Enzyme-Based Analytical Chemistry

Nitrate and the U.S. EPA

ELLEN CAMPBELL, CEO & VP

NECI SUPERIOR ENZYMES


Michigan's
Upper
Peninsula
Lake Linden, MI
NECi Superior Enzymes

BIOTECHNOLOGY *CHEMISTS* CAN USE

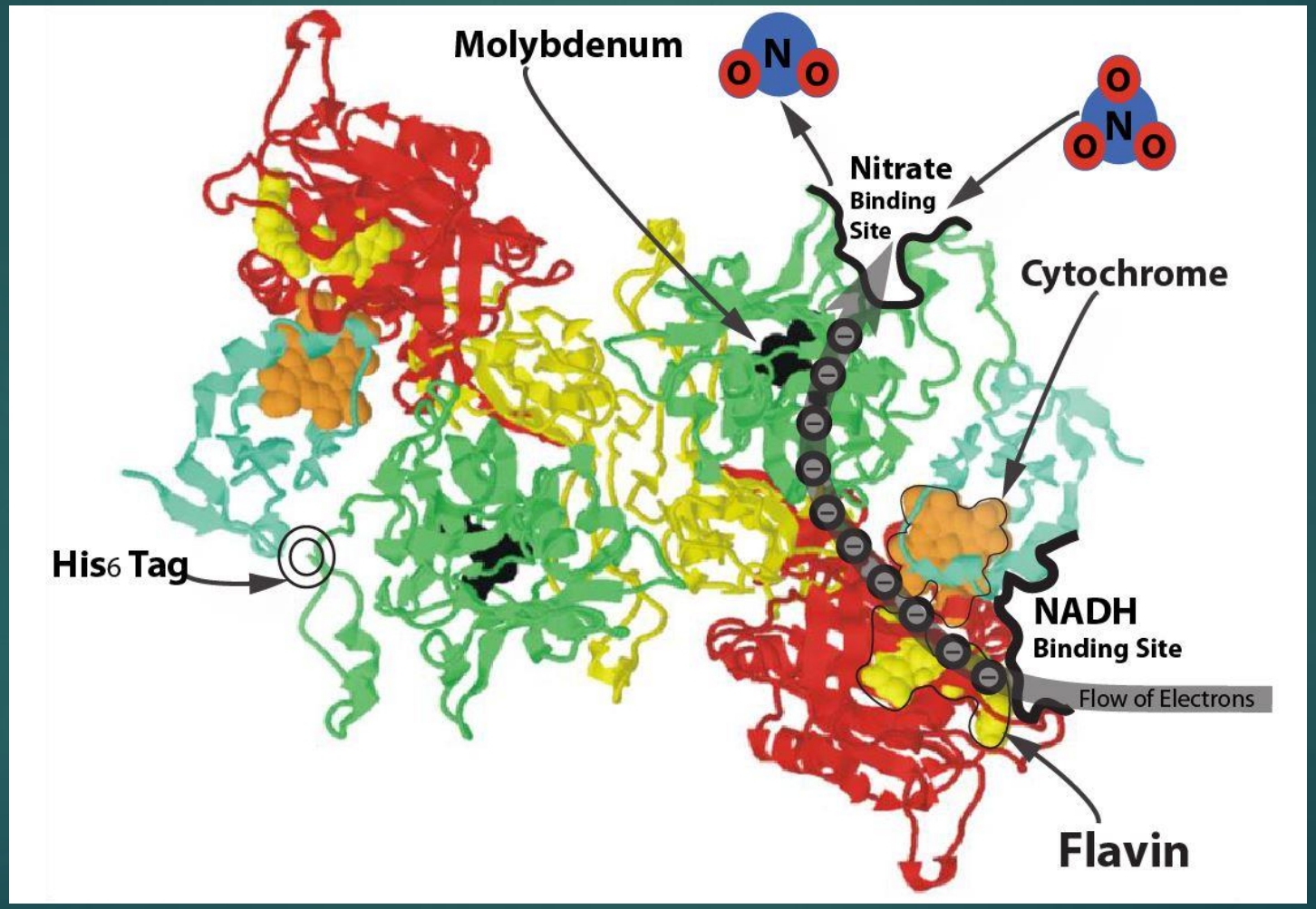
- Enzymes are catalysts made of protein
- Chemical reactions in biological systems are made possible by enzymes
- Enzymes have been used for medical analysis for decades
- State of the art in biotechnology enables reliable enzyme production
- Enzymes for **Green Analytical Chemistry**

ANALYTICAL ADVANTAGES OF ENZYMES

- **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

NITRATE REDUCTASE



TIGHT QC ENSURES ENZYME QUALITY

- Highest purity media components
- Optimized SOPs
- Computer controlled fermentation

RECOMBINANT ENZYME PRODUCTION WORKFLOW

Recombinant NaR is produced in the Pichia pastoris protein expression system by fermentation.

Cells are mechanically lysed to release contents. Cell debris is removed by centrifugation.

One step affinity chromatography purification of the clarified extract.

Enzyme is stored at -80C until ready for processing into products.

EARLY NITRATE REDUCTASE PUBLICATIONS

CAMPBELL, WA H. PhD NECi President, MTU Professor Emeritus

- **Feb 1986:** Plant Physiology: *Regulation of Corn Leaf Nitrate Reductase*
- **Aug 1990:** Trends in Biochemical Sciences: *Functional domains of assimilatory nitrate reductases and nitrite reductases*
- **Feb 1996:** Plant Physiology: *Nitrate Reductase Biochemistry Comes of Age*
- **Mar 1998:** Analytical Chemistry: *Construction and Characterization of Nitrate Reductase-Based Amperometric Electrode and Nitrate Assay of Fertilizers and Drinking Water*
- **Jun 1999:** Annual Review of Plant Physiology and Plant Molecular Biology: *Nitrate Reductase Structure, Function and Regulation: Bridging the Gap between Biochemistry and Physiology*
- **Jun 2000:** Plant Physiology: *Recombinant Expression of Molybdenum Reductase Fragments of Plant Nitrate Reductase at High Levels in Pichia pastoris*
- **Jan 2002:** Environmental Science & Technology: *Corn Leaf Nitrate Reductase – A Nontoxic Alternative to Cadmium for Photometric Nitrate Determinations in Water Samples by Air-Segmented Continuous-Flow Analysis*

**The above publications are only a selected few of the comprehensive list published by Dr. Bill Campbell on Nitrate Reductase*

NITRATE REDUCTASE REPLACES CADMIUM

$NO_3 + NADH + H^+ \xrightarrow[\text{Nitrate Reductase}]{} NO_2 + NAD^+ + H_2O$

NO_2 + Sulfanilic Acid $\longrightarrow$ Diazonium Salt

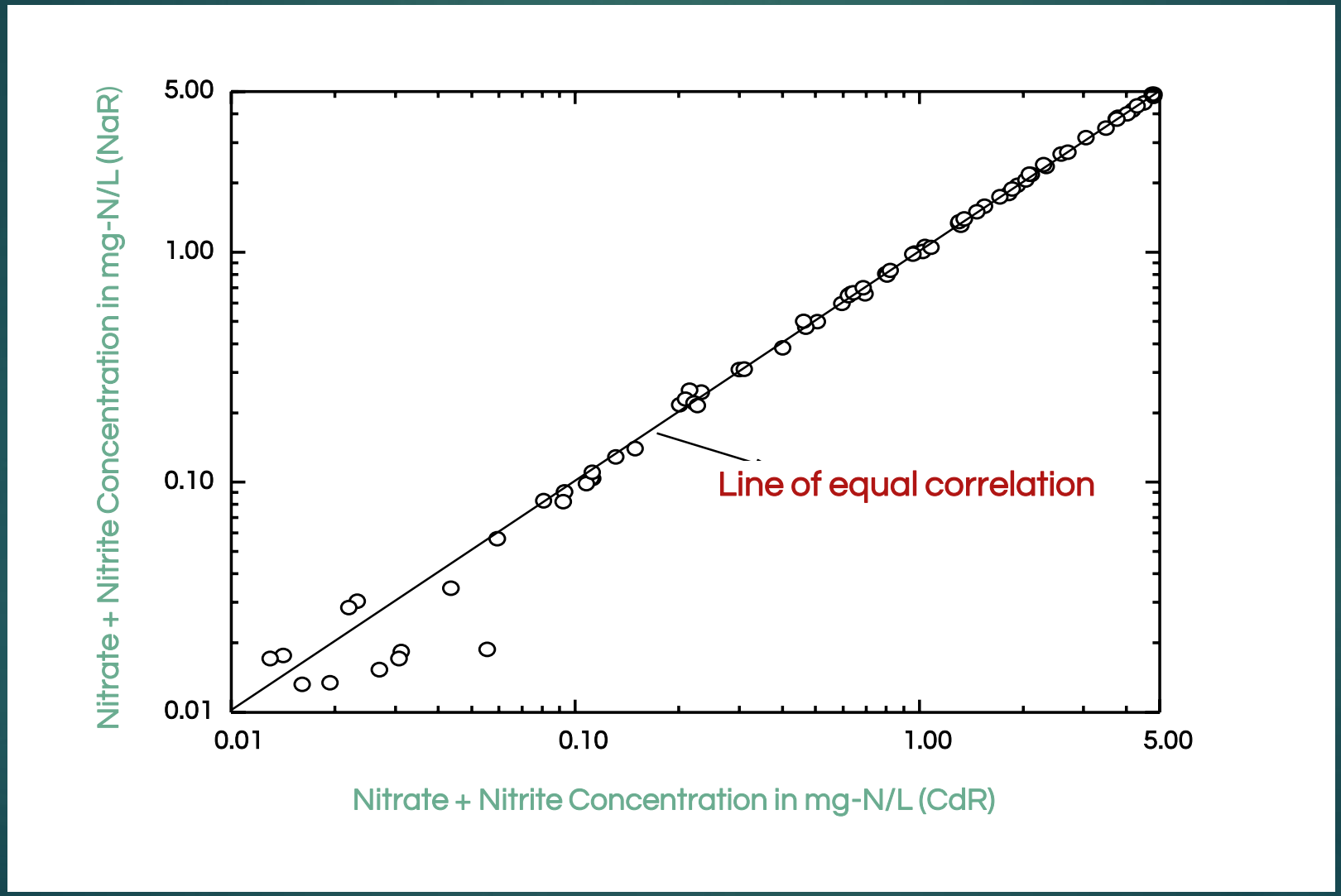
Diazonium Salt + NED $\longrightarrow$ Pink dye for measuring A 540 ± 20nm

Samples are measured against a standard nitrate curve to determine results in ppm Nitrate-N

NITRATE ANALYSIS USING NITRATE REDUCTASE

- Nitrate Reductase (NaR) catalyzes the reduction of nitrate to nitrite
- NADH is the electron donor for driving the reaction
- Resulting Nitrite reacts with Griess color reagents to produce an AZO dye
- Samples are analyzed photometrically
- NaR simply replaces Cadmium in conventional methods. The rest of the chemistry is the same.

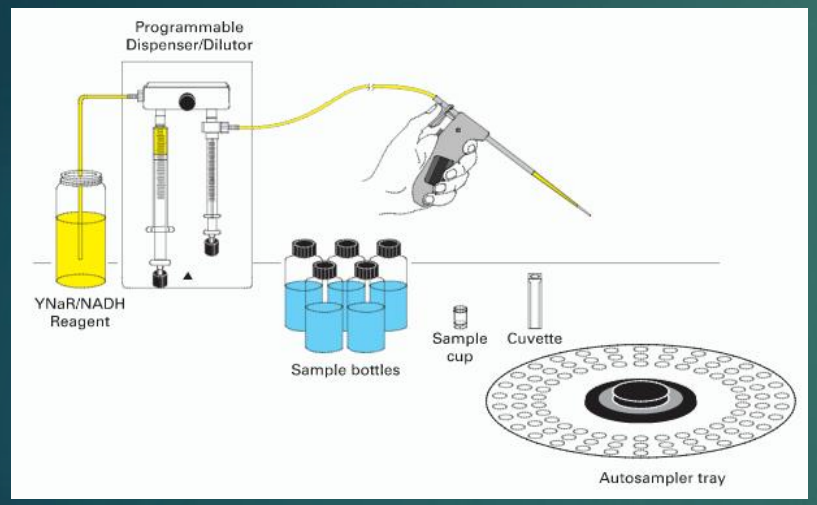
CADMIUM VS. NITRATE REDUCTASE

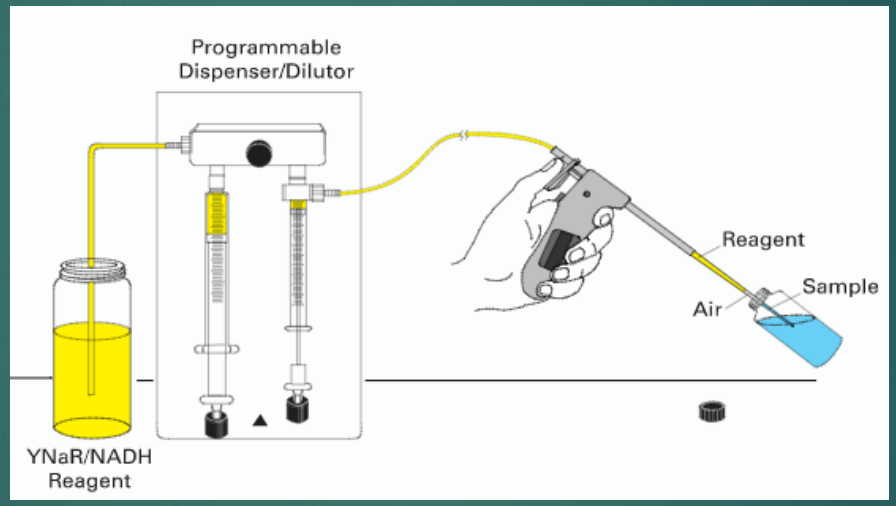


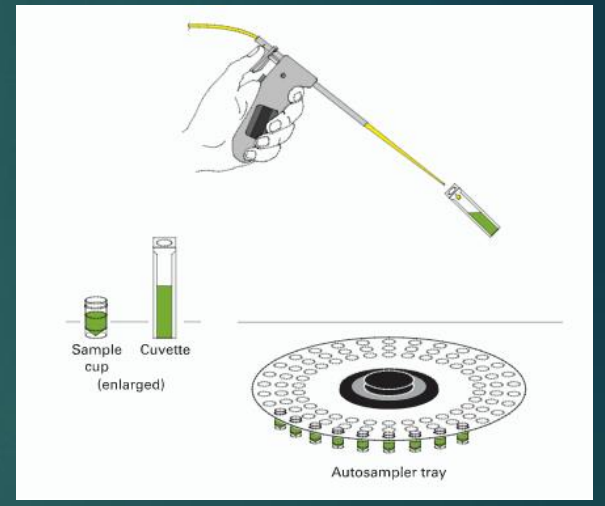
NITRATE REDUCTASE ASSAY FOR NITRATE ANALYSIS

- Small sample size in relation to total assay volume
- Little sample preparation required
- High specificity reduces false positives and false negatives
- Highly purified and freeze-dried protein glass
- $0.21-$0.25 per sample for any Discrete Analyzer
- Competitive cost per sample for most FIA & SFA Systems

Semi-Automated Batch Reduction System for Enzymatic Nitrate Determinations







RECOMBINANT ENZYME PRODUCTION

- **Guaranteed lot-to-lot reproducibility**
- **Increased production capacity**
- **Reasonable production costs**
- **Engineered for Improved Stability**
  - Stable during analytical runs
  - Shipping at Ambient
  - Storage at controlled RT

GUARANTEED ENZYME PURITY

One unit will reduce 1.0 µmol nitrate to nitrite per minute in NADH system at pH 7.5 and 30°C

Stable indefinitely at 20°C; Min 2 years at 4°C (freeze dried)

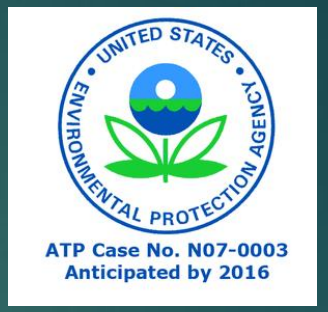
Expected Purity: 20 40 units/mg protein

All additives are reagent grade, no animal derived or hazardous materials

NITRATE REDUCTASE METHOD VALIDATIONS

- Validated in 2011 for DA
- Collaborative study with NWQL
- Statistically equivalent to Cd reduction method

- Validated in 2014
- Replaces nitrate methods D1254 and D992
- Can be used in place of D3867 (Cd Reduction)



- Validation study submitted 2013
- ATP Letter received April 2014
- No negative comments to address
- Methods Update Rule for CWA Reporting in 40 CFR Part 136.6

SAMPLES TESTED FOR VALIDATION STUDIES

Sample Type	Filtered	Acid
Denver area treatment plant Influent wastewater	Yes	Yes
Denver area treatment plant Wastewater effluent #1	Yes	Yes
Denver area treatment plant Wastewater effluent #2	Yes	Yes
Michigan paper mill waste stream effluent	Yes	Yes
Denver area metal finisher waste stream effluent	Yes	Yes
Denver area Commercial laundry waste stream effluent	Yes	Yes
Environmental Resources Associates #507 Hardness WasteWatR reference material	Yes	Yes
Michigan Confined Animal Feeding Operation (CAFO) effluent from tiled field	Yes	Yes
Low-nutrient seawater (collected offshore Hawaii)	Yes	No
ERA # 608 Reference Standard	Yes	Yes
USGS PE N-116 (low nutrient-fortified river water)	Yes	No
USGS PE N-115 (high nutrient-fortified river water)	Yes	No
Tap water at each lab		
Tap water plus added Chlorine		

ENZYMATIC REDUCTION EFFICIENCY SUMMARY

- Acceptance Standard is 90% or greater reduction efficiency
- 2nd Source = Nitrate Standard in mg N/L
- Nitrate Standard = mg N/L

			A-540 nm	mg N/L	Reduction Efficiency
Lab 1	2nd Source	2.50	0.35217	2.51	102.1%
	Nitrite Std	2.50	0.34507	2.46	
Lab 2	2nd Source	2.50	0.36664	2.48	101.3%
	Nitrite Std	2.50	0.36190	2.45	
Lab 3	2nd Source	2.50	0.2456	2.548	107.2%
	Nitrite Std	2.50	0.2292	2.376	
Lab 4	2nd Source	2.50	0.295	2.59	116.6%
	Nitrite Std	2.50	0.252	2.22	
Lab 5	2nd Source	2.50	0.33422	2.3576	94.9%
	Nitrite Std	2.50	0.3514	2.4833	
Lab 6	2nd Source	2.50	0.37078	2.557	103.8%
	Nitrite Std	2.50	0.35867	2.464	
Lab 7	2nd Source	2.50		2.506	103.7%
	Nitrite Std	2.50		2.417	
Lab 8	2nd Source	2.50	0.358	2.4872	102.3%
	Nitrite Std	2.50	0.350	2.4310	
Lab 9* [550nm]	2nd Source	3.04	0.2127	3.00	98.6%
	Nitrite Std	3.04	0.2151	3.04	
Lab 10	2nd Source	2.50	0.146	2.598	105.0%
	Nitrite Std	2.50	0.140	2.475	

TABLE 5 FROM EPA VALIDATION REPORT: INITIAL PERFORMANCE AND RECOVERY (IPR) SUMMARY

Lab	# Analyses	Mean Recovery (%)	RSD (%)	Minimum Recovery (%)	Max Recovery (%)
1	4	100.67	0.71	99.67	101.30
2	4	101.82	0.54	101.29	102.40
3	4	96.16	2.23	93.50	98.70
4	4	106.38	0.73	105.82	107.52
5	4	101.79	0.78	100.63	102.37
6	4	102.76	0.92	101.77	103.93
7	4	100.50	1.13	98.97	101.66
8	4	98.45	0.66	97.77	99.21
9	4	101.11	0.95	100.17	102.43
10	4	99.34	2.45	95.85	101.50

TABLE 7: METHOD DETECTION LIMIT (MDL) SUMMARY

- DA = Discrete Analyzer
- NA = Not Analyzed
- One replicate discarded due to issue with blank

Lab	Method	MDL	Replicates	Spike	Spike/MDL
		mg N/L		mg N/L	Ratio
1	DA	0.0079	8	0.040	5.068
2	DA	0.0148	8 (7)*	0.040	2.701
3	DA	0.0130	7	0.050	3.832
4	DA	0.0055	7	0.025	4.522
5	DA	0.0226	7	0.050	2.215
6	DA	0.0310	7	0.050	1.615
6	DA	0.0260	7	0.075	2.881
8	DA	NA			
9	DA	0.0060	7	0.045	7.541
10	DA	0.0463	7	0.100	2.160

NITRATE + NITRITE BY DA: ENZYME VS. CADMIUM

Sample	NaR	CdR	RPD
	mg N/L	mg N/L	%
WW-1*	0.03	0.03	0.0000
WW-2	7.8	7.6	+2.5974
WW-3	0.23	0.26	-12.7656
WW-4*	0.04	0.03	+28.5714
WW-5	270.8	272.6	-0.6625
WW-6	4.8	4.8	0.0000
WW-7*	0.05	0.06	-18.1818
WW-8	13.77	14.1	-2.3681
SW-1*	0.027	0.030	-10.5263
SR-1	6.80	7.02	-3.1838
SRM-1	0.45	0.48	-6.4516
SRM-2	2.28	2.36	-3.4188

ONGOING AND FUTURE ENZYME-BASED NITRATE METHOD VALIDATIONS

- Validation Study for EPA SDWA
  - Submitted last week
- Currently Seeking Joint Task Group Chair for Standard Methods
- Suggestions?

EPA Drinking Water Study

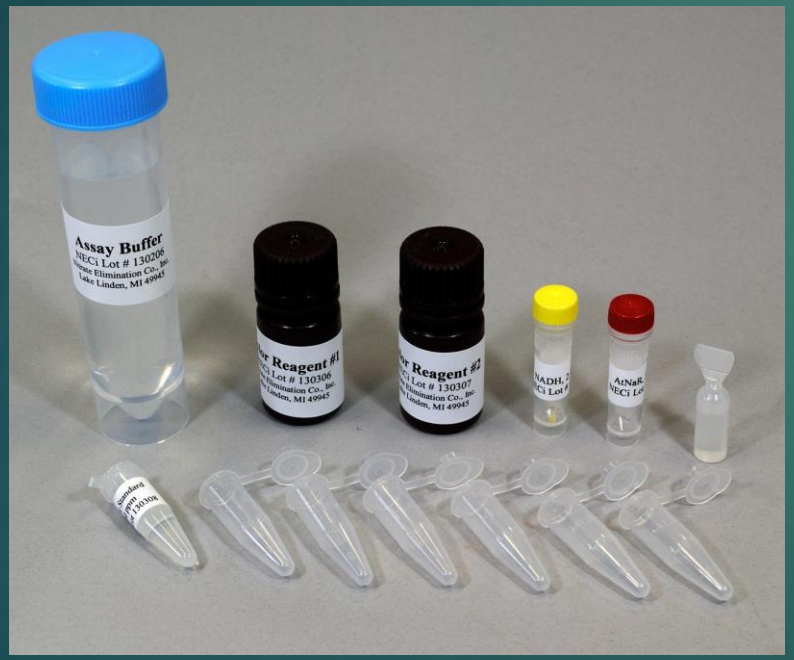
	Lab 1		Lab 2		Lab 3*	
	NaR-R	Cd-R	NaR-R	Cd-R	NaR-R	Cd-R
Catalytic Reduction Efficiency (NO_3/NO_2) Percent	93.2551	93.9279 98.0040	105.9603 104.7569 103.0221 102.5518 100.2798	96.7139 97.0086 98.5727 99.8509 93.3010	94.3464 101.8339	96.7978 104.0904

EPA Drinking Water Study

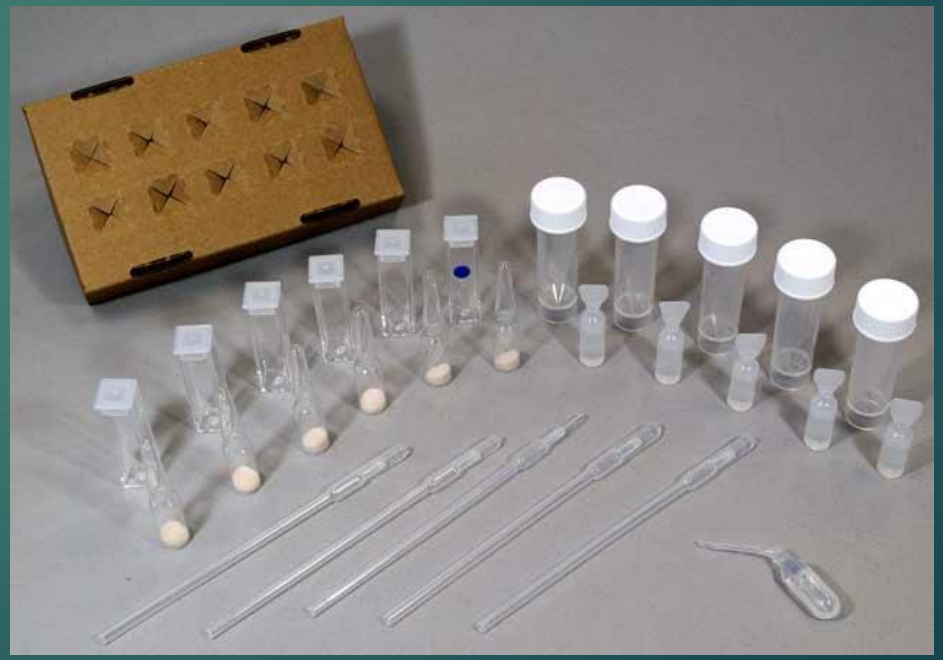
	Standard Deviation mg Nitrate N/L	Final mg Nitrate N/L	Compared to ERA
Lab 1 NaRR	0.03375	5.3989	105.0195%
Lab 1 CdR	0.07998	5.0937	99.0992%
Lab 2 NaRR	0.07552	4.9795	96.8783%
Lab 2 CdR	0.02132	5.0640	98.5214%
Lab 3 NaRR	0.005100	5.1258	99.7218%
Lab 3 CdR	0.01816	5.1800	100.7782%

SAME METHOD
DIFFERENT FORMATS

LABORATORY TEST KITS



SIMPLIFIED TEST KITS



NECI HANDHELD PHOTOMETER

NECI HANDHELD PHOTOMETER

- Other devices use round vials or outdated chemistry methods which are inaccurate or toxic
- NSF Grant in collaboration with Dr. Pearce at MTU resulted in PLOS ONE Publication in August 2015 for open-source technology
- Open source technology makes science more accessible to a wide audience
- Makes simplified test kits more accurate for field measurement

NECI HANDHELD PHOTOMETER

- Same data and technologies in the field as in the laboratory
- Enzyme assay standard curve programmed in
- LEDs for specific wavelengths
- Blank function for calibration
- Uses standard square cuvettes to eliminate optic distortion

NECI HANDHELD PHOTOMETER

GPS and Time Stamp

Data Collection

Export of CSV

Cell Phone Battery

RECENT RESEARCH

2012

ANALYTICAL CHEMISTRY

Enzyme-Catalyzed Oxygen Removal System for Electrochemical Analysis under Ambient Air: Application in an Amperometric Nitrate Biosensor

2015

PLOS ONE

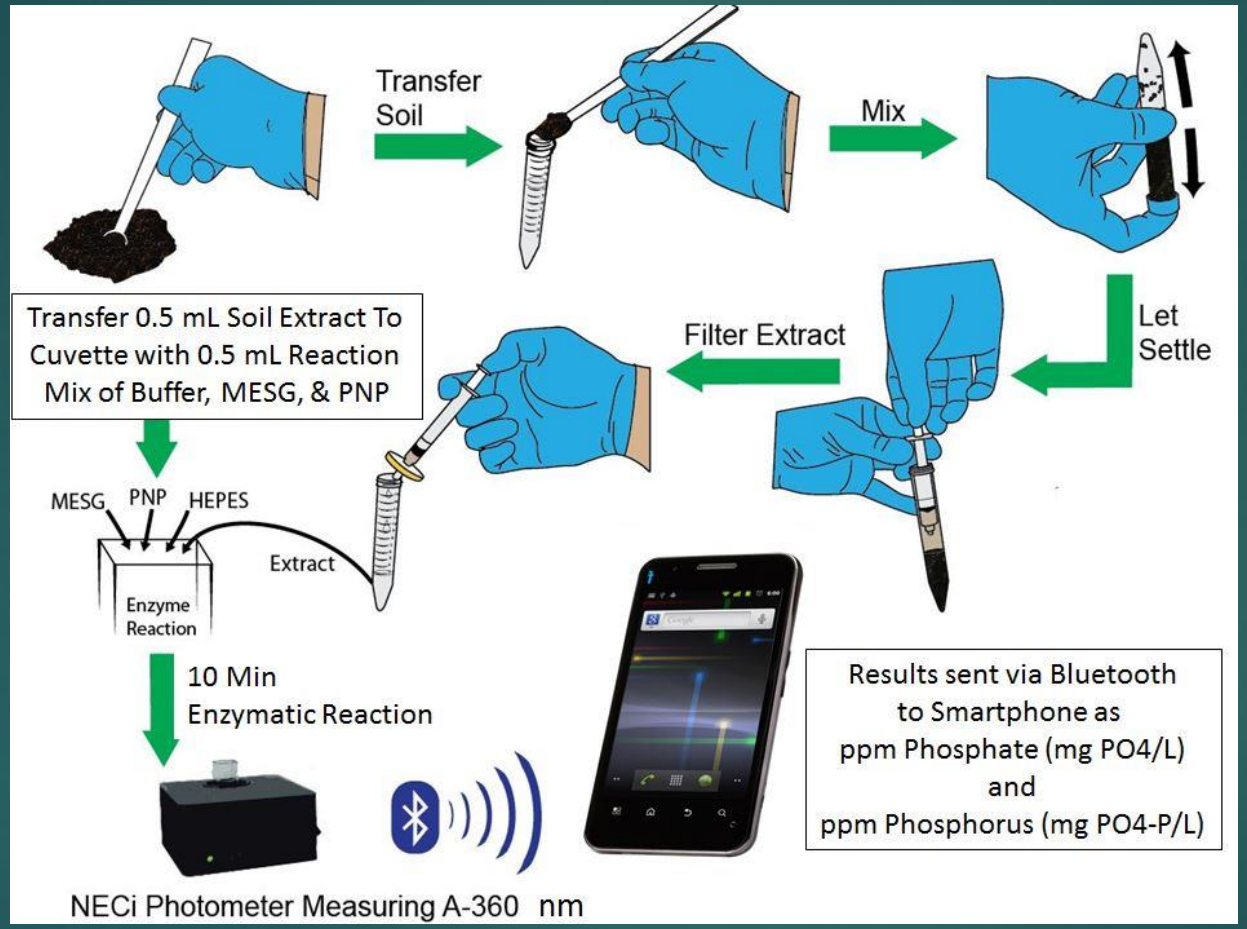
Open-Source Photometric System for Enzymatic Nitrate Quantification

2015

ELSEVIER METHODS X

Determination of Phosphate in Soil Extracts in the Field: A Green Chemistry Enzymatic Method

PNP FOR PHOSPHATE MEASUREMENT



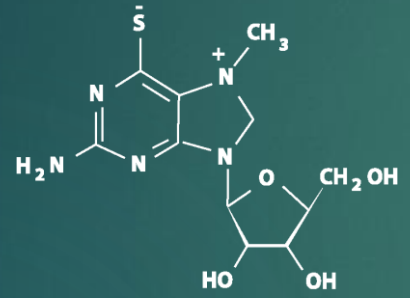
Graphical Abstract: Determination of Phosphate in Soil Extracts in the Field: A Green Chemistry Enzymatic Method

PNP ASSAY FOR MEASURING PHOSPHATE

- Reaction mixture is prepared:
  - **Buffer** (200 mM HEPES, pH 7.6, 20 mM $MgCl_2$) + **MESG** (80 nmol) + **Recombinant PNP Enzyme** (1 unit)
- Sample is added to reaction mixture and mixed
- Reaction goes to completion within 20 minutes
- Absorbance at 360 nm is measured after blanking spectrophotometer with HEPES buffer
- Absorbance of sample is compared with standard curve (prepared in advance with certified 1000 ppm KH_2PO_4 standard diluted in deionized water to the desired range)

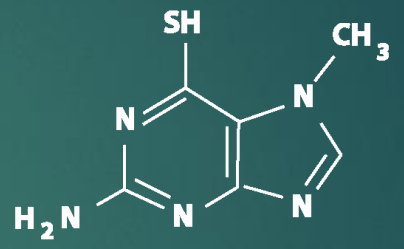
PNP ASSAY CHEMISTRY

$H_2PO_4^-$ + MESG



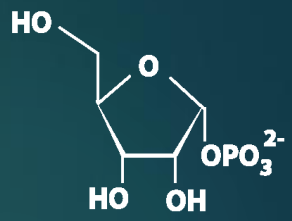
2-amino-6-mercapto-7-methylpurine ribonucleoside

Purine Nucleoside Phosphorylase



2-amino-6-mercapto-7-methylpurine

+



Ribose-1-phosphate

The purine product yields an increase in absorbance at 360 nm in equal molar ratio to the inorganic phosphate in the reaction mixture

After background absorbance of reagent blank is subtracted, the linear standard curve, ppm phosphate is easily determined

TOTAL NITROGEN AND TOTAL PHOSPHORUS

- We’re validating methods for analyzing Total N and Total P
- Involves one sample preparation method prior to analysis using enzyme based test kits for nitrate and phosphate
- Application Notes coming soon
  - Ask us about priority notification

ENZYME FOR MEASURING GLYCEROL

- High glycerol content in biodiesel may cause problems during storage, in the fuel system, injector fouling, and formation of deposits.
- Important to monitor glycerol content during biofuel production
- Pure Glycerol QA/QC
- Thanks to USDA funding, we have successfully made a recombinant enzyme and are researching properties

ENZYME FOR MEASURING ETHANOL

- Alcoholic beverage QA/QC
- Growing biofuels demand & market
- Ethanol content analysis in conventional fuels
  - Concerns of Vehicle Compatibility: Meeting E10 requirements?

NITRATE BIOSENSOR

- Publication in 2012 on oxygen removal system for electrochemical analysis under ambient air
- USDA grant application submitted for the development of a Nitrate Biosensor
- This technology will further develop accessibility of science to the general public
- More samples analyzed, more data collected, more environmental analysis means better resource management and protection

THANK YOU TO OUR SUPPORTERS

Dayle Frame & MELA for inviting us to present

Small Business Innovation Research Programs of the USDA, NIH, and NSF. Michigan MEDC SBIR matching funds and the UofM FCP.

USEPA CWA and SDWA Offices, ASTM, USGS, TNI, Standard Methods, Equipment Manufacturers

Charles Patton, William Lipps, Lemuel Walker, Lynn Egan, J Kevin Roberts, and many others