

CORN LEAF NITRATE REDUCTASE: A NONTOXIC ALTERNATIVE TO CADMIUM FOR PHOTOMETRIC NITRATE DETERMINATION IN WATER SAMPLES

Ellen R Campbell*, Charles J Patton[†], Wilbur H Campbell*, and Jennifer R Kryskalla[†]

*NECi, The Nitrate Elimination Co., Inc., 334 Hecla Street, Lake Linden, MI 49945 USA

[†]US Geological Survey, National Water Quality Laboratory, PO Box 25046, MS 407, Denver Federal Center, Denver, CO 80225-0046 USA

Biographical Sketch of Authors

Ellen R Campbell is Vice President and Bill Campbell is president of NECi. NECi is an environmental biotechnology company, dedicated to the development of enzyme-based technologies for water testing and water treatment. Ms Campbell directs and coordinates NECi's R&D projects, which have been funded by the Small Business Innovation Research (SBIR) programs of the EPA, NIH, the Dept of Energy, and the USDA. Bill Campbell is also professor of biochemistry at Michigan Technological University, Houghton, MI and is an international expert on nitrate reductase. Charlie Patton is Research Chemist in the Methods R&D Program of the USGS, Water Resources Division. Jennifer Kryskalla is a chemistry technician in the same division, and served as technician for this project. Dr Patton helps develop new analytical methods for the USGS's nutrient monitoring programs. He is one of the developers of ion autoanalyzer equipment and an expert in this field. The work presented in this paper is the result of a collaboration between NECi and the USGS. We thank the SBIR program of US Dept of Agriculture for their support of this project.

Abstract

Recent studies have demonstrated the feasibility and practicality of replacing toxic cadmium with nitrate reductase purified from corn leaves (NaR, E.C. 1.6.6.1) as the reagent of choice for routine photometric and colorimetric determination of nitrate in water. In contrast to bacterially-derived nitrate reductase (E.C. 1.9.6.1), which is oxygen sensitive and requires a toxic cofactor, NaR is fully functional in air-saturated solutions. Furthermore, its cofactor, the reduced form of β -nicotinamide adenine dinucleotide (NADH), is nontoxic and costs much less than the β -nicotinamide adenine dinucleotide phosphate (NADPH) cofactor required by enzyme derived from fungi (E.C. 1.6.6.2). In this presentation, we describe how NaR has replaced cadmium in field test kit methods, automated, bench-top air-segmented continuous flow (CFA) methods such as US EPA method 353.2 and USGS method I-2545-90, and in manual batch-reduction procedures followed by automated CFA or flow injection analysis (FIA) finishes. Finally, we discuss how cheaper or reusable NaR in the form of immobilized enzyme reactors might impact the future of laboratory and in situ nitrate analysis.

Introduction and Background

The Nitrate Problem

Nitrate is listed as a primary contaminant under the Safe Drinking Water Act and Clean Water Act. An early edition of the US EPA's publication explaining the SDWA to consumers talks about nitrate in this way: "Only two substances for which standards have been set pose an immediate threat to health whenever they are exceeded: Bacteria and Nitrate" (USEPA OW #WH-550, 1991). Consumption of excess nitrate is considered to be dangerous for infants and a potential health hazard to older children and adults. Excess nitrate can also be toxic to livestock. This is because nitrate can be converted to nitrite in the gut, and nitrite can bind to hemoglobin thus preventing the blood from carrying enough oxygen. There were infant fatalities from this condition, called methemoglobinemia, in agricultural regions of the US in the 1950's, as the use of chemical fertilizers became widespread. Nitrate helped demonstrate the need for regulations to monitor and control water quality. Today, most nations regulate nitrate in ground and drinking waters.

Nitrate can cause other problems. Long term consumption of excess nitrate may be involved in other human diseases, such as certain types of cancer, Type I diabetes, and even childhood asthma. Excess nitrate can encourage microbial growth where it's not wanted - for example, in swimming pools, or algal blooms in coastal environments (Natl Research Council 2000). Sources of nitrate contamination include runoff of excess fertilizer from farms, golf courses, and lawns, industries such as paper and munitions manufacturing, and untreated or poorly treated human and animal wastes. Another source is "acid rain": NO_x gas species produced during combustion tend to become nitrate when in solution. Basically, wherever population density is high and/or there has been long-term agricultural activity, there is likely to be excess nitrate in the soil and water.

Nitrate is a key nutrient to plants and microbes — think of it as protein to these organisms. The environment is geared to turn decaying living matter and wastes into nitrate. What makes nitrate particularly troublesome is that it is so stable and unreactive: it does not bind to soil minerals or particles, nor react with other compounds to change into a more benign chemical, and is not volatile (does not evaporate). It is extremely soluble in water and so tends to move with the flow of ground water, both horizontally in contaminant "plumes", and vertically into the aquifers which supply local wells.

Nitrate Analysis

Nitrate is one of the most widely determined anions in water and in the environment. Effective technology for monitoring and determination of nitrate is available. All of the available methods suffer from specific shortcomings, which we are attempting to address with enzyme-based methods.

Current instrumentation for nitrate *monitoring* is based on 1) simple UV detection, 2) chemical reduction of nitrate to nitrite followed by colorimetric determination of nitrite, or 3) the nitrate ion selective electrode (ISE). UV detection of nitrate is nonselective and therefore prone to interference: many compounds absorb at 214nm, the range used for nitrate monitoring equipment. Devices based on colorimetric detection are mechanically complex (thus subject to equipment failure), require large volumes of reagent solutions, and can leach cadmium (the nitrate reduction catalyst) into the monitored environment. The nitrate ISE is subject to interference by ions in the Hofmeister series, which includes nitrite, chlorate, chloride, bicarbonate, phosphates, and sulfate. Nitrate ISE membranes are short-lived and subject to fouling. Thus available technologies are limited in selectivity, and none is completely satisfactory for monitoring applications, particularly for environmental or biological process monitoring. NECi is currently developing an enzyme-based nitrate biosensor for nitrate monitoring applications.

The EPA Standard Methods for nitrate *analysis* in the lab are: manual or automated reduction of nitrate to nitrite using cadmium followed by colorimetric nitrite determination, the nitrate ion selective electrode (ISE), and ion chromatography (IC). Cadmium-based methods expose the user to the toxic heavy metal and generate large volumes of hazardous waste. NECi has begun adapting our enzyme-based test kit technology for on-line nitrate analysis in autoanalyzers; this would eliminate the cadmium problem. Preliminary work, done in collaboration with a USGS lab, has been published in *Environmental Science & Technology*, February 2002 (Patton et al 2002).

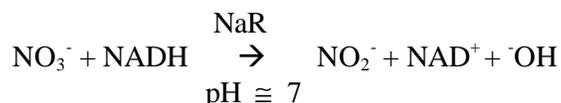
The ISE is subject to interference from a number of ion species, and the sensing modules have a relatively short useful life. IC requires extensive sample preparation, highly skilled operators, and expensive equipment. A nitrate biosensor will find ready markets in a number of industries and disciplines.

Field methods for nitrate determination include test kits and ISE meters. NECi markets nitrate test kits for Field use, and this has given us a good overview of the wide array of users and their needs. For example, we have developed test kits that provide accurate nitrate determination at concentrations below 1 ppm nitrate-N (a range of 5 – 75 μM nitrate) in salt or fresh water samples using standard cuvetts and a colorimeter. This kit is gaining interest among oceanographers and environmental scientists.

Our work with nitrate test kits and on-line nitrate analysis (Patton et al 2002, Campbell et al 2001) demonstrates that enzyme-based nitrate analysis is able to provide the sensitivity needed for environmental applications. We have also shown that nitrate reductase-based nitrate analysis is very selective: better selectivity means fewer interferences or false positives. Our main customer base for lab nitrate test kits has been the biomedical research community. These users are measuring nanomolar concentrations of nitrate in biological samples – extremely complex and challenging matrices. This demonstrates the superior accuracy of the enzyme-based nitrate determination system (Campbell & Campbell 1998). Enzyme-based nitrate analysis has the potential to be of equal value to the environmental and water science communities.

How the method works

NECi introduced commercial grade NADH:Nitrate Reductase isolated from corn (*Zea mays*) seedlings in 1994 (Hyde et al, 1989). This nitrate reductase (NaR) from higher plants catalyzes the reduction of nitrate to nitrite. The catalytic rate of NaR is approximately 200 nitrate-to-nitrite conversions per second per molecule of enzyme - one of the fastest enzymatic reactions known (Campbell 1999). The biological electron donor for the reaction is NADH (β -nicotinamide adenine dinucleotide, reduced form), a benign and readily available biochemical reagent. The reaction is irreversible and goes to completion as shown below:



The resulting nitrite subsequently reacts with the Griess reagents—sulfanilamide and N-(1-Naphthyl)ethylenediamine—to form a stable and highly colored product that is ideal for determination by photometry. The nitrate reduction step is catalyzed by cadmium (or occasionally zinc) in test kits and in manual and automated nitrate analysis methods used today. The Griess reagent step used for enzyme-based nitrate analysis is the same as the reagent system used in EPA and other standard methods.

The advantages of NADH NaR versus commercially available fungal and bacterial NADPH-dependent NaR forms for use as nitrate analysis reagents are:

1. NADPH costs ten times more than NADH.
2. Bacterial NaR forms require methyl viologen (a teratogen and potential carcinogen) and sodium dithionite (an unstable and highly reactive chemical) for activity. This means that the reaction conditions for the nitrate assay are more complex, and less user-friendly.
3. The NaR manufactured at NECi is supplied in a purified form and is therefore a more reliable analytical reagent, with reduced potential for interfering reactions.
4. Plant forms of NADH NaR are **not** oxygen sensitive.

Reports of use of the enzyme nitrate reductase for nitrate determination have been in the literature for a number of years (Senn & Carr 1976; Granger et al 1996; etc.). NaR-based nitrate analysis is currently the method of choice for assay of nitric oxide (NO, as nitrate or nitrite in solution) in biomedical research. Sample matrices include serum, tissue culture fluid, spinal fluid, saliva and urine, and typical required sensitivity is in the

nanomolar (**ppb**) range. Clearly, the robustness and selectivity of our NaR reagent in complex samples has been well proven.

NECi began developing NaR-based nitrate test kits for agricultural and environmental applications in 1995, with the assistance of an earlier USDA award. We have developed formulations of NaR from corn (*Zea mays*) with sufficient stability to be used at ambient temperatures in field conditions. NECi's nitrate test kits are competitive in price to conventional nitrate test kits, are safer to use, and the spent reagents can be sewerred without contaminating the environment. Publications describing the utility of this NaR form for nitrate determination include Glazier et al 1998, Campbell & Campbell 1998, Campbell et al 2001, and Patton et al 2002.

The advantages of corn NaR include: 1) high purity, 2) fast reaction time, 3) high selectivity for nitrate, 4) simple reaction conditions, 5) no hazardous reagents needed. These qualities are just as important to automated nitrate analysis as they are to test kits. We show here that NaR is a viable replacement for cadmium in automated nitrate analysis.

Automated methods for nitrate determination, U.S. EPA method 353.2 for example, are widely used in the United States and throughout the world. In such methods, nitrate is reduced to nitrite by small, packed-bed, cadmium reactors. Nitrite is subsequently reacted with Griess reagents—sulfanilamide and N-(1-Naphthyl)ethylenediamine—to form a stable and highly colored product that is ideal for determination by photometry. Cadmium required by these methods, however, is a highly toxic heavy metal. Its use exposes analysts to a potent health hazard, and generates a cadmium contaminated waste stream, which must be collected for proper disposal.

Enzymatic nitrate reduction is an attractive alternative to the cadmium-based chemistry. Enzymatic reactions are rapid, extremely selective, and nontoxic. They are safer for the user and for the environment, and can improve lab economy by reducing costs of hazardous waste disposal. NECi has developed reliable nitrate analysis tools based on its proprietary NADH:nitrate reductase (NaR) enzyme, which is isolated from corn seedlings. Our company is also developing new and potentially less expensive NaR reagent tools using genetic engineering and protein expression technologies, under funding from the SBIR program of NIH (Phase II award #R44 GM56598).

Results

Nitrate test kits: NECi has developed NaR-based nitrate test kits for a variety of applications and skill levels. Test kit descriptions can be found on the website (www.nitrate.com).

Kits designed for **lab** use are available in test tube and 96-well microplate formats. These kits require a moderate level of technical skill, accurate measuring devices (automatic pipettors or glass pipets), and a colorimeter or microplate reader capable of reading absorbance at 540 nm (± 10 nm). Users prepare a nitrate standard curve based on the nitrate standards provided with the kits, and then determine the nitrate content of their samples from the curve. The lab kits use 3N HCl in the color reagents, and can produce data of accuracy comparable to ion chromatography; e.g., the lab kits produce quantitative data. A standard curve generated from a Low Range lab kit is shown below, Figure 1. This is an example of ultra-low nitrate analysis – levels below 0.1ppm nitrate-N. Nitrate below 1.0 μ M (0.07ppm nitrate-N) was determined by using a longer pathlength cuvette (5cm in this example). The reagent blank absorbance of 0.002 was subtracted. This assay works for Seawater with minor modifications.

Field kits should be of most interest for citizen monitoring groups and in educational settings. These kits require minimal technical training or skill and contain all needed mixing and measuring devices. We provide step-by-step instructions. A CD with a 5 minute video demonstrating all the steps is available for the FNTK-200 series kits. Nitrate standards are provided and are run alongside user samples. This is an internal check that all reagents are working, and that instructions have been followed. Nitrate content of samples is read by comparing the color of the samples to that of the nitrate standards. Data quality is qualitative to semi-quantitative. Color density is

strong enough that colorblind users are still able to easily see the differences. Users can read the results using a colorimeter if available, for semi-quantitative data.

The color reagent system in the Field kits is based on a less aggressive acid. The reagents are therefore safer for inexperienced users and in field settings. For example, spills on site or in a boat or car pose no hazard.

Ultra Low Range Nitrate Assay with NADH/NaR

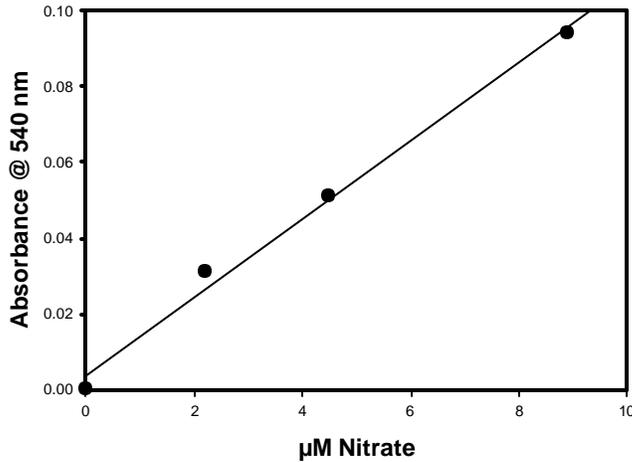


Figure 1: Low Range Lab Kit (L-202) was used to determine very low nitrate levels in water. This graph was generated at NECi, using a Hewlett-Packard 8452 UV-Vis spectrophotometer.

An interesting example of an on-site application of enzyme-based nitrate analysis is shown in Figure 2. This graph was generated by Mark Quilter, an inspector for the Utah Dept of Agriculture and Foods. He was engaged in a project to test drinking water wells for safety at private homes throughout the state. He and an assistant gathered samples all day and analyzed them in the motel room in the evening, using the Lab Kit reagent system. An inexpensive handheld colorimeter with 550nm filter was used, and at least 200 wells were tested in the course of his study. Mr Quilter compared the NECi test kit with those of three competitors and found that the NaR-based kits were the only ones capable of providing him with useful data

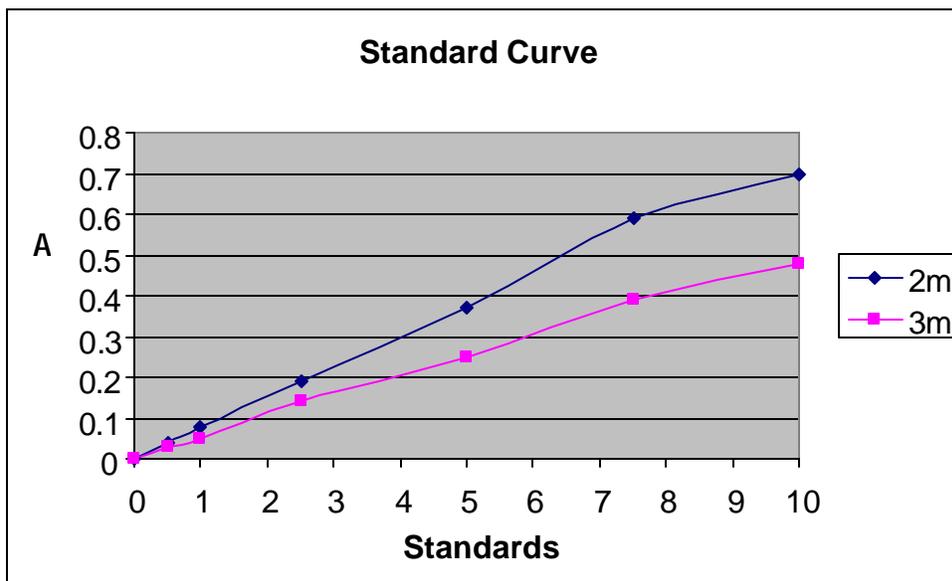


Figure 2: Nitrate standard curve from 0 – 10 ppm nitrate-N generated in on-site situation.

NECI has validated the lab and field kits for a number of sample types:

1. Marine waters
2. Plant extracts and soil samples, including those extracted with 2M KCl
3. Biological samples including tissue culture media
4. Manures
5. A wide array of potable, ground and surface water samples from many regions of the US.

Automated nitrate analysis

Automated methods for nitrate (NO_3^-) determination generally involve reduction of nitrate to nitrite with a suitable reagent followed by reaction with the Griess reagents. Granular cadmium metal, a reliable reagent for reducing nitrate to nitrite, is prescribed or recommended for use in current methods of the American Public Health Association (APHA), the American Water Works Association (AWWA), the Water Environment Federation (WEF), the U.S. Geological Survey (USGS), and the U.S. Environmental Protection Agency (EPA), *e.g.* EPA 353.2.

Automated cadmium reduction methods for nitrate determination are widely used in the United States and worldwide. While such methods are accurate and reliable, the extreme toxicity of cadmium poses challenges to worker safety and environmental hygiene. At column manufacturing sites and in analytical laboratories, for example, workers are exposed to cadmium whenever columns are handled or packed. Furthermore, millimole per liter quantities of cadmium leach from packed bed cadmium reactors continuously during use (Nydahl 1974, 1976). In the past, this amount of Cd has been considered to be negligible and innocuous to most users and regulators. More recently, however, growing awareness of cadmium's toxicity makes its continued use questionable to analysts and regulators alike. These factors, coupled with the rising costs of cadmium-containing waste stream disposal, has produced a climate in which regulators and laboratory managers are seeking "green chemistry" alternatives to cadmium-reduction methods for nitrate determination. We initiated this collaboration for these reasons.

The initial work has focused on development of enzyme-based nitrate analysis in segmented-flow equipment. We chose the CF approach in preference to FIA because it is the technique of choice when long reaction times (> 60 s), low flow rates (typically, 0.5 mL/min), and high rates of analysis (> 90/hr) are required (Patton & Wade 1997, Patton & Crouch 1986).

NECI and the U.S. Geological Survey National Water Quality Laboratory (NWQL) demonstrated proof of concept for this approach during 2000, using enzyme in solution. These experiments were performed with a multi-channel, air-segmented continuous flow analyzer configured for simultaneous determination of nitrate with cadmium reduction. NaR reduction set-up is shown in Figure 3. A detailed description of this work is available in *ES&T*, Patton et al 2002.. More than 100 natural water samples along with numerous duplicates, spikes, calibrant, and check standards were analyzed using both the cadmium reduction column and NaR in solution. Agreement between the two nitrate reduction methods is excellent, and the work is now published (Patton et al 2002).

Additional samples were run in 2001 and 2002. To date, the only interfering substance found has been chloride, which is known to be a partial inhibitor of nitrate reductase. This inhibition can be overcome by allowing the nitrate reduction step to proceed for a longer time, or by using additional enzyme.

Figure 3 below shows the set-up for using nitrate reductase and NADH in place of a cadmium column in a standard CFA instrument.

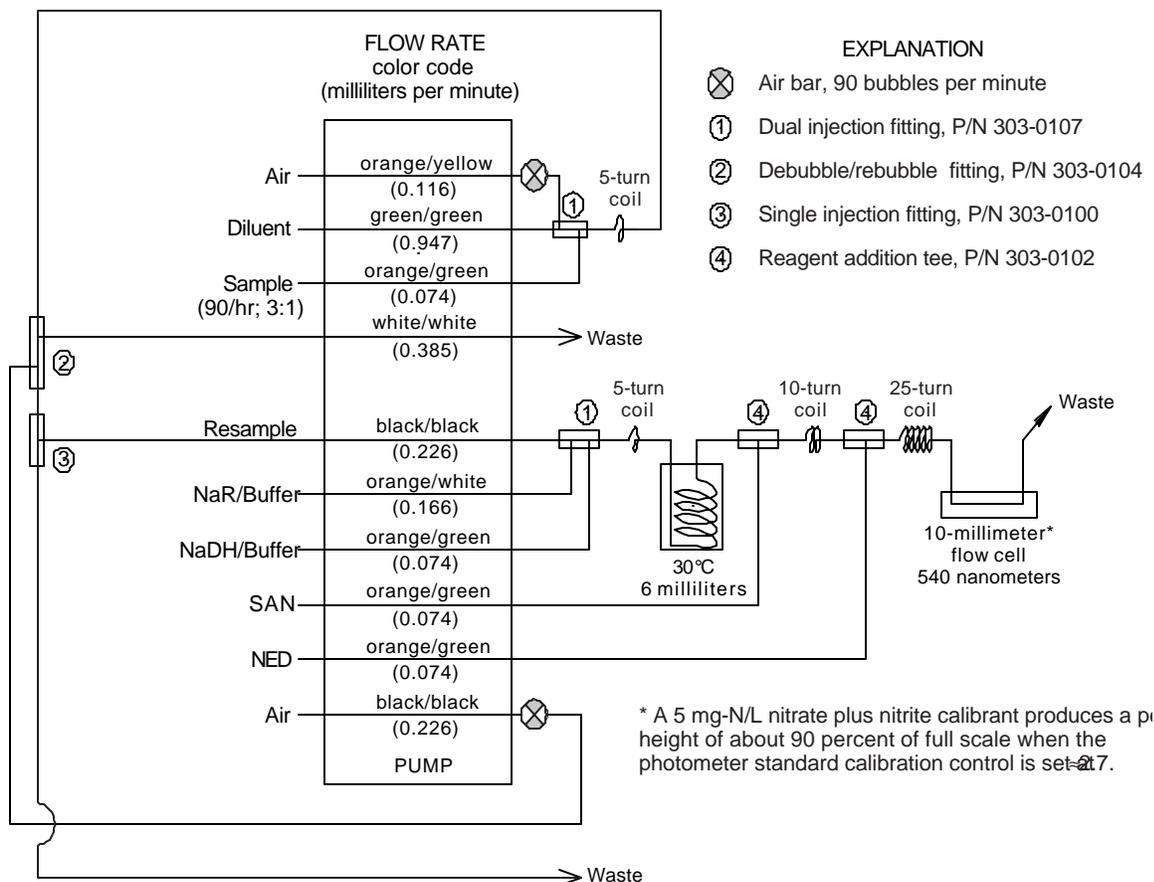


Fig. 3: Analytical cartridge diagram for determination of nitrate plus nitrite by the soluble NaR method.

Experiments using inexpensive FIA equipment

We are interested in developing alternative and inexpensive methods for monitoring of nitrate or for processing large numbers of samples. For these experiments, a FIALab-2000 was used for NADH:NaR reduction of nitrate to nitrite. The carrier stream (NaR + NADH in MOPS buffer) flow rate was 2.0 ml/minute. Samples were injected at 2-minute intervals (30 samples per hour). A Gilson Microfractionator fraction collector was used to isolate and collect the analytical stream resulting from each 50 µl injection into individual test tubes. After a reaction time of 10-30 minutes, nitrite concentrations in the collected fractions were determined by Griess reaction. Nitrate standards were diluted from 1000 ppm Labchem Standard (KNO₃).

We also analyzed “real” samples: spiked tap water, and water from a pond and a river. A concurrent project at NECi involved development of a simple nitrate test kit for on-site testing of farm crops. We tested some of the corn tissue samples as well. Agreement between the FIA method and the NECi lab standard nitrate analysis method was good. Figure 4 shows a typical nitrate standard curve generated using the system as described above.

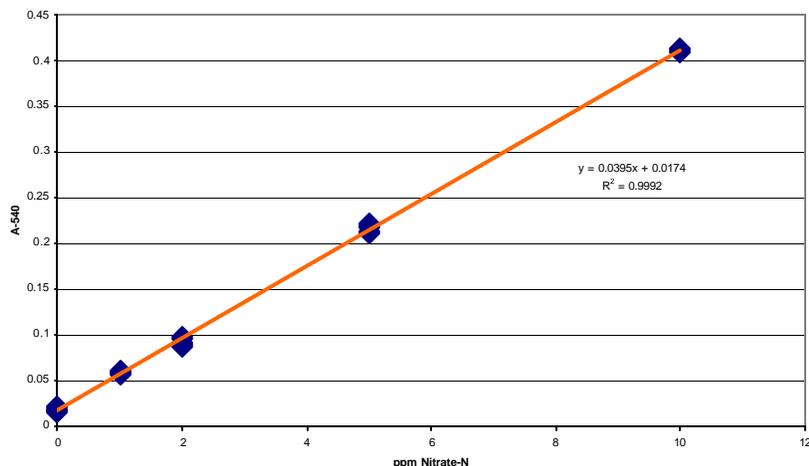


Figure 4. Nitrate Standard curve. Volume of the analytical stream collected for each sample is 2 ml. Sample rate, 30/hr.

Conclusions

Enzymatic nitrate reduction is an attractive alternative to cadmium or zinc chemistry for nitrate analysis. Enzymatic reactions are rapid, extremely selective, and nontoxic. They are safer for the user and for the environment, and can improve lab economy by reducing costs of hazardous waste disposal. These studies demonstrate the potential for nitrate reductase as a replacement reagent for nitrate analysis as a wet chemistry method in the lab or in the field, as well as in automated nitrate analysis.

A major thrust of our R&D efforts at this time is to develop a less expensive source of reliable nitrate reductase. We are making good progress in this area. Enzyme-based nitrate analysis is green, reliable, selective, and accurate. Less expensive enzyme will make the method more desirable: as more enzyme can be used per assay, we can reduce the assay time. This is the last step in attaining the criteria that users look for: faster, better, and cheaper. With all three, it will be more difficult for the analytical community to resist this environmentally benign method.

References:

- Campbell ER, TPK Skidmore, LA Winowiecki, & WH Campbell (2001) A new trend in nitrate analysis: enzyme-based field test for nitrate. *American Environmental Laboratory*, Feb 2001: 90-93.
- Campbell ER & WH Campbell (1998) Nitrate assays using nitrate reductase. In: *Current Protocols in Field Analytical Chemistry*, First Supplement, John Wiley & Sons, pp. 5A1 - 5A15.
- Campbell, W.H. (1999) Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. *Annual Review Plant Physiology Plant Molecular Biology* 50: 277-303.
- Glazier SA, Campbell ER & Campbell WH (1998) Construction and characterization of nitrate reductase-based amperometric electrode and nitrate assay of fertilizers and drinking water. *Anal Chem* 70: 1511-1515.
- Granger DL, RR Tainor, KS Broockvar & JB Hibbs (1996) Measurement of nitrate and nitrite in biological fluids using nitrate reductase and Griess reagents. *Meth. Enzymol.* 268: 142-151.

Hyde G.E., J.A. Wilberding, A.L. Meyer, E.R. Campbell and W.H. Campbell (1989) Monoclonal antibody-based immunoaffinity chromatography for purifying corn and squash NADH:nitrate reductases. Evidence for an interchain disulfide bond in nitrate reductase. *Plant Molecular Biology*, **13**: 233-246.

National Research Council (2000) Clean Coastal Water: Understanding and Reducing the Effects of Nutrient Pollution. National Academy Press, Wash., DC. ISBN 0-309-06948-3.

Nydahl F (1976) *Talanta* **23**: 349-357.

Patton CJ, AE Fischer, WH Campbell, ER Campbell (2002) Corn leaf nitrate reductase – a nontoxic alternative to cadmium for photometric nitrate determinations in water samples by air-segmented continuous-flow analysis. *Environmental Science & Technology* 36:729-735.

Patton CJ and E.J. Gilroy, 1998, USGS Nutrient Preservation Experiment—Experimental Design, Statistical Analysis, and Interpretation of Analytical Results, Water Resources Investigation Report 98-4118.

Patton CJ & AP Wade (1997) *Continuous Flow Analyzers* (Chapter 4) in **Analytical Instrumentation Handbook - 2nd Ed.**, GW Ewing, ed.: New York, Marcel Dekker, Inc. PP125-220.

Patton CJ & SR Crouch (1986) Experimental comparison of flow injection analysis and air-segmented continuous flow analysis: *Anal. Chim. Acta* **179**: 189-201.

Senn DR and P.W. Carr, *Anal. Chem.* **48**, 954-958 (1976).