

A New Cadmium Reduction Device for the Microplate Determination of Nitrate in Water, Soil, Plant Tissue, and Physiological Fluids

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A reusable catalytic reductor consisting of 96 copperized-cadmium pins attached to a microplate lid was developed to simultaneously reduce nitrate (NO_3^-) to nitrite (NO_2^-) in all wells of a standard microplate. The resulting NO_2^- is analyzed colorimetrically by the Griess reaction using a microplate reader. Nitrate data from groundwater samples analyzed using the new device correlated well with data obtained by ion chromatography ($r^2 = 0.9959$). Soil and plant tissue samples previously analyzed for NO_3^- in an interlaboratory validation study sponsored by the Soil Science Society of America were also analyzed using the new technique. For the soil sample set, the data are shown to correlate well with the other methods used ($r^2 = 0.9976$). Plant data correlated less well, especially for samples containing low concentrations of NO_3^- . Reasons for these discrepancies are discussed, and new techniques to increase the accuracy of the analysis are explored. In addition, a method is presented for analyzing NO_3^- in physiological fluids (blood serum and urine) after matrix modification with Somogyi's reagent. A protocol for statistical validation of data when analyzing samples with complex matrixes is also established. The simplicity, adaptability, and low cost of the device indicate its potential for widespread application.

instrumentation, such as ion chromatography (IC), LC, and GC/MS, have found application in environmental NO_3^- monitoring studies and biomedical research (1, 4). Other commonly used techniques for direct NO_3^- measurement include colorimetric determination using reaction with chromotropic acid (CTA; 5), potentiometric analysis using nitrate-specific electrodes (6), and quantification by UV absorbance spectrometry (7, 8). Unfortunately, many of these techniques are severely compromised by common sample matrix components, such as excess salt, phosphate, colored pigments, soluble proteins, enzymes, humic substances, etc., precluding their use unless expensive and time-consuming cleanup procedures are used.

Currently, NO_3^- is most often determined indirectly as nitrite (NO_2^-) following chemical or enzymatic reduction using one of the many colorimetric Griess assays (9). The Griess reaction (10) is specific for NO_2^- , and is sensitive enough that many of the interfering matrix components mentioned above can simply be diluted out before beginning the analysis. The reduction process has long been combined with the colorimetric analysis using automated continuous-flow systems such as Technicon autoanalyzers (now Seal Analytical, Mequon, WI), and flow-injection analyzers. The reduction of NO_3^- to NO_2^- is accomplished using an in-line column usually packed with Zn or Cd metal, or Cd plated with Cu. Other chemical reduction methods using hydrazine, titanium chloride, or vanadium oxide, or enzymatic methods using nitrate reductase, are also widely used, either manually or with the automated systems (1).

An increasingly popular alternative to continuous-flow analytical systems involves the use of disposable 96-well microplates to perform scaled-down versions of various manual colorimetric methods. Calibration standards and samples are pipetted into microplate wells, then color reagents are added sequentially using multichannel pipets. After final color development, the optical densities (ODs) are determined using a computer-controlled microplate reader. The instrument software can then produce analytical results, with statistical analysis, in as little as 12 s. Several authors have adapted reduction techniques for analysis of NO_3^- as NO_2^- to the microplate reader format (11, 12). However, these techniques are often not well suited for use in laboratories performing routine

In agricultural, food, and environmental sciences, nitrate (NO_3^-) is one of the most important and frequently analyzed chemical species (1). There are hundreds of published procedures, beginning with the early work of Harper (2), who determined NO_3^- directly in soil extracts using the colorimetric phenoldisulfonic acid method. Later, Bremner and Keeney (3) made the determination by steam distillation after reduction to ammonium (NH_4^+) with Devarda's alloy. More recently, chromatographic techniques using sophisticated and costly

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analysis of NO_3^- in a variety of matrixes. They tend to require close attention to empirically derived protocol restrictions, and can produce undetected, erroneous results if the NO_3^- concentration in the sample exceeds a preselected range.

All these methods have to be thoroughly evaluated before reliable NO_3^- determinations can be made, and they are in no way universal in their application. Potential methods are usually validated either by comparing data sets obtained from different methodologies (e.g., colorimetry and chromatography), or by selecting a commercially available standard reference material (SRM) in a matrix similar to that of the experimental samples, and then performing the appropriate comparison studies. However, chromatographic techniques for NO_3^- analysis are especially prone to matrix interferences, and the biological instability of NO_3^- has prevented the National Institute of Standards and Technology from ever providing a certified SRM. Thus, no simple means exists for determining if a method is applicable for a specific study. Statistical validation techniques, such as the standard addition method (SAM) or sample spiking are often imprecise, time-consuming, and of dubious value when the composition of the matrix varies within a sample set. Nevertheless, these validation techniques are in general use and seem to be the best alternative available (13).

For many years, NO_3^- analysis remained largely the province of agricultural and environmental researchers. Minor problems with procedural validation were often ignored because experimental results could reasonably be expressed as relative differences in sample populations rather than as absolute NO_3^- values. However, with the discovery of the importance of nitric oxide (NO) in biological systems (14, 15), biomedical researchers are now focusing on the study of NO metabolites, specifically NO_3^- and NO_2^- , in physiological fluids (16, 17); thus, a need for more absolute accuracy in determining NO_3^- has become apparent. The correlation between in vivo NO activity and concentrations of NO_3^- and NO_2^- in blood serum and urine has been established (16), but studies using different analytical techniques to determine the basal concentrations in test subjects have led to widely divergent results and conflicting conclusions (18). Because of their versatility and excellent sensitivity, Griess reaction-based techniques are commonly used in these studies, but no definitive reduction technique has emerged. [A thorough review of the analytical procedures involved in determining NO_3^- as NO_2^- by the Griess reaction has been published by Tsikas (18).]

The increase in demand for NO_3^- analysis has led to the development of commercially available Griess reaction-based assay kits compatible with microplate reader systems to analyze NO_3^- in blood serum and urine (19). Although widely used for their convenience, these kits suffer from the same interferences with the reduction

process as other procedures and offer no guarantee of accuracy (18). To minimize these interferences, extensive, preanalytical sample matrix modification must be employed using procedures such as ultrafiltration or coprecipitation of interfering substances with either ZnSO_4 (20) or Somogyi's reagent ($\text{ZnSO}_4/\text{NaOH}$; 21).

The Somogyi's reagent cleanup procedure was found (in this work) to be a practical means for clarifying not only physiological fluid samples, but various highly-colored plant tissue extracts as well. The procedure involves adding stoichiometric concentrations of ZnSO_4 and NaOH to the sample to remove, by co-precipitation with $\text{Zn}(\text{OH})_2$, many interfering substances including soluble proteins, colored pigments, phosphate, ascorbate, sulfur-containing amino acids, and thiols (22, 23). After centrifuging, the supernatant contains a clear, neutral solution of Na_2SO_4 that does not interfere with the subsequent NO_3^- analysis.

This report introduces a simple, new technique for reducing NO_3^- to NO_2^- that is compatible with a microplate reader analytical system, but does not require purchase of an assay kit. A reusable, copperized-Cd reductor quantitatively converts NO_3^- to NO_2^- , simultaneously, in all wells of a standard 96-well microplate. The resulting NO_2^- is then determined colorimetrically at 542 nm using the Griess reaction. The procedure is much more efficient than conventional methods. No dedicated instrumentation or special expertise is required to perform the analysis; the reagents are easily prepared and stable, expenses are reduced by using disposable supplies, and minimal hazardous waste is generated. The reductor can be cleaned and reactivated immediately after completing a sample set, and it can be used to perform several hundred discrete NO_3^- analyses/day. Several thousand samples can be analyzed over the lifetime of the device.

Procedural validation can be accomplished by SAM following a protocol that allows the relationship of water standards to statistically derived sample concentrations for a subset of samples to be established in a single operation. The procedure has been applied to analysis of NO_3^- in agricultural and environmental samples, such as natural water and extracts of soil and plant tissue. In addition, a procedure for screening NO_3^- concentrations in equine physiological fluids (i.e., blood serum and urine) has been developed. Some modification in the general technique is required for each of these various analyses.

Experimental

Test Samples

Groundwater samples collected from lysimeters installed under agricultural test plots on the University of Kentucky (UK) Spindletop research farm in Lexington, KY, were used in procedural validation studies. These

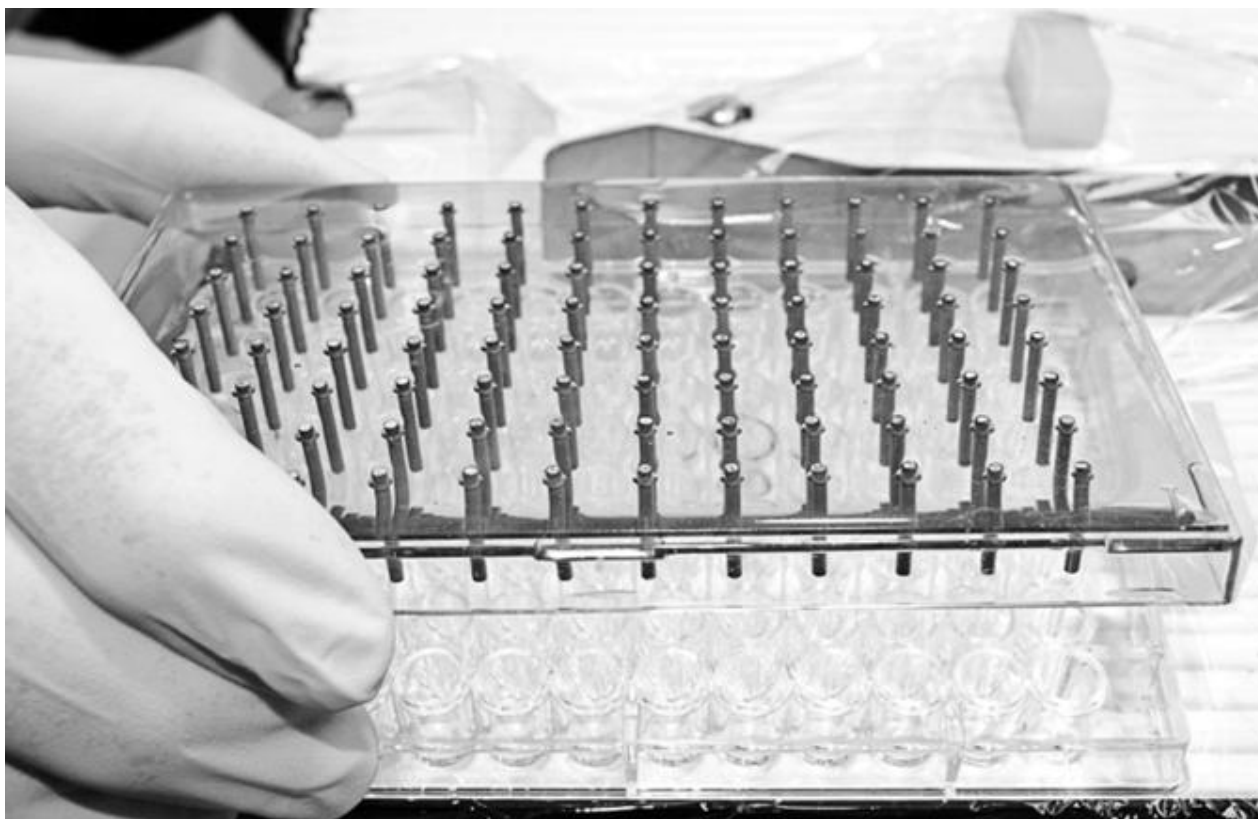


Figure 1. Nitrate reductor consisting of 96 copperized-Cd pins attached to a microplate lid.

samples were filtered through 0.45 μm syringe filters (Osmonics Inc., Fisher Scientific; Pittsburgh, PA) into sterilized bottles and refrigerated.

Soil and plant tissue samples, previously analyzed for NO_3^- by laboratories participating in the 2007 Soil Science Society of America (SSSA) North American Proficiency Testing (NAPT) program were supplied by the UK College of Agriculture, Division of Regulatory Services. These samples had been stored at room temperature in sealed containers for several months and were analyzed as received. Other types of dried plant tissue (i.e., corn stover and tobacco), obtained from on-going agronomic research projects within the UK Experiment Station, were used to evaluate matrix effects on the technique.

Frozen serum samples originally taken from mares located on the UK Maine Chance research farm in Lexington, KY, and fresh serum and urine from horses under treatment by UK veterinarians were used to develop the method for analysis of physiological fluids. These samples were preserved by refrigeration prior to analysis.

Reductor Fabrication

A catalytic reductor consisting of an 8×12 array of Cd wire pins was fabricated to match the dimensions of a 96-well microplate. The prototype (Figure 1) was constructed by first drilling holes on 9 mm centers in a standard polystyrene microplate lid (ICN, Costa Mesa,

CA), then positioning pieces of 1.59 mm Cd wire (Kapp Alloy, Oil City, PA), cut to identical lengths, into the holes so that when the lid was fit onto a standard-depth microplate, the wires would not touch the optical surface at the bottom. Finally, the back was sealed with epoxy resin (Devcon, Danvers, MA) to hold the wires in place.

Reagents

Chemicals used to prepare reagents were purchased from Fisher Scientific and used as received. Solutions were prepared on a w/v basis unless otherwise noted. Deionized (DI) water was prepared by passing reverse-osmosis purified water through a mixed-bed resin tank (Culligan, Rosemont, IL), then through two additional Model D8911 and D8922 cleanup cartridges (Fisher Scientific) to remove organics. Stock solutions of certified 1000 mg/L NO_3^- -N and NO_2^- -N were purchased to prepare calibration standards (AS-N03N9-2Y and AS-N02N9-2Y; Fisher Scientific). Somogyi's reagent was prepared in two separate parts: a 0.300 M NaOH solution and a 5.00% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ solution, both in DI water. (A plastic flask was used to contain the Zn reagent to eliminate potential Zn interaction with glass.) A 1.0% NH_4Cl buffer in DI water was prepared and adjusted to pH 8.5 with concentrated NH_4OH . A separate 1.0% NH_4Cl buffer was prepared and adjusted to pH 10. These solutions are indefinitely stable at room temperature.

A Griess reagent stock solution (A) of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED) was prepared in DI water. This solution is stable for several weeks if refrigerated. A Griess reagent stock solution (B) containing 1.0% sulfanilamide in 3 M HCl was prepared for use with the pH 8.5 buffer system. A Griess reagent stock solution (C) containing 1.0% sulfanilamide in 6 M HCl was prepared for use with the pH 10 buffer system. These two solutions are also indefinitely stable at room temperature. Griess reagent (24) was prepared immediately before use by combining reagent A with reagent B or C in equal volumes as appropriate. For selected applications, it is advisable to dissolve a further quantity of sulfanilamide, ranging from 200 to 500 mg, in 10 mL combined reagent. This serves to decrease postreduction matrix effects, improve sensitivity, and increase the linear range of the calibration curve. These concentrations result in sulfanilamide/NED ratios of from 50/1 to 110/1 rather than the commonly used 10/1 ratio as prepared above.

Instrumentation and Supplies

A Molecular Devices VERSAmax microplate reader interfaced with a Gateway PC (Molecular Devices Corp., Sunnyvale, CA) was used to make the colorimetric determinations. Calibration standards and standard addition solutions were prepared using a digital dilutor (Hamilton Microlab 500; Fisher Scientific). Sample preparation was facilitated by using racks of disposable 1.2 mL polypropylene cluster tubes (Corning/Costar; Fisher Scientific). A centrifuge (Eppendorf Model 5810 with A-2-DWP head; Fisher Scientific) was used with the cluster-tube rack assembly to prepare samples for analysis in lieu of filtration.

Reductor Activation

For the initial activation, each new reductor was first submerged in a 10 $\mu\text{g/mL}$ solution of CuSO_4 in 1.0 M HCl contained in a sealable, plastic box placed inside an ultrasonic cleaner (Branson 2510; Fisher Scientific) and sonicated for 2 min. The reductor was then rinsed with DI water, blotted dry, and placed in a holding trough (Nunc omnitray; Fisher Scientific) containing pH 8.5 buffer. A microplate containing 20 μL of a 200 $\mu\text{g/mL}$ NO_3^- -N solution and 200 μL pH 8.5 buffer in each well was then prepared and positioned on a titer-plate shaker (Lab-Line Instruments; Fisher Scientific) over a sheet of self-sealing plastic film (Reynolds film; Fisher Scientific). The reductor was removed from the holding trough, blotted on a paper towel, and immediately fit onto the microplate. The plastic film was then folded over and sealed to exclude air. The whole assembly was then shaken for 60 min. After shaking, the reductor was thoroughly rinsed with DI water, and the process was repeated (the CuSO_4 in the

acid cleaning solution was replenished). After activation, the reductor was blotted dry and stored in a desiccator.

Calibration Check

To establish the level of precision for each individual device, a calibration check was performed by analyzing aliquots of the same sample using the basic technique described as follows: The reductor was sonicated briefly (approximately 60 s) in the Cu-HCl solution to remove surface oxidation, rinsed with DI water, blotted dry, and placed in a holding trough containing pH 8.5 buffer. A 5 $\mu\text{g/mL}$ NO_3^- -N standard was prepared, and 20 μL aliquots were pipetted into each microplate well; 200 μL pH 8.5 buffer was added, and the reduction process was performed using the reductor in the manner described above. After a 60 min reduction period, 60 μL Griess reagent (A+B) was pipetted into each well, and the microplate was shaken for 5 min to allow color development. Finally, OD values were determined on the microplate reader at 542 nm. If necessary, the activation process and the calibration check were repeated. When all OD values were determined to be within the pipetting error ($\pm 3\%$ of the mean), the device was placed in service.

Analysis of Water Samples or KCl Extracts of Soil

A microplate containing prediluted samples and calibration standards was prepared. Aliquots of 20 μL were normally used to produce a linear calibration curve in the range 0–5 $\mu\text{g/mL}$ NO_3^- -N. (The scale can be adjusted, as needed, by changing the aliquot volumes.) The analysis was performed as previously described using 200 μL aliquots of pH 8.5 buffer in each well. A reduction time of 45 to 60 min was sufficient for most water samples and soil extracts.

The 2007 NAFT program samples used in the soil NO_3^- validation study (Table 1) were extracted with 1.0 M KCl (5 g/25 mL) for 1 h on a Eberbach Model 6000 reciprocal shaker (Eberbach Corp., Ann Arbor, MI), allowed to settle, pipetted into cluster tubes (Fisher Scientific; Part No. 07-200-317), and centrifuged at maximum speed, 3700 rpm ($380 \times g$), for 20 min prior to analysis. Calibration standards were prepared in the KCl extraction solution in the range 0–5 $\mu\text{g/mL}$ NO_3^- -N. The samples were diluted as necessary, and the analysis was performed using 20 μL aliquots of samples and standards with a 60 min reduction time.

Analysis of Plant Tissue Extracts

The reductor was prepared as before using pH 8.5 buffer in the holding trough. An appropriate standard range was selected, and the analytical process described previously was repeated using the pH 10 buffer. The Griess reagent made specifically for this buffer system

Table 1. Comparison of soil NO₃⁻ data obtained using the new method with 2007 NAPT program data^a

Sample	Soil series	State	Cd Rd ^b <i>n</i> = 71	ISE ^c <i>n</i> = 19	CTA ^d <i>n</i> = 5	IC ^e <i>n</i> = 2	Weighted mean <i>n</i> = 97	New method	SAM ^f calculated value
2007-101	Quincy	OR	23.0	22.2	23.6	24.3	22.9	22.8	
2007-102	Green Canyon	UT	9.50	9.40	9.60	16.0	9.60	9.58	
2007-103	Whitaker	IN	140	122	136	137	136	140	
2007-104	Cache Junction	UT	80.7	81.7	87.5	91.8	81.5	90.3	
2007-105	Blue Weeks	UT	9.13	8.44	9.90	9.77	9.18	9.19	9.19
			<i>n</i> = 63	<i>n</i> = 18	<i>n</i> = 5	<i>n</i> = 2	<i>n</i> = 88		
2007-106	Orono	ME	19.0	16.3	21.8	20.4	18.6	17.5	
2007-107	Warden	MI	11.2	11.0	12.2	10.9	11.2	9.95	8.81
2007-108	Morrow	OR	24.0	22.3	22.8	24.1	23.6	23.7	
2007-109	South Weeks	UT	6.65	11.0	8.60	6.60	7.65	6.62	
2007-110	Cache Junction	UT	10.7	14.3	12.0	12.6	11.6	11.2	
			<i>n</i> = 55	<i>n</i> = 21	<i>n</i> = 5	<i>n</i> = 2	<i>n</i> = 83		
2007-111	Gilford	IL	16.2	19.2	16.0	14.7	16.9	15.8	
2007-112	Black Weeks	UT	4.76	5.00	6.00	5.28	4.91	5.24	
2007-113	N. Logan	UT	3.67	6.90	4.80	4.06	4.56	3.40	
2007-114	Horneck	OR	24.5	24.2	23.8	22.2	24.3	22.1	
2007-115	Immoklee	FL	6.00	6.30	6.21	5.33	6.07	5.51	4.29
			<i>n</i> = 51	<i>n</i> = 17	<i>n</i> = 5	<i>n</i> = 1	<i>n</i> = 74		
2007-116	Hermiston	OR	24.0	22.3	23.3	21.9	23.5	23.3	
2007-117	Andres	IL	13.8	11.1	14.6	12.8	13.2	13.5	13.2
2007-118	Randall	IN	10.7	11.2	11.6	9.29	10.9	9.72	9.50
2007-119	Wasatch	UT	43.1	37.2	43.6	45.5	41.8	41.5	
2007-120	Nibley	UT	8.60	8.40	9.25	8.12	8.59	8.39	8.12

^a Data presented as mg N/kg soil as received.

^b Cadmium reduction.

^c Ion-specific electrode.

^d Chromotropic acid.

^e Ion chromatography.

^f Standard addition method.

(A+C), and containing excess sulfanilamide, was used to achieve proper acidity for color development. When analyzing highly colored extracts of plant material, the technique described below for physiological fluids, using Somogyi's reagent, was used to decolorize the samples prior to analysis. The optimum reduction time for various plant species was determined empirically.

The 2007 NAPT program samples used in the plant NO₃⁻ validation study (Table 2) were extracted by shaking with DI water (500 mg/10 mL) for 60 min, diluted as necessary, and decolorized by reaction with Somogyi's reagent (*see below*) at volume ratios of two parts sample (300 µL): 1 part NaOH (150 µL): 1 part ZnSO₄ (150 µL). Cluster tubes were used to expedite

the process. After centrifuging, the NO₃⁻ analysis was performed using the pH 10 buffer system with 20 µL aliquots of samples and standards (0–5 µg/mL NO₃⁻-N), 200 mg excess sulfanilamide/10 mL Griess reagent, and a 90 min reduction time.

Analysis of Physiological Fluids Using Cluster Tubes

To analyze NO₃⁻ in blood serum or prediluted urine, the samples were first clarified using Somogyi's reagent as follows: two parts (300 µL) 0.300 M NaOH was pipetted into each cluster tube; then one part (150 µL) sample was pipetted directly into the NaOH. (A more complete

transfer was made by drawing the sample plus NaOH back into the pipet tip and expelling it at least twice. This also served to mix the sample with the reagent.) Next, two parts (300 μL) 5.00% ZnSO_4 solution were added to each sample using a single-channel pipet, and the cluster tube was immediately vortexed. (If this reagent was added to multiple samples before vortexing, precision was decreased.) After cooling for approximately 10 min in an ice bath, the cluster tube rack assembly was centrifuged for 20 min at maximum speed. Calibration standards in DI water were prepared according to the range selected, and the analysis was performed as before using the pH 10 buffer and a 60 min reduction time. Griess reagent (A+C) with added sulfanilamide (200 mg/10 mL) was used for the final color development.

The equine physiological fluid samples under study were first clarified with Somogyi's reagent, establishing a dilution factor of five, and then analyzed as follows using DI water standards in the range 0–2 $\mu\text{g/mL}$ NO_3^- -N (i.e., 0–143 μM): 50 μL aliquots of standards and samples were pipetted, in triplicate, into a microplate; then 170 μL of pH 10 buffer was added, and the analysis was performed as previously described.

LOD

The LOD for this method in a DI water matrix was calculated using the SD of 32 blank determinations and the t -value appropriate for a 99% confidence level (25). The test was performed using data generated with all sample-processing steps included.

SAM Validation Protocol

To assess the absolute accuracy of the method, a simple single-microplate validation protocol using the SAM (26) was devised. Six samples were selected from a sample set and analyzed in triplicate following addition of aliquots of the blank and three NO_3^- standard solutions of appropriate concentrations. The analyte configuration in the microplate consisted of a blank and seven calibration standards prepared in DI water in three rows, with the remaining wells divided into six blocks of 12 to contain the 3×4 arrays of SAM test aliquots. For soil samples, the additions were made pre-extraction, and the analysis was performed in the conventional manner. However, with plant tissue extracts and physiological fluid samples, NO_3^- interaction with the sample matrix is possible, and the SAM test was performed after the Somogyi's reagent cleanup procedure using the following technique: 50 μL sample aliquots were pipetted into the microplate wells of the 3×4 arrays for each of the six samples; triplicate 50 μL aliquots blank and three low-range water standards were then added to the appropriate wells. The volume in each well containing the calibration standards was then adjusted to 100 μL with DI water, and the analysis

performed as before using 120 μL aliquots of pH 10 buffer.

This scheme enabled simultaneous collection of data showing the relationship between the data calculated conventionally from the standard curve (using the 0 added NO_3^- values) and the data obtained from the SAM protocol (after subtraction of the blank). Also, sample-spiking data can be obtained using this technique.

Results and Discussion

To evaluate the utility of the proposed reduction procedure, comparison studies were performed using sets of NO_3^- samples in increasingly complex matrixes.

Determination of NO_3^- in Pure Solutions

To test the baseline efficiency of the reductor, identical sets of certified NO_3^- (x) and NO_2^- (y) standards ranging from 0 to 1 $\mu\text{g/mL}$ N were prepared in DI water and analyzed in triplicate using the basic procedure with the pH 8.5 buffer system and a 1 h reduction time. A comparison of the data showed a close statistical correlation:

$$y = 1.0207x + 0.0017 \quad (r^2 = 0.9998)$$

When working with pure solutions, the procedure can be tuned to achieve near 100% conversion of NO_3^- to NO_2^- by merely establishing a sufficient reduction time. The LOD was experimentally determined to be 3.588 $\mu\text{g/L}$ NO_3^- -N. The usefulness of this calculation, however, is limited when applied to samples with more complex matrixes. The reactivity of NO_2^- at low pH can interfere with the Griess reaction chemistry (see discussion below), leading to false-negative results, and thus, making the calculation of the LOD problematic.

Determination of NO_3^- in Natural Water

As sample matrixes increase in complexity, the exact relationship between standards and samples becomes less certain, and the absolute accuracy of the data comes into question. For a simple matrix, such as groundwater, IC techniques (e.g., U.S. Environmental Protection Agency Method 300) are widely considered to be the "gold standard" for accurate NO_3^- measurement, and can be used with confidence in validation studies to quantify matrix effects on the test procedure. A set of 32 lysimeter samples was analyzed by IC and the new method (data not shown). The autoanalyzer method previously used in the laboratory, which employed an in-line Cu-Cd reduction column, was also included in the study. The same NO_3^- standards prepared in DI water were used to calibrate all three analyses. A Student's t -test indicated no difference in the data means at the 0.05 level when comparing IC (x) with the new method (y):

$$y = 1.0171x - 0.0046 \quad (r^2 = 0.9959)$$

The sum of the autoanalyzer method data exhibited a low bias of approximately 6% when compared to the sums of both other methods, thereby providing a measure of the matrix effect on the accuracy of the column-reduction method.

Determination of NO_3^- in Soil Extracts

When analyzing samples with more complex matrixes (e.g., KCl extracts of soil), independent confirmation techniques, such as IC, become problematic. An alternative means for evaluating a prospective method is to participate in a collaborative study with other laboratories. The SSSA distributes a series of soil and plant tissue samples on a quarterly basis to Regulatory Service Laboratories throughout the country as part of the NAPT program. These samples are analyzed by many laboratories using a variety of methods, and the collated data are then made available to participating analysts as a QC check. The NAPT program NO_3^- data for the 2007 set of 20 soil samples and the new method NO_3^- data are compared in Table 1. (The NAPT program data are median values rather than mean values to mitigate the influence of extreme outliers.) Most of the participating laboratories used Cd reduction methods (Cd Rd. column), but ion-specific electrode (ISE), CTA, and IC techniques

were also used. The values are generally consistent for most laboratories and show that use of the new method should provide acceptably precise NO_3^- data for a wide variety of soils. A Student's *t*-test indicated no difference in the data means at the 0.05 level when comparing NAPT program weighted mean data (*x*) with the new method data (*y*):

$$y = 1.054x - 1.180 \quad (r^2 = 0.9976)$$

To assess the accuracy of the comparison data, a blank and three standard addition solutions of appropriate NO_3^- -N concentrations were prepared in 1.0 M KCl and used to extract replicates of a geographically diverse subset of six of the 20 soil samples. The SAM validation protocol was employed as described previously using 20 μL aliquots of standards and SAM solutions. The data shown in the last column of Table 1 are the calculated values corresponding to the negative intercepts of the six individual SAM curves (not shown), and are assumed to represent the most accurate values generated by statistical methods. The SAM data (*x*) are in good agreement with data using the new method (*y*):

$$y = 0.904x + 1.394 \quad (r^2 = 0.9737)$$

Table 2. Comparison of plant NO_3^- data using the new method with 2007 NAPT program data^a

Sample	Plant species	Cd Rd ^b	ISE ^c	Other	New method	SAM ^d calculated value
		<i>n</i> = 31	<i>n</i> = 7	<i>n</i> = 4		
2007-201	Avocado	27.0	134	34.3	11.3	11.9
2007-202	Grape petiole	507	798	506	554	685
2007-203	Organic basil	5298	6112	5142	5509	
		<i>n</i> = 23	<i>n</i> = 9	<i>n</i> = 6		
2007-204	Grape petiole	522	725	627	508	
2007-205	Wheat	30.0	95.0	68.6	2.27	5.23
2007-206	Lettuce	4935	5620	3925	5018	
		<i>n</i> = 20	<i>n</i> = 9	<i>n</i> = 6		
2007-207	Potato	231	318	396	198	263
2007-208	Citrus	77.4	180	500	62.9	67.2
2007-209	Grape petiole	487	726	488	511	
		<i>n</i> = 22	<i>n</i> = 9	<i>n</i> = 4		
2007-210	Blueberry	37.0	115	392	3.20	3.52
2007-211	Grape petiole	495	756	621	506	
2007-212	Safflower	1297	1690	1340	1444	

^a Data presented as mg N/kg plant tissue as received.

^b Cadmium reduction.

^c Ion-selective electrode.

^d Standard addition method.

Determination of NO₃⁻ in Plant Tissue Extracts

The NAPT program NO₃⁻ data for the 2007 set of 12 plant tissue samples and the new method NO₃⁻ data are compared in Table 2. Both buffer systems were tried, but in every case the pH 10 buffer gave higher values. This buffer dissolves Cd from the reductor at a much faster rate and probably maintains the reactive surface area to a greater extent than does the pH 8.5 buffer. The higher pH also stabilizes NO₂⁻ as it is formed by minimizing side reactions that can occur at lower pH (27, 28). Again, Cd reduction was the most widely used technique, with some laboratories using ISE and a few using other unspecified methods. The data illustrate the level of uncertainty that is inherent in plant tissue analysis. Those samples containing higher levels of NO₃⁻ were most comparable by the different methods, but as the concentration of NO₃⁻ decreased, matrix interferences could no longer be diluted out, and the comparison data began to diverge. The samples analyzed by the new method were, in most cases, comparable to the values in the Cd Rd. column, but less so with the other techniques. However, the values for the three lowest samples (avocado, wheat, and blueberry) were considerably lower with the new method.

The SAM validation protocol was applied to six of the plant tissue samples. The three samples mentioned above were included to discover the source of discrepancy in the comparison data. When the standard additions were included in the extraction solutions (as with the soil samples), interactions with matrix components of the plant tissue obviously occurred at some point in the analytical process, and little useful data resulted. However, when the NO₃⁻ standard additions were added after pretreatment of the samples with Somogyi's reagent, better recoveries were obtained. The calculated SAM-NO₃⁻ values are shown in the last column of Table 2. Assuming these values are more nearly correct, the new method data appear to be more accurate than the NAPT program data, although the possibility that the sample NO₃⁻ concentrations changed during storage cannot be disregarded.

The blueberry and wheat samples were especially interesting and probably represented a worst-case scenario for plant tissue NO₃⁻ analysis. In the preliminary SAM test using the pH 8.5 buffer system, only about 2/3 of the added NO₃⁻ was recovered from the blueberry sample extract using a 60 min reduction time. The pH 10 buffer system and increased reduction times were required to produce approximately 90% recovery of added NO₃⁻. The wheat sample presented a different challenge. Less than 90% recovery of added NO₃⁻ was achieved, even with longer reduction times and the pH 10 buffer. Plant tissue samples of this type contain extractable, complex carbohydrates that are not removed by the Somogyi reagent. These compounds have been hypothesized to attach to the metal surface during the reduction process,

resulting in a decrease in efficiency of the reduction of NO₃⁻ to NO₂⁻ relative to the calibration standards (9). Other, more dilute plant tissue extracts (i.e., corn stover and dark tobacco) containing higher levels of NO₃⁻ produced nearly identical results using either the pH 8.5 buffer after cleanup with Somogyi's reagent or without cleanup using the pH 10 buffer (data not shown). The implication is that each type of sample matrix may be different in its response to the analytical process, and preliminary testing using statistical methods is essential before the most effective protocol for determining NO₃⁻ can be selected.

Determination of NO₃⁻ in Physiological Fluids

The equine physiological fluids used in this study presented a particularly difficult sample matrix. In most cases the NO₃⁻ levels were extremely low, making it necessary to minimize sample dilution. Also, blood serum and urine typically contain large molecules (soluble proteins, hemoglobin, enzymes, lipids, etc.) that physically and chemically interfere with the reduction process, and additional substances that may interfere with the colorimetric assay (18). Ultrafiltration is commonly used to remove proteins and other large molecules, but it may not filter out certain amino acids, enzymes, or other interfering compounds of lower MW. Some researchers have used the classic techniques of acid deproteinization with mineral acids or trichloroacetic acid, but if NO₂⁻ is present in the sample and NO₃⁻ is to be calculated by difference, these methods are not reliable (18). A widely recommended method (20) that uses only a more concentrated ZnSO₄ solution to clarify the samples was found to cause excessive formation of bubbles in the microplate wells and offered no advantage. The use of the original Somogyi method (21) at a dilution ratio of 1/5, along with the pH 10 buffer system, was found to give the most reproducible results.

Because the NO₃⁻ content of equine physiological fluid has not been studied extensively, no context for data comparison exists. Attempts to analyze split samples by different techniques, such as ISE and commercially available kits, produced unacceptably imprecise data. Therefore, reliance on statistical methods was the only option available for procedural validation. The SAM protocol was employed as previously described using six randomly selected mare serum samples (obviously hemolytic samples were excluded). The resulting NO₃⁻ data are presented graphically in Figure 2. A comparison of the data generated by the SAM analysis (*y*) versus data relative to water standards (*x*) is shown in Figure 3. The calculated linear regression equation was:

$$y = 1.0913x + 0.8219 \quad (r^2 = 0.9969)$$

One of the samples (mare 45 in Figure 2) was an obvious outlier and was not included in the regression

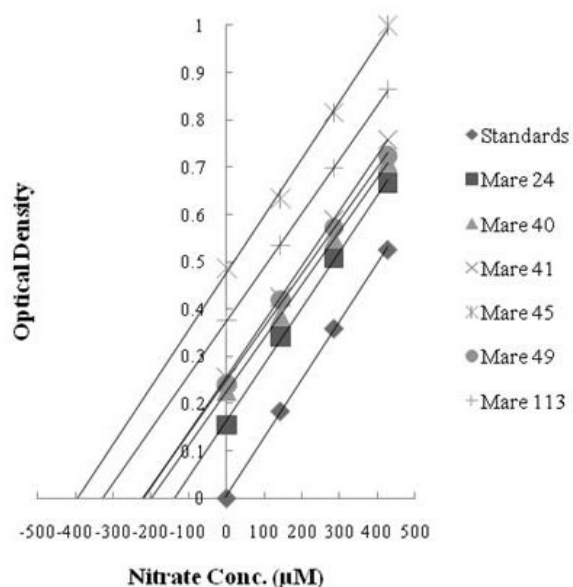


Figure 2. Determination of NO_3^- in blood serum by the SAM for six mares on pasture.

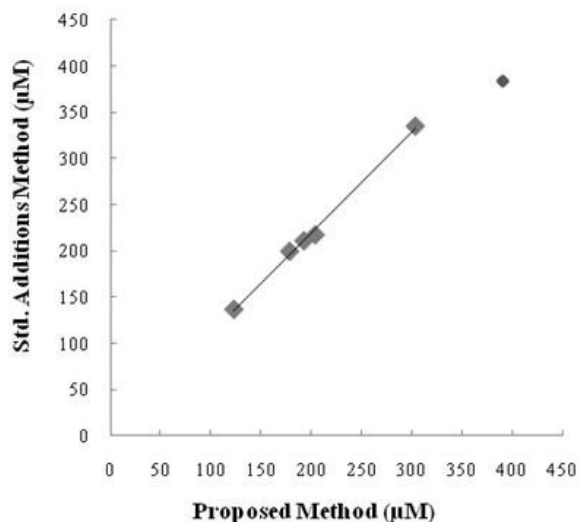


Figure 3. Comparison of NO_3^- data calculated using water standards with statistically derived data using the SAM. Unfit datum is for mare 45.

calculation. The water standard data for the five mares averaged 91.2% (CV = 1.95%) of the calculated SAM data, indicating a fairly stable matrix effect for this sample population. The outlier sample, however, showed no matrix effect at all. An examination of the SAM curve for this sample illustrates the unresolved limitations of the SAM analysis. The 0 addition point caused most of the deviation from linearity and was proven to be inaccurate upon reanalysis. Thus, the seeming lack of matrix effect may be explained by a single, imprecise

data point. Attempts to reduce the matrix effect by further purification of the equine serum samples using graphitized carbon black to remove steroids and other organics (29) showed promise, but require further study.

Matrix Effects on the Griess Reaction

The primary focus of this paper is on the reduction of NO_3^- to NO_2^- ; however, when evaluating the method, the possibility of error associated with the Griess reaction chemistry, after the reduction process has been completed, must be considered. Ideally, NO_2^- reacts first with acidified sulfanilamide, forming a diazonium cation that then reacts with NED to form the desired azo dye. However, at low pH, NO_2^- will react indiscriminately with many matrix components (27), resulting in a decreased color response relative to the calibration standards. The probability of this type of interference increases with the complexity of the sample matrix (18). Quantifying this interference is possible by performing the SAM protocol using NO_2^- standards and standard additions instead of NO_3^- . This extra test may be performed on the same prepared subset of samples. Interferences with the Griess reaction were observed with the avocado and blueberry plant tissue extracts. These extracts turned bright yellow upon addition of NaOH during the Somogyi's reagent cleanup procedure, indicating the possible presence of highly reactive polyphenols (28). Adding excess sulfanilamide to the mixed Griess reagent was intended to reduce the effect of competing reactants by simple mass action, but the 50/1 sulfanilamide/NED ratio (i.e., 200 mg/10 mL of mixed reagent) was not sufficient for these particular samples. When the sulfanilamide/NED ratio was increased to 110/1 (i.e., 500 mg/10 mL of mixed reagent), the interference with both samples was eliminated. No postreduction interference of this type was observed with the mare serum samples after treatment with Somogyi's reagent. The decreased response for these samples was, therefore, presumed to be caused by interference with the reduction process or lack of precision in the analysis.

Conclusions

The catalytic reduction system described in this paper is unique in that it enables 96 discrete NO_3^- analyses to be performed simultaneously, with the sample matrix having no interaction with the calibration standards. The relationship between standards and samples can, therefore, be determined more accurately than with conventional column-reduction systems in which detector response may be influenced by continuous column degradation. Using a combination of preanalytical matrix modification with Somogyi's reagent, higher pH during the reduction process, an increase in sulfanilamide concentration during color development, and application of the SAM protocol, it should be possible to make more accurate

NO₃⁻ measurements in a variety of complex matrixes, including plant tissue extracts and physiological fluids.

Also, the technique does not suffer from the time constraints imposed on the reduction process by various automated column-reduction systems. The reduction time can be extended as needed to effect near complete conversion of NO₃⁻ to NO₂⁻. The common assumption that the NO₃⁻ reduction rate of calibration standards and samples is identical in column-reduction systems, and that it is unnecessary to accomplish complete reduction, probably applies only to the simplest matrixes. This assumption can lead to significant error, especially when appreciable levels of NO₂⁻ are present in the sample.

The data presented in this paper were obtained using reductors made with pure Cd wire. However, a reductor made with 95% Cd and 5% Ag wire, although more expensive, was also found to give reproducible results. Because this alloy is much harder than Cd wire, it is easier to work with and may prove to be a better alternative (30).

Safety and Waste Disposal

With continuous use, the acid cleaning solution will become saturated with Cd, and due caution should be taken to avoid skin contact. The waste Cd generated with this technique is small, but it is still advisable to empty the used microplates and spent cleaning solution into a suitable container and dispose of it as heavy metal-contaminated waste.

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