Enzyme method for Orthophosphate: Phosphate & Total P

NEMC 2021 Virtual Presentation

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NECi Superior Enzymes™

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Easy Green Method for Phosphate & Total P

NECi develops and produces recombinant enzymes for analytical chemistry.

Enzymes catalyze difficult chemical reactions under gentle conditions in complex matrices.

Common lab methods can be done with nonhazardous reagents and low cost instrumentation when the methods are enzyme-based.

Standard lab instrumentation, increased safety, reduced waste.

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Why Enzymes?
They're great for analytical chemistry

- **Selectivity**
  "Find" target in complex mixtures
- **Sensitivity**
  Low detection limits in complex mixtures
- **Specificity**
  False negatives ***and*** false positives are rare
- **Safety**
  For shipping, storage, handling, and disposal.

Reagent Grade Enzymes are accurate, reliable, and environmentally benign.

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PNP: Purine Nucleoside Phosphorylase

- NECi's interest in a new method for phosphate based on our work for the USDA on nutrient testing for soil and water.
- Common methods require concentrated acids and heavy metals. Sample prep an additional complicating factor.
- Found references in the academic literature on PNP:
  - Webb, MR *PNAS* 1992, *89,* 4884-4887
  - Wedler et al, *Anal. Biochem,* 1994, *218*, 449-453
  - Cheng et al, *Bioorg Chem,* 1999, *27*, 307-325
  - Medical R&D vendor Molecular Probes test kit

So we developed a recombinant PNP and Validated the method in an array of ag materials, including runoff.

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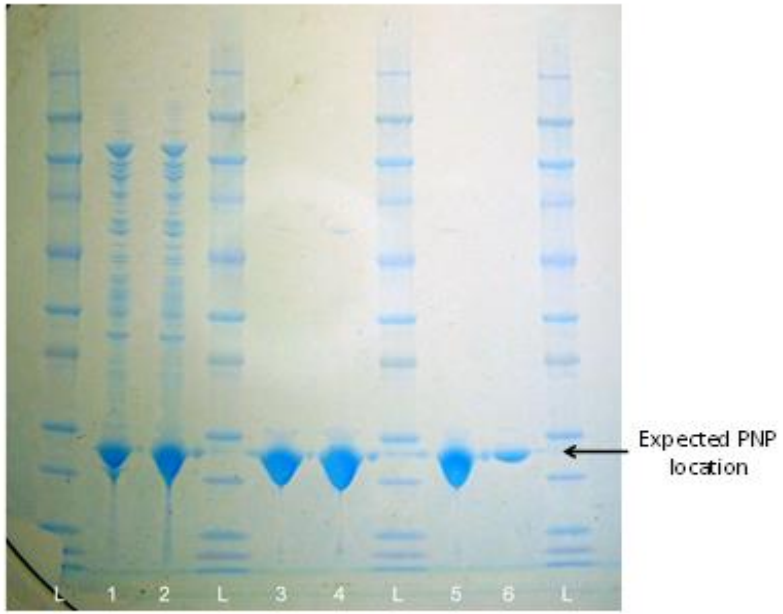
Enzyme Purity

- This is a standard purity test for proteins: SDS-PAGE
- Proteins are “pushed” through a hydrogel matrix by electrical charge.
- The SDS detergent renders the proteins neutral. Separation is by protein size.
- Lanes L are protein standards.
- Lanes 1 & 2 are PNP as extracted.
- Lanes 3,4,5 are the fully purified PNP.



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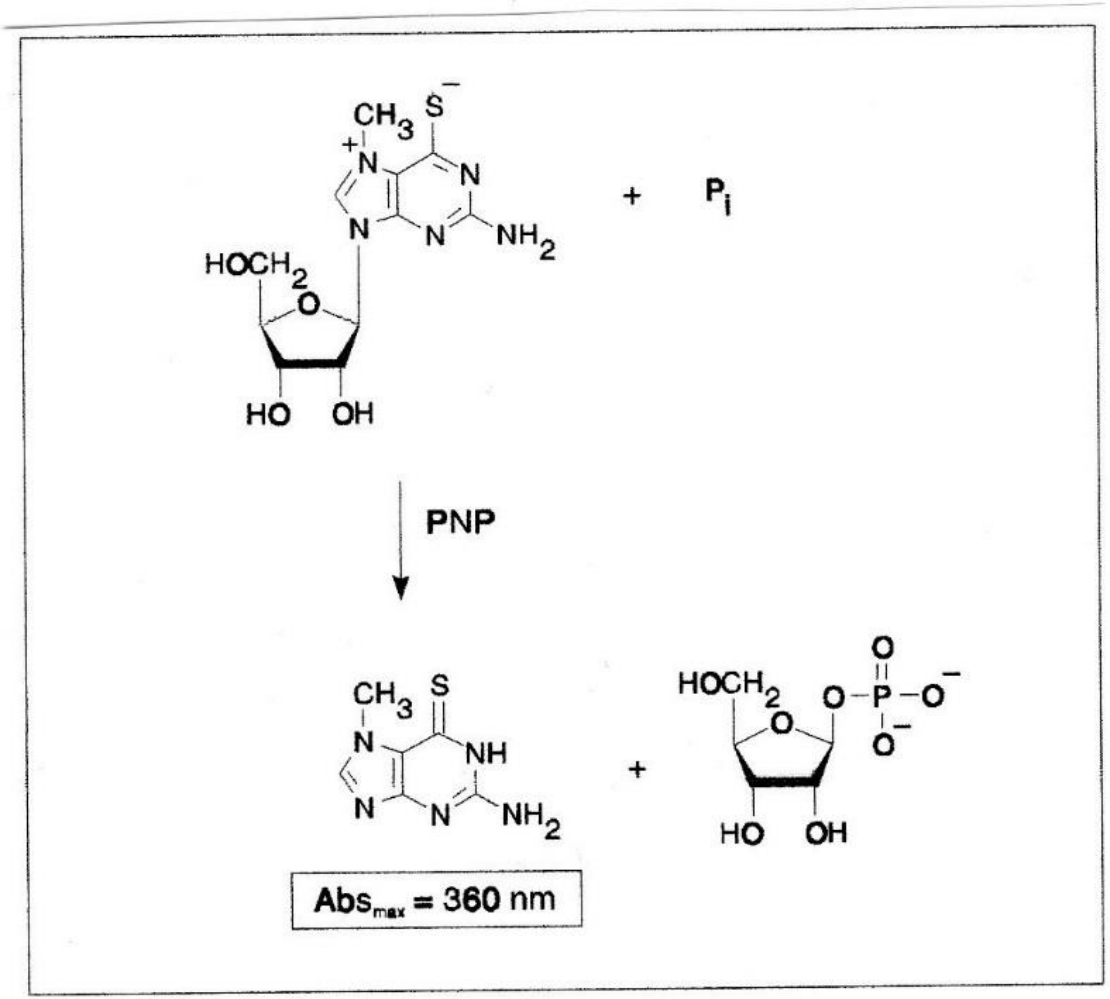
Clinical Research application: phosphate release kinetics



Webb (PNAS 1992) developed this method to measure phosphate release kinetics. Think ATP to ADP, for example.

Simplified, this is a clean method for assaying orthophosphate.

PNP catalyzes the reaction of orthophosphate with MESG.

The reaction product, AMMP, absorbs at 360nm.

PNP: Purine nucleoside phosphorylase
MESG: 7-methyl-6-thioguanosine
AMMP: 2-amino-6-mercapto-7-methylpurine

Graphic from Molecular Probes EnzChek E-6646

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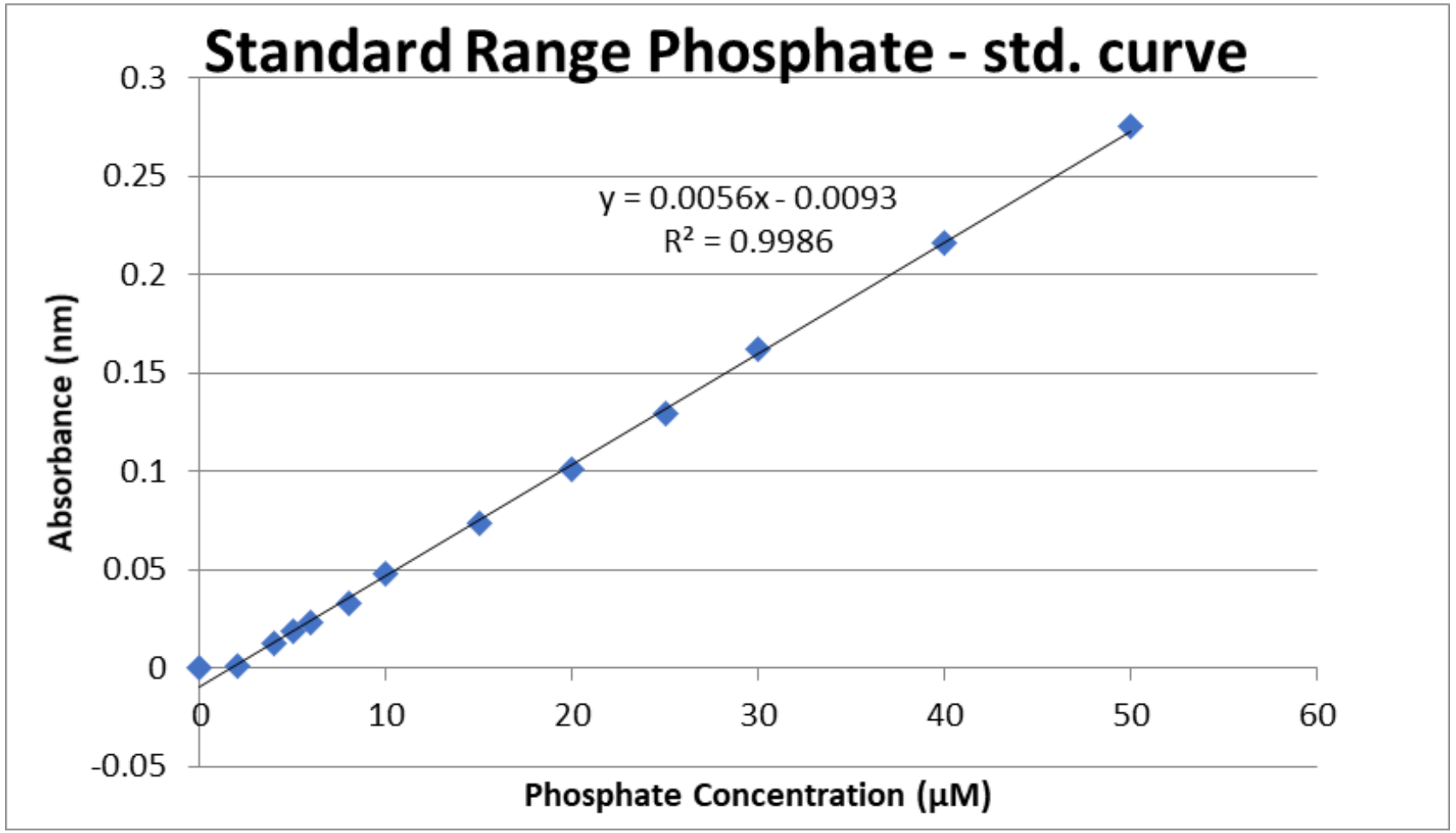
Method Validation: Standard Curve



10 µM Pi = 0.31 ppm P

NECi has Lab or OnSite kits
For Low Range: 0.5 – 5 ppm
Std Range: 5 – 20 ppm

Low Range for most Water Applications.

Std Range for Wastewater or Soil Fertility.

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Method Validation: Spiking Studies

Interference	Concentration in sample	Measured Unspiked (2ppm Pi)	Measured Spiked (5ppm Pi)	Measured Change	Recovery %
Fe 2+	1 ppm	1.73	6.07	4.34	86.8%
Fe 3+	1 ppm	1.89	6.45	4.56	91.2%
Ca 2+	1 ppm	1.92	6.68	4.76	95.2%
Pb 2+	1 ppm	1.73	6.07	4.34	86.8%
Mn 2+	1 ppm	1.59	6.38	4.79	95.8%
Mg 2+	1 ppm	2.21	6.9	4.69	93.8%
Zn 2+	1 ppm	1.93	7.37	5.44	108.8%
Al 3+	1 ppm	1.57	7.07	5.5	110.0%
SO4 2-	500 ppm	2.17	7.34	5.17	103.4%
SO2 2-	500 ppm	5.55	8.34	2.79	55.8%
NaCl	100 mM	2.12	6.74	4.62	92.4%
Arsenic	5 ppm	1.92	7.46	5.54	110.8%

Recoveries in the presence of Ions common in water and soil. Sulfite seems to be a problem.

Excellent recovery for other ions.

The method also works in high NaCl media for marine algae.

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Sample Types/Matrices

- NECi has standards, standard samples, and real world sample types from nitrate validation studies stored for use in new method development.
- Agriculture: soil and plant samples
- Environmental: Drinking water, waste water, ambient water, natural water, industrial effluents, process water
- Phosphate validation studies were performed using many of these samples.
- Now we are testing PNP with the new Alkaline Persulfate digestion matrix.

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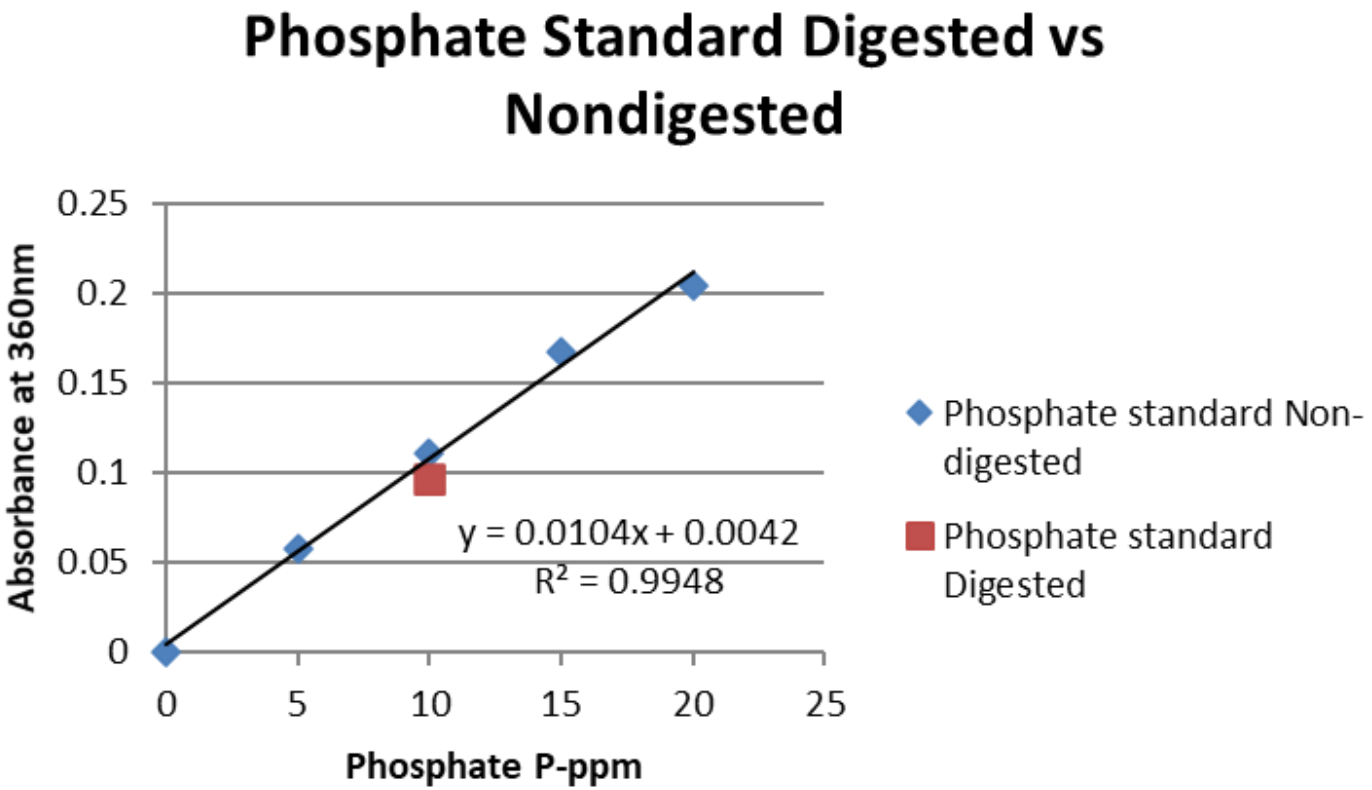
Total P by Alkaline Persulfate Digestion



Method based on US Dept of Interior/USGS Water-Resources Investigations 03-4174 Patton and Kryskalla

Here's the basic yes/no:
We can do that!

Red data point is the average of ten 10ppm standards assayed after digestion.

The digestion reagents are compatible with the PNP method for phosphate detection.

We'll proceed with various real world samples Fall 2021.

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Standard Method Status: PNP

- We have not pursued Standard Method status with any agency or association at this time (Summer 2021).
  - Is this something we should consider?
- We have developed the assay in formats for Lab use
  - Test tube and microplate
  - Happy work with other instrumentation, esp Discrete Analyzers and Flow Analyzers. Detection at 360nm is required.
- OnSite test kits for water, soil, plant extracts
  - Results are not in the visible range (A360). Photometer required.
  - NECi's pocket photometer designed for this and Nitrate.

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Thanks!

- **NECi thanks all the individuals, labs, companies, funding sources, and regulatory agencies who've helped us bring enzyme-based analytical chemistry to the environmental world.**
  - **SBIR Programs of the USDA, Natl Science Foundation, and NIH**
  - **US EPA and USGS**
  - **Dr William Lipps, Shimadzu, ASTM, and StdMethods.org**
  - **Economic development organizations: MEDC and KEDA**
  - **Our customers**

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