Field Analytical Methods for Hazardous Wastes and Toxic Chemicals

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Field Determination of Nitrate using Nitrate Reductase

Ellen R. Campbell, James S. Corrigan, and Wilbur H. Campbell
The Nitrate Elimination Company, Inc.
334 Hecla Street
Lake Linden, MI 49945

ABSTRACT

Nitrate is routinely measured in a variety of substrates - water, tissues, soils, and foods - both in the field and in laboratory settings. The most commonly used nitrate test methods involve the reduction of nitrate to nitrite via a copper-cadmium reagent, followed by reaction of the nitrite with the Griess dye reagents. The resulting color is translated into a nitrate concentration by comparison with a calibrated color chart or comparator, or by reading the absorbance in a spectrophotometer. This basic method is reliable and sufficiently sensitive for many applications. However, the cadmium reagent is quite toxic. The trend today is for continued increase in concern for worker health and safety; in addition, there are increasing costs and logistical problems associated with regulatory constraints on transport and disposal of hazardous materials. Some suppliers have substituted a zinc-based reagent powder for the cadmium in an effort to reduce toxicity. We describe here an enzyme-based nitrate detection method as an improvement on the basic Griess method that demonstrates equal or superior sensitivity, superior selectivity, and is more environmentally benign. Comparisons between the enzyme-based method and some standard field test kits being used today are made.

INTRODUCTION

Nitrate is present in water and soil world wide. Field testing applications for nitrate determination include environmental site assessment and monitoring, soil and crop consultants in agricultural, on-site testing of potable water by vendors of water quality improvement equipment, and in aquaculture.

Nitrate testing has a long history. The most commonly used detection method, involving reduction of nitrate to nitrite, uses diazo chemistry developed in the 1950's. Ion selective electrodes for nitrate became available in the 1960's. Methods based on direct nitration of aromatic compounds such as salicylate and brucine became popular in the 1970's. The reduction of nitrate to nitrite using a Cu-Cd reductant and nitrite detection by diazo chemistry and various nitration systems are probably the most widely used methods in analytical laboratories and field test kits. As early as 1971 (1), the enzyme nitrate reductase (NaR) was suggested as an alternative catalyst for the reduction of nitrate to nitrite. The enzymatic reduction method offers several advantages over the chemical reduction method: it is sensitive, since the catalyst is effective in small sample volumes; it is specific; and it can be formulated to utilize non-toxic reagents, which is a distinct advantage over the chemical reductant methods.

NaR is found in bacteria, algae, fungi and higher plants (2). NaR exists in several forms in these organisms with most of the bacterial sources having a membrane bound form while the eukaryotes have a soluble pyridine nucleotide dependent form. The research studies of NaR over the past forty years have led to a rather complete characterization of both the bacterial membrane enzyme (called dissimilatory) and the eukaryotic enzyme (called assimilatory). Genes for both forms have been cloned and sequenced from a number of sources (2, 3). WH Campbell began research on NaR in 1974 and maintains a strong academic program in this field. His research has focused mainly on higher plant NaR, especially forms using NADH as electron donor (4). In 1989, a monoclonal antibody immunoaffinity chromatography purification method was developed in Dr. Campbell's laboratory. This method is a single-step direct purification of the enzyme from crude leaf extracts and yields essentially homogeneous protein (5).

The major obstacle to the use of NaR as the catalyst for reduction of nitrate prior to analysis has been the lack of a standardized and stable nitrate reductase preparation. This technical barrier has been removed by NECi. NaR can be purified from maize seedlings in sufficient quantity and purity for use as a dependable analytical reagent. NECi has also successfully extended the "shelf-life" of NaR so that activity is retained for at least one year under standard storage conditions (6); see Fig. 1. With this reagent, we are developing nitrate test kits in formats suitable for use in the laboratory, in the field, and in the home by consumers.

Here we compare results generated with prototype test kit formulations using the NaR reagent to results generated by some of the commonly used kits on the market today. The Orion nitrate ISE was used for comparison and validation. Standards as well as "real" samples collected in the field are evaluated.

EXPERIMENTAL METHODS

Purchased kits were used in accordance with their instructions as supplied. Orion ISE protocols for low nitrate samples were followed. Buffers were purchased from Research Organics, Cleveland, OH; additional reagents were from Fisher Scientific, Pittsburgh, PA, and Sigma Chemical Company, St. Louis, MO; all were reagent or biotechnology grade. Enzyme-based nitrate assays were performed by our standard protocol.

Nitrate reductase-based nitrate assay: 1.7 ml NaR Assay Buffer (50 mM MOPS/1 mM EDTA , pH 7.4); 100 µl NO_3^- Standard or water sample; and 100 µl NaR enzyme (0.025 Unit/test) were combined in a test tube and vortexed. 100 µl of 2 mM NADH (reduced nicotinamide adenine dinucleotide, a common electron carrier in biological systems) was added. After a 20 minute incubation, the Griess color reagents were added: 1 ml of color reagent #1 (Sulfanilamide 1g/100 ml 3N HCl) was added, followed by 1 ml color reagent #2 (N-(1-Naphthyl)ethylenediamine-diHCl, 20 mg/100 ml H_2O); the tube is again vortexed. After a color development period of 15 minutes, absorbance is read at 540 nm. Figure 2 shows a typical nitrate standard curve produced by this method. This method is a variation of the EPA 40 CFR 141 standard method for determination of nitrate in drinking and waste water.

Standards in the range of 0-15 ppm Nitrate-N (or Nitrite-N) were prepared by dilution of LabChem 1000 ppm Nitrate-N (Nitrite-N) Standard (LabChem Inc., purchased through Fisher Scientific) with nitrate/nitrite-free, 18 megaohm deionized water. Samples were used as is, without preparation or pretreatment, except for dilution as required for one of the kits.

RESULTS AND DISCUSSION

With the stable and well-characterized NaR enzyme reagent now available to us, we have begun to develop nitrate test kits for a number of applications. We began by modifying the assay used for determining nitrate reductase enzymatic activity. This assay measures the amount of nitrite produced per minute for an unknown amount of enzyme; the Griess color reagents are used to determine the quantity of nitrite. The results are defined as units of nitrate reductase activity. Variations of this method are commonly used in plant and microbial biochemistry studies of nitrate reductase. A set number of enzyme units will reduce a predictable quantity of nitrate to nitrite in a predictable number of minutes at a fixed temperature. This allows us to determine the quantity of enzyme reagent required for reduction of nitrate in a sample within a desired time frame. It is important that there is sufficient enzyme for reduction of all of the nitrate expected to be in the sample within a convenient period of time, and in the expected ambient temperature range. If more enzyme is used, less time is required for the reduction to reach end point. We employed cost/benefit analysis to determine the minimum quantity of enzyme required to quantitatively reduce the nitrate to nitrite in samples containing 0 - 15 ppm nitrate-N within a convenient time frame.

For the purpose of this paper, two of the most commonly used field test kits for Nitrate-N were purchased. The kits were given to a competent technician who was told to compare them with the enzyme-based nitrate assay used in our laboratory. Kits were used as instructed, and results were read using the manufacturers' supplied color comparators. Assay results for the NaR method were read in a

colorimeter (Pharmacia Novaspec II) at 540 nm. Kits were compared for accuracy, ease of use, and exposure to hazardous materials.

Figure 3 shows results of each method. As is apparent from Figures 2 and 3, the NaR assay gives clear and accurate information over the useful range of 0 to 15 ppm nitrate-N. Linearity is good even between 0 to 1 ppm, and color development does not fall off at the higher concentrations. Sample dilution is not required until nitrate concentration exceeds 15 ppm.

The NaR-based nitrate assay offers excellent recovery, or efficiency of conversion, of nitrate to nitrite. As shown in Figure 4, conversion efficiency of NaR approaches 90%. This is an improvement over the cadmium or zinc methods. Another advantage is the small sample volume relative to the total reaction volume. This tends to mitigate any adverse effects from possible interfering substances. For example, highly colored samples, e.g. those containing tannins or humic acids, can be used in the NaR method without inhibition of nitrate to nitrite conversion, and also without interfering with or masking subsequent color development.

In order to demonstrate that the NaR method gives accurate response for real water samples collected in the field, not only with spiked laboratory water, we show in Figure 5 a comparison of our enzyme-based method with the kits and with the nitrate ISE. The NaR method shows greater sensitivity in the low ranges, and is more consistent with the ISE values in all of the samples tested. The samples were collected into sterile 50 ml capped tubes. The samples came from a variety of sources including wells, streams, and public water supplies at highway rest stops. During the course of our Phase I project with the USDA, we tested approximately 50 water samples from such sources. Results from the NaR nitrate assay matched those from the nitrate ISE in all cases, although the nitrate electrode consistently gives a response about 1 ppm higher than the enzyme assay. This is most likely due to other ions in the samples; the nitrate ISE is subject to interference from a number of ions.

We have also begun to adapt this method for assaying nitrate levels in foods and plant tissues, for applications in laboratory and field by feed and crop consultants, food quality assurance, and by plant physiology researchers. While we did not compare test results from the kits with this type of sample, the data might be of interest to some readers. A pilot study was performed using prepared foods purchased from local supermarkets. The samples were prepared as described in the Table legend, and assayed by the NaR method and by nitrate ISE. Table 1 shows typical results.

The current thrust of our development efforts is to formulate a nitrate test kit that is environmentally neutral; that is, the use and disposal of component reagents results in as little negative environmental impact as possible. Initial studies coupling our NaR-catalyzed nitrate reduction step with color reagent tablets supplied with a number of other commercially available kits show promise. These tablets are composed of Griess-type color reagents and are compatible with our system. Color intensity is reduced, but sensitivity, linearity and accuracy are retained. We are optimizing tablet formulations for improved color development under the Small Business Innovation Research program of the USDA.

CONCLUSIONS

The Nitrate Elimination Company, Inc., (NECi) has been developing nitrate test kits based on the enzyme nitrate reductase (NaR; E.C. #1.6.6.1) isolated from maize seedlings. Enzymatic nitrate measurement is quick and sensitive, very specific and easy to perform. Enzyme based assays in general are less subject to interferences or false positives than more conventional techniques such as ion selective electrodes. In addition, use of toxic and hazardous reagents can also be reduced. NECi will begin marketing a nitrate test kit for water in the microtiter plate format for use in research laboratories in 1997, and has been developing a consumer test kit for home nitrate testing with the assistance of USDA funding. Here we describe the application of this nitrate measurement technique to field testing.

Several companies market nitrate/nitrite kits for field and lab use; these are sold for water testing, to soil and crop consultants, and for environmental surveys. In general, nitrate is reduced to nitrite by cadmium or another hazardous reagent. The nitrite is reacted with the Griess diazo reagents to form a colored product, and the nitrite is quantified by measuring the absorbance in a spectrophotometer or by

visually comparing to a color chart or color comparator. While these methods may provide accurate nitrate quantification, the spent reagents may present disposal problems after use. In addition, the most commonly used cadmium reagent can vary in conversion efficiency, leading to underestimation of nitrate and a degree of uncertainty in the results. Clearly, there is a need for an accurate, easy-to-use, and environmentally safe nitrate test kit, especially for use in the field .

The test kits we are developing replace the chemical reduction step with enzymatic reduction, requiring only a buffer, a minute quantity of nitrate reductase, and NADH. Our system reduces exposure to toxic and hazardous materials without compromising on sensitivity or accuracy. It provides comparable or superior ease-of-use, sensitivity, selectivity, and cost, with dramatically reduced levels of exposure to harmful compounds for the user. Safety issues and transportation costs mandate this type of improvement in environmental assays, especially for field and consumer uses. A key advantage of this reagent system is that the contents of the reaction tubes are virtually harmless: expended reagents and samples can be disposed of on site with little or no adverse impact to the environment.

Enzymes offer the ultimate in selectivity and sensitivity to their target substrates. Enzymatic reactions are in general quite fast and very specific. The recent advances in protein chemistry and biotechnology are enabling biochemists to begin to adapt these delicate molecules for practical uses. Other enzymes may be suitable for adapting to quantitation of compounds of environmental concern; possibilities include enzyme-based mercury and sulfur detection kits.

Nitrate reductase can replace the more hazardous reagents currently used for the detection of nitrate in water and many other aqueous media. NECi is adapting this technology for a nitrate test kit in the convenient microtiter plate format for use in laboratories. We are also developing a safe nitrate test kit for home use by well-owners, farmers, and other consumers.

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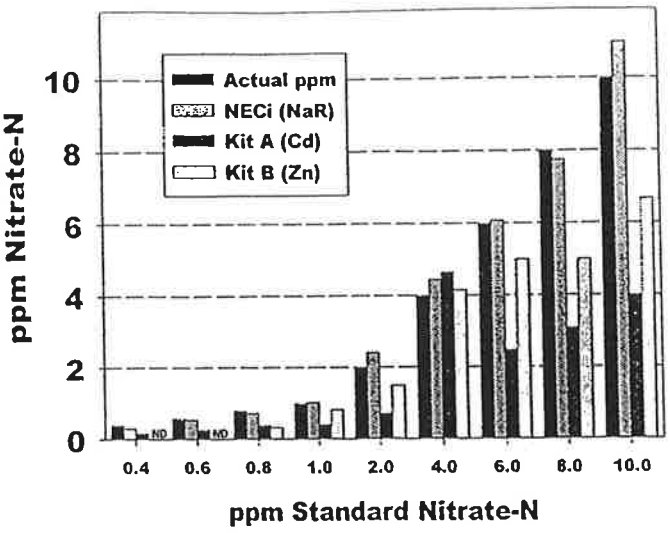
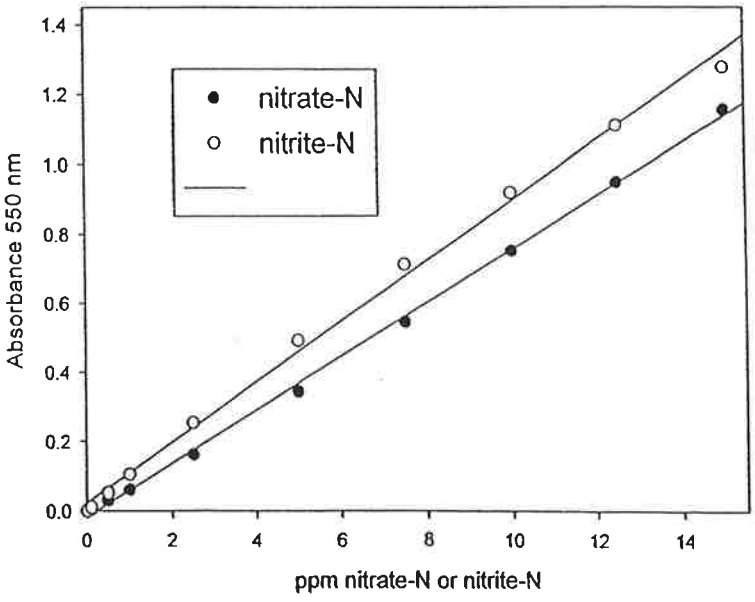
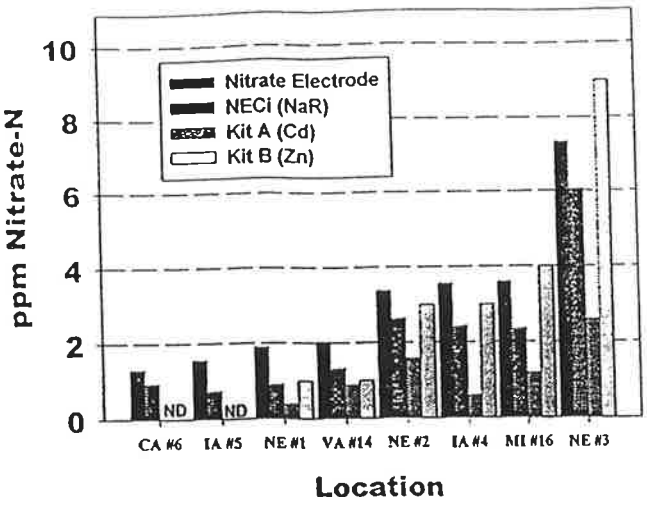


3. Results of analysis of nitrate standards: comparison between NaR method and two nitrate test kits. Assays were run as described in Methods.



4. Comparison of standard curves for NaR catalyzed nitrate determination versus a nitrite standard, showing conversion efficiency of the method.



5. Comparative analysis of water samples from different regions of the US.

Table 1. Nitrate in foods assayed enzymatically and by nitrate ISE.
Values expressed as ppm Nitrate-N

Sample Description	Nitrate by Nitrate Reductase Method		Nitrate by ISE
	No prep	Samples boiled *	Samples boiled*
Vegetable juice	31	57	68
Tomato juice A	5	8	22
Carrot juice A	34	176	187
Carrot juice B	32	66	29
Baby foods:			
applesauce	0.3	2.5	Not determined
sweet potatoes	4	7	Not determined
green beans	13	17	Not determined
peas	0.5	3	4
squash	23	63	82
carrots	20	25	28
carrot/beef	18	22	28
broccoli/chicken	24	103	132

* Sample preparation for juice and food samples: 1 ml of sample was boiled in a microcentrifuge tube for 10 minutes, then centrifuged 10 min @ 7000 rpm to remove particulates. Volume was restored to 1ml before assaying 100µl of the supernatant. For ISE analysis, 10ml aliquots were prepared.

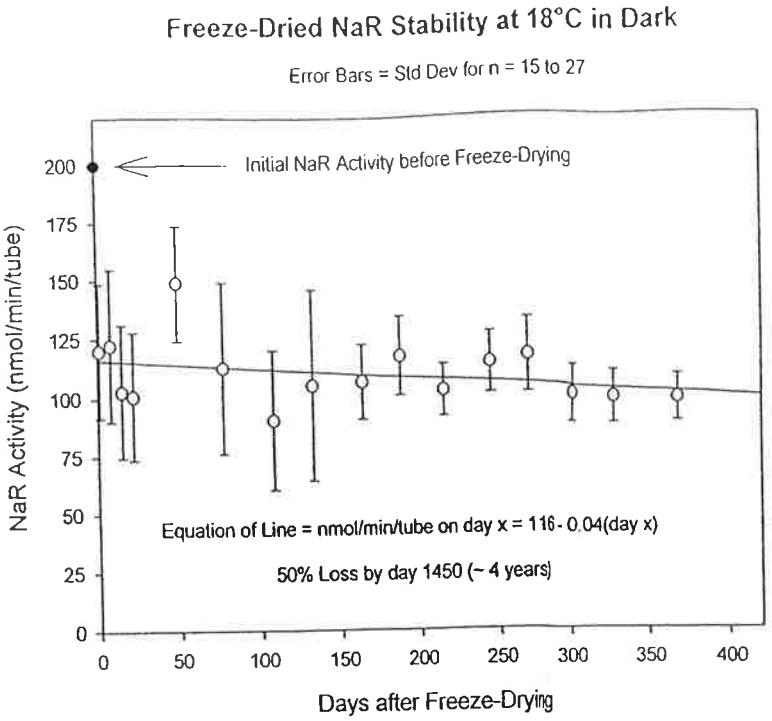


Figure 1. Graph demonstrating stability of NaR activity

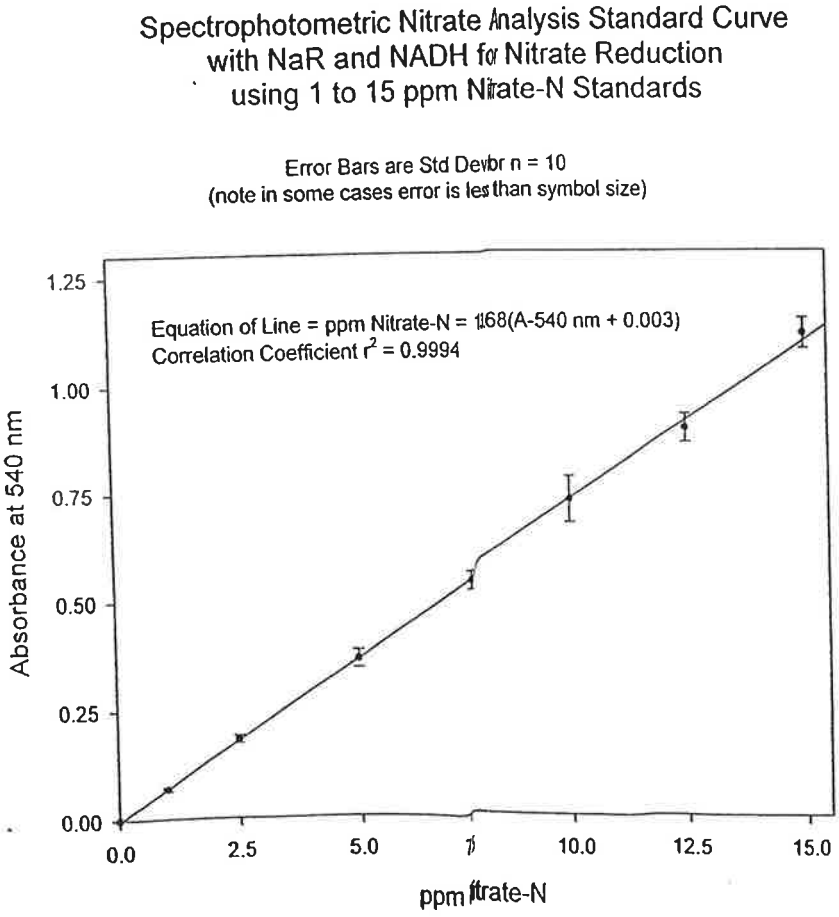


Figure 2. A typical nitrate-N standard curve generated by the NaR-based nitrate assay.