uncertainties in Sequential Chemical Extraction of Soil Phosphorus Using XANES Spectroscopy. Environmental Science & Technology 54, 2257-2267.

Hedley, M.J., Stewart, J.W.B., Chauhan, B.S., 1982. Changes in Inorganic and Organic Soil Phosphorus Fractions Induced by Cultivation Practices and by Laboratory Incubations. Soil Science Society of America Journal 46, 970-976.

Klotzbücher, A., Kaiser, K., Klotzbücher, T., Wolff, M., Mikutta, R., 2019. Testing mechanisms underlying the Hedley sequential phosphorus extraction of soils. Journal of Plant Nutrition and Soil Science 182, 570-577.

Tiessen, H., Moir, J.O., 2007. Characterization of Available P by Sequential Extraction, In: Carter, M.R., Gregorich, E.G. (Eds.), Soil Sampling and Methods of Analysis, 2nd ed., Boca Raton: CRC Press, pp. 293–306.

Suggested reading:

HEDLEY, M.J., WHITE, R.E. and NYE, P.H. (1982), PLANT-INDUCED CHANGES IN THE RHIZOSPHERE OF RAPE (BRASSICA NAPUS VAR. EMERALD) SEEDLINGS. New Phytologist, 91: 45-56. https://doi.org/10.1111/j.1469-8137.1982.tb03291.x

Cross, A.F., Schlesinger, W.H., 1995. A literature review and evaluation of the. Hedley fractionation: Applications to the biogeochemical cycle of soil phosphorus in natural ecosystems. Geoderma 64, 197-214. https://doi.org/10.1016/0016-7061(94)00023-4

Citation:

SOP: Hedley fractionation. 2021. 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