Committee D19 0n Water
Subcommittee D19.05 on Methods for Analysis of Inorganic Substances in Water

Research Report

Supporting Data for D7781 Test Methods For Nitrite-Nitrate in Water by Nitrate Reductase

Table of Contents

PURPOSE 3

SCOPE AND APPLICABILITY 4

REACTION CHEMISTRY 4

SELECTIVITY 7

SAMPLE PRESERVATION AND STORAGE 12

INTERFERENCES 14

INSTRUMENT CALIBRATION 17

CALIBRATION VERIFICATION 19

REDUCTION EFFICIENCY 20

INITIAL DEMONSTRATION OF LABORATORY CAPABILITY (IDC) 21

DETERMINATION OF THE METHOD DETECTION LIMIT (MDL) 23

PRECISION 25

DRINKING WATER VALIDATION 25
WASTEWATER VALIDATION 26

BIAS 29

PREPARATION OF LABORATORY FORTIFIED MATRICES 29
DRINKING WATER SPIKE AND SPIKE DUPLICATE RECOVERIES 30
WASTEWATER SPIKE AND SPIKE DUPLICATE RECOVERIES 31

PARTICIPATING LABORATORIES 33

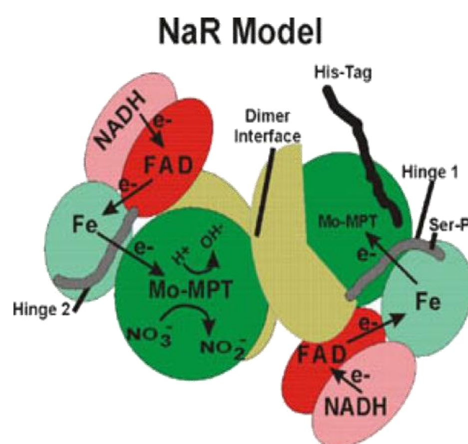
Purpose

This research report describes the validation of a new method for the determination of nitrate plus nitrite nitrogen in drinking water and in wastewater. The intent of the new method is SDWA and CWA compliance reporting. Nitrate is regulated at 10 mg/L in drinking water. Nitrate is measured as part of the nutrient removal component during wastewater treatment and is measured as an essential nutrient in ambient water criteria. There are several existing EPA approved methods used for the determination of nitrate and nitrate plus nitrite nitrogen. For instance, EPA 300.0 determines nitrate by ion chromatography (IC). While IC is certainly a “green” method, a typical IC analysis may take up to 12 minutes per sample. This new ASTM method determines nitrate using discrete analyzers capable of very fast determinations equaling or exceeding 60 determinations per hour. Other method for nitrate plus nitrite, such as EPA 353.2 and EPA 353.1 determine nitrite after a reduction of nitrate to nitrite using cadmium metal or hydrazine respectively. This new method, ASTM D7781 reduces nitrate to nitrite using an environmentally benign enzyme making the new ASTM method a “green” chemistry.

Scope and Applicability

Enzymes are proteins produced by living organisms that function as biochemical catalysts. They act by bringing reactants closer together allowing reactions to occur at a faster rate than if the enzyme were not there. The well-defined shape of an enzyme (Figure 1) makes the reaction more specific than most other catalysts. Most enzymatic reactions occur under narrow pH and temperature ranges.

Figure 1 Nitrate Reductase Enzyme

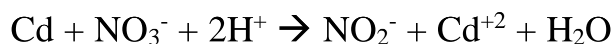


Nitrate reductase is an enzyme derived from upper level plants that together with the natural reducing agent NADH specifically catalyze the reduction of nitrate to nitrite nitrogen[1]. This enzymatic reaction can be utilized in conjunction with the sensitive Greiss colorimetric reaction to quantitatively measure nitrate + nitrite in environmental samples. Nitrate reductase represents an environmentally friendly, cost-effective alternative to methods employing cadmium metal as a reduction catalyst.

Reaction Chemistry

Time honored methods for the determination of Nitrate + Nitrite Nitrogen employ granular cadmium columns, or tubular cadmium coils reducing NO_3-N to NO_2-N by the following equation

[1] http://www.nitrate.com (accessed January 6, 2009)



$$Cd + NO_3^- + 2H^+ \rightarrow NO_2^- + Cd^{+2} + H_2O$$

Although the cadmium catalyst is a solid, the cadmium ion formed as a result of the reduction of nitrate is soluble and becomes part of the waste stream. Dissolved oxygen, always present even in degassed reagents, can exceed the nitrate concentration by 100 to 1000 times. The reaction of cadmium metal with dissolved oxygen proceeds at a rate 30 times faster than the reduction of nitrate to nitrite. Also, because hydrogen ions are consumed in the reduction reaction the pH increases during reduction.[2] Without proper degassing of reagents the amount of cadmium in the waste solution can be as high as 200 ppm. Even with degassed reagents the dissolution of cadmium by oxygen cannot be prevented[3]. Considering that cadmium is a TCLP target analyte it makes sense to measure nitrate with nitrate reductase.

Nitrate Reductase is an environmentally benign, commercially available reagent that can be used to reduce nitrate nitrogen to nitrite for routine colorimetric determination of nitrate plus nitrite nitrogen in environmental samples. Nitrate reductase is a “green” chemistry, and overcomes various interferences experienced when using cadmium.

Nitrate Reductase reacts according to the following equation

$$NO_3^- + NADH + H^+ \xrightarrow{NaR} NO_2^- + NAD^+ + H_2O$$

Extensive testing has demonstrated equivalency between enzymatic reduction and cadmium reduction on a wide variety of matrices including ground water, surface water, drinking water, municipal wastewater, industrial effluents, agricultural soil extracts, and seawater. The method is especially suited for complete automation by discrete analyzers; however, off-line semi-automated batch reduction followed by continuous flow or manual methods may be carried out as well.

---

[2] Patton, C.J. *Design, Characterization, and Applications of a Miniature Continuous Flow Analysis System,* PhD Dissertation, Michigan State University, 1982, p 93.

[3] Gal, Frenzel, and Moller, *Re-examination of the cadmium Reduction Method and Optimization of the Conditions for the Determination of Nitrate by Flow Injection Analysis*, Microchim Acta 146, 155-164, 2004

This new ASTM nitrate method is a discrete analyzer method. Discrete analyzers are defined by the standard as " a programmable, computer-controlled instrument that automates wet- chemical analysis by using one or more robotic arms interfaced to high-precision volumetric dispensers to aspirate and dispense samples, standards, diluents and reagents. ".

Analytical methods using nitrate reductase have been previously reported,[4,5,6] however, these investigators limited testing to surface and ground waters without evaluating complex matrices such as wastewater.

[4] Campbell WH, Kinnunen-Skidmore T, Campbell ER, *New and Improved Nitrate Reductase for Enzymatic Nitrate Analysis,* American Laboratory, September 2004, 12

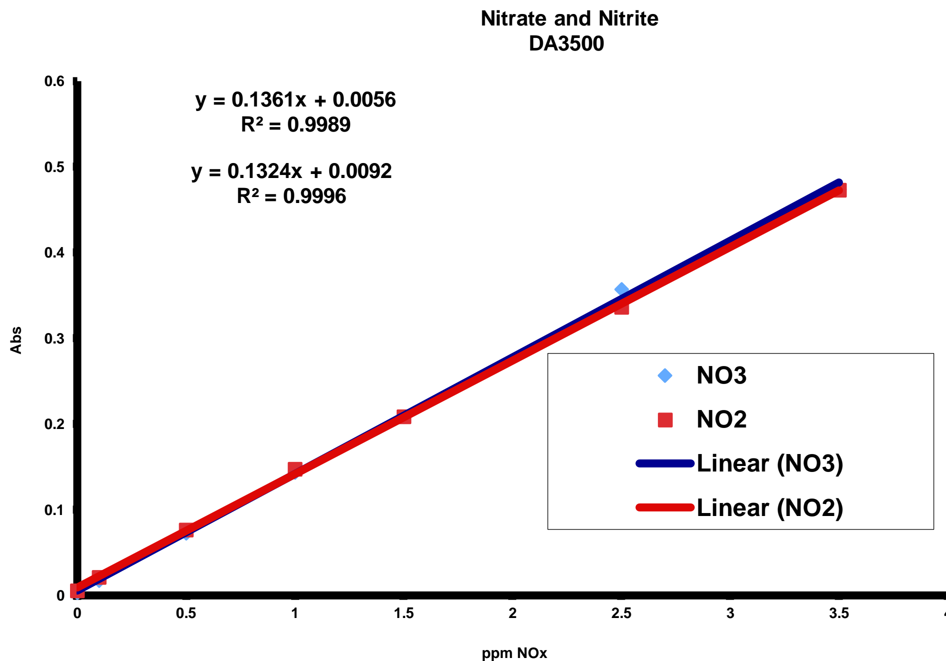
[5] Campbell WH, Song P, Barbier GC, *Nitrate Reductase for nitrate analysis in water,* Environ Chem Lett (2006), 4:69

[6] Patton CJ, Fischer AE, Campbell WH, and Campbell ER, *Corn Leaf Reductase-A nontoxic Alternative to Cadmium for Photometric Nitrate Determinations in Water Samples by Air-Segmented Continuous Flow Analysis, Environ*. Sci. Technol. 2002, 36, 729 - 735

Selectivity

A nitrate reductase method was developed on an OI DA3500 discrete analyzer. Nitrate and Nitrite were independently calibrated (Figure 2). The almost identical slopes and intercepts of the calibration curves demonstrate the accuracy of determining nitrate by subtraction of nitrite from the nitrate plus nitrite values.

Figure 2 Nitrate and Nitrite Nitrogen Calibration Curves



Seven matrices of acid preserved and analyzed samples were obtained from a commercial laboratory. These samples were also analyzed by OI Analytical by the cadmium reduction method on the DA3500 discrete analyzer and compared to results obtained using the nitrate reductase method developed on the same DA3500 discrete analyzer. Non-preserved portions of the samples were also obtained and analyzed using reductase for comparison. Table 1 is a summary of the results.

Table 1 Summary of Nitrate Analysis on Real World Samples

Sample #	Commercial Laboratory Results, Analysis Method EPA 335.2 (mg NO_3+NO_2-N/L)	OI Results, Analysis by Cd Reduction on the DA3500 (mg NO_3+NO_2-N/L)	OI Results, Analysis by Reductase on the DA3500 (mg NO_3+NO_2-N/L)	OI Results, Analysis by Reductase on the DA3500 (mg NO_3+NO_2-N/L) Non-Preserved
1	0.8	0.96	0.94	0.88
2	<0.1	0.04	0.05	0.06
3	<0.1	0.32	0.24	0.55
4	0.66	0.68	0.68	0.58
5	11.8	10.1	11.6	Lost
6	0.78	0.75	0.79	0.77
7	2.4	2.5	3.11	2.88

Sample #7 contains sulfide. Sulfide reacts with cadmium forming cadmium sulfide decreasing the efficiency of the reduction.

Twelve additional matrices were acquired from another commercial laboratory, analyzed by the reductase method on an OI DA3500 and then spiked with known nitrate concentrations. An OI analyst then analyzed the spiked samples using the OI Nitrate Reductase method with the commercially available reagents and accompanying instructions[7]. Table 2 is a summary of the results.

[7] http://nitrate.com/

Table 2 Single Laboratory validations of 12 matrices by Nitrate Reductase on the OI DA3500

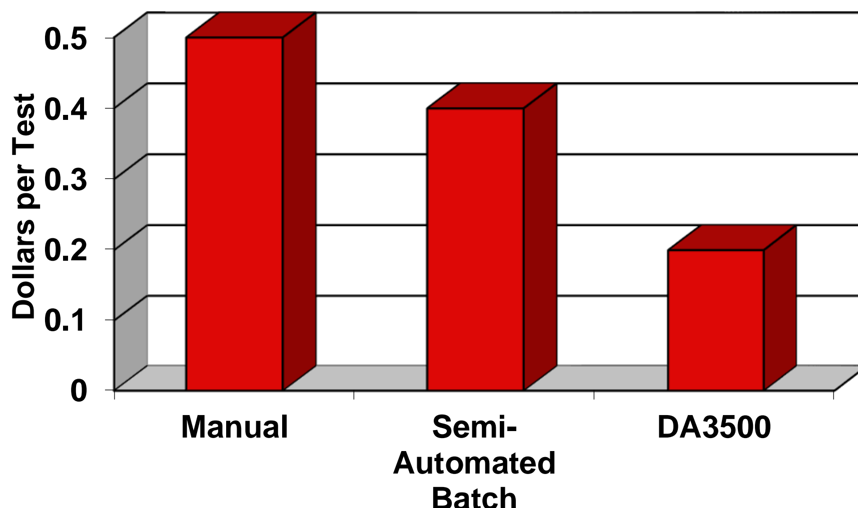
Matrix	Expected (mg NO_3-N/L)	Found (mg NO_3-N/L)	% Recovery
Industrial Effluent	1.03	0.95	93
	5.03	5.19	103
POTW Influent	0.10	0.10	100
	5.00	4.64	93
POTW Effluent	1.48	1.61 (10)*	110
	5.48	5.19 (2.7)	95
Septic System	0.12	0.11	93
	5.02	5.34	106
Reagent Water	1.06	1.15	109
	5.06	5.22	103
Tap Water	5.04	4.95	98
Seawater	0.15	0.16	107
	1.05	1.10	105
	5.05	4.71 (10)	93
Monitoring Well	1.06	0.99	93
	5.06	5.07	100
Industrial Effluent 1	1.74	1.77 (4.0)	102
	5.74	5.03 (4.4)	88
Soil Extract	1.74	1.62	88
Industrial Effluent 2	1.78	1.80	103
	5.78	4.92	83
Industrial Effluent 3	2.43	2.50	107
Industrial Effluent 3 (diluted)	1.71	1.73	102
	5.71	5.14	89

* RPD in parentheses

The results of these preliminary tests conducted by OI Analytical indicate that the test adequately detects nitrate equivalently to the cadmium reduction method used on the same instrument and obtained similar results to EPA 353.2 analyzed at a commercial laboratory. The one sample (Table 1 sample 7) whose results seemed to vary indicates that the reductase method is capable of overcoming the sulfide interference that plaques the cadmium reduction method.

Reagent costs for the manual methods are well within the costs of typical single analyte test kits. In the manual methods, the analyst measures the sample aliquot, adds reagents, sets a timer, adds color reagent, sets a timer and then reads the absorbance of each standard and sample on a spectrophotometer. Using a flow analyzer for the determination step lowers the analyst interaction and the cost per sample becomes dependent on flow rates and sample throughput. The use of a semi automated batch reduction enhances productivity in flow methods and keeps costs down, however, the analyst must still pipet aliquots of sample into cuvettes and then add reagents. The manual addition of reagents increases the chance for human error. Discrete analyzers are capable of fully automated reductase methods from start to finish without significant reagent cost. This is because discrete analyzers are not injecting samples into a constantly flowing stream of reagents. Instead, discrete analyzers inject only the required amount of reagent needed into individually contained aliquots of sample. Figure 3 is a comparison of the estimated analytical cost of the reductase method by manual, semi-automated batch, and discrete analyzer methods.

Figure 3 Comparisons of Analytical Costs for Nitrate by Reductase by Method.



Nitrate plus Nitrite analysis by reductase obtains equivalent results to cadmium reduction methods in complex matrices. By replacing cadmium the method is safer and decreases hazardous waste generation. Discrete analyzers fully automate the reduction and analysis lowering analytical costs below manual or semi-automated batch continuous flow methods. Since reagent costs and the potential for analyst error are significantly decreased by completely automating the entire method, D19.05 decided that the new ASTM standard should be limited to discrete analyzers only.

Sample Preservation and Storage

As indicated in Table 1, preliminary results of preserved samples were determined equivalent to results obtained by the EPA approved cadmium reduction method. The pH range for the enzyme is limited to 6 - 8, however the buffer reagent in the method is capable of handling up to 50 mM acid or base and keep the pH in the range of the enzyme. If higher concentrations of acid or base were needed the buffer concentration would need to be increased. As long as the instructions for the reagents are followed, pH 2 preserved samples can be analyzed. Laboratories should use caution if attempting to decrease detection limits by increasing the sample volume.

The following excerpt is from the sampling and preservation section of the new standard:

8.2 When nitrite ion is to be determined separately, analyze within 48 hours after sampling. Even when sterile bottles are used, bacteria naturally present in the water may cause conversion of all or part of nitrite ion to other forms such as nitrate or ammonia. Ammonia and natural amines, which are frequently present in natural waters, may react with nitrites to form nitrogen. If samples are to be stored for 48 h or less, preserve the sample by refrigeration at 2 - 6°C. If the sample must be stored for more than 48 h, preserve it by the addition sulfuric acid to pH 2 in addition to refrigeration at 2 - 6°C.

Note 2—Use sulfuric acid for preservation of nitrite-nitrate nitrogen only. Samples for nitrite must be analyzed within 48 hours.

Note 3—Sulfuric acid does not necessarily inhibit oxidation and mercury compounds should be avoided to prevent environmental pollution.

Note 4 – Residual chlorine does not interfere, however, attempts to remove residual chlorine (such as addition of ascorbic acid) interfere by inhibiting reduction of nitrate to nitrite. Do attempt to remove residual chlorine.

Since the method is for an existing EPA pollutant (nitrate plus nitrite) with established sampling and preservation guidelines, D19.05 did not perform a holding time study. The ability of the method to analyze preserved samples has been established and the standards ability to accurately measure nitrate in chlorinated tap water has been verified as well. Therefore, this standard assumes the 28-day EPA required holding time will be applied.

Interferences

Trace metals will inhibit the reduction so EDTA is added to the buffer reagent. As in all other colorimetric nitrate/nitrite methods that use the Greiss reaction, excess metals can also interfere with the color development. The sample is diluted 20 fold in the buffer reagent making metal interference almost moot except for the most polluted samples. Potential interferences are listed in Table 3.

Table 3 Determination of Nitrate in the Presence of Potential Interferences

Species	Concentration Added (mg/L)	Unspiked Sample Result (mg/L)	Spiked Sample Results (mg/L)	Spike Added (mg/L)	% Recovery
Cl^-	500	0.02	0.23	0.200	105
		0.17	2.54	2.50	95
F^-	500	0.01	0.22	0.200	105
Br^-	500	<0.01	0.21	0.200	100
		0.15	2.65	2.50	100
PO_4^{-3}	500	0.01	0.22	0.200	105
		0.14	2.54	2.50	96
SO_4^{-2}	500	<0.01	0.21	0.200	105
		0.14	2.53	2.50	96
Fe	500	0.17	2.60	2.50	97
	1.0	<0.01	0.21	0.200	105
		0.168	2.59	2.50	96
Zn	1.0	<0.01	0.22	0.200	110
		0.14	2.64	2.50	100
Al	1.0	<0.01	0.21	0.200	105
		0.14	2.53	2.50	96
BrO_3^-	1.0	<0.01	0.22	0.200	110
		0.17	2.64	2.50	99
ClO_2^-	1.0	0.01	0.22	0.200	110
		0.14	2.54	2.50	96
ClO_3^-	1.0	0.23	2.45	2.50	89
$CHCl_3$	> Miscibility	<0.01	0.21	0.200	105

High phenolic content humic acid substances may inhibit some nitrate reductase reagents. The reagents used in this ASTM standard were developed specifically to overcome humic acid interferences. One of the inter-laboratory study samples

was synthetically prepared to contain about 2 ppm humic acid known to interfere with some reductase reagents. The standard warns against use of unknown enzyme reagents with the following precaution:

—For some NaR forms, high phenolic content humic substances (> 2 mg dissolved organic carbon /L) have little affect on the NaR activity in the temperature range of 5 – 15 C, but become increasingly inhibitory in the temperature range of 20 – 30 C. Humic substances at the operation temperatures specified in this standard do not inhibit other forms of NaR[8]. If humic acids are expected to be present the user must verify reduction efficiency of the NaR is use by analysis of Quality Control checks that approximate the sample matrix.

During the inter-laboratory trial, each lab testing drinking water was asked to analyze a tap water from their own facility and then re-analyze another aliquot spiked to contain 3-ppm residual chlorine above the concentration of residual chlorine originally present in the sample. The data, presented in Table 4, illustrates that residual chlorine does not interfere. This is important because residual chlorine does interfere with cadmium reduction methods. The method appears to have less interference than the cadmium reduction method.

[8] NaR available from the Nitrate Elimination Company Inc. (NECi), www.nitrate.com, has been found suitable.

Table 4 Determination of Nitrate in Drinking Water Samples spiked with 3-ppm residual chlorine.

Sample Matrix	Mean mg N/L	Std Dev mg N/L	Spike 0.5 % Rec	Spike 1.0 % Rec	Spike 2.5 % Rec	Lab #	Source
DW-1	0.313	0.013	101.2%	100.1%	100.8%	1A	Lk Linden
DW-1 + Cl	0.300	0.015	104.6%	97.9%	99.3%	1A	Lk Linden
DW-2	0.536	0.016	90.3%	108.0%	90.5%	1A	Houghton
DW-2 + Cl	0.589	0.016	101.4%	103.5%	106.5%	1A	Houghton
DW-3	0.546	0.012	100.4%	98.1%	106.2%	1A	Houghton
DW-3 + Cl	0.603	0.010	92.2%	98.0%	108.2%	1A	Houghton
DW-4	0.113	0.003	100 ± 0.4	100 ± 0.8	102 ± 1	2	Denver
DW-4 +Cl	0.119	0.006	98.8 ± 1.2	99.7 ± 0.9	103 ± 3	2	Denver
DW-5	0.113	0.004	96.7 ± 0.4	96.5 ± 0.4	97.6 ± 0.4	3	Denver
DW-5 +Cl	0.121	0.005	98.9 ± 0.4	97.0 ± 0.4	98.4 ± 0.4	3	Denver

Instrument Calibration

Calibration curves on discrete analyzers are prepared automatically by dilution from a single, or multiple, stock solutions. The instrument prepares diluted aliquots of the known concentration stock solution corresponding to pre-set values established in the operating software/method. The instrument then adds reagents, allows the reaction to proceed, and then measures the response of the colored product. The software then prepares a calibration curve plotting response versus concentration to which all subsequent sample analyses will be compared. One of the advantages of discrete analyzers is this automatic preparation of the calibration curve. Figure 2 shows an example calibration curve generated by OI Analytical. Table 5 summarizes the calibration results submitted by each of the participating laboratories.

Table 5 Summary of Calibration Results

Lab	Slope at 540nm	Intercept	Correlation Coefficient (r2)
1	0.1392	0.0027	0.9999
2	0.1468	0.0019	0.9999
3	0.0954	0.0026	0.9999
4	0.1159	- 0.0006	0.9994
5	0.1420	- 0.0048	0.9963
6	0.1361	0.0148	0.9994
7	0.1352	- 0.0015	0.9997
8	0.1328	0.0014	0.9980
9	0.0643*	0.0027	0.9986
10	0.0530	0.0084	0.9999

* Measured at 550nm

Each laboratory had a correlation coefficient (r2) greater than 0.995 demonstrating the ability of each individual discrete analyzer to prepare precise calibration curves.

Each laboratory that participated in the inter-laboratory trial was provided with a 15-mg/L stock solution from which to prepare their calibrations. There were a total of 10 laboratories each using a different discrete analyzer. Four different discrete analyzer manufacturers were represented. One manufacturer had several different models of discrete analyzer with at least one of each model used in the study. Laboratories 3, 9, and 10 were each different manufacturers while the rest of the laboratories used discrete analyzers from the same manufacturer.

Calibration Verification

Each laboratory was asked to calibrate with specific calibrant concentrations ranging from 0.05 - 5.0 mg/L NO3-N according to manufacturer's instructions but all using the stock solution provided. Each laboratory verified the accuracy of the calibration using a 2.5 mg/L NO3-N second source standard obtained from their own laboratory. Table 6 summarizes the calibration verification results.

Table 6 Calibration Verification Results

Lab	Mg/L NO3-N ICV	Mg/L NO3-N (found)	% Recovery
1	2.50	2.51	100
2	2.50	2.48	99.2
3	2.50	2.55	102
4	2.50	2.59	104
5	2.50	2.36 2.62	94.4** 105
6	2.50	2.56	102
7	2.50	2.51	100
8	2.50	2.49	99.6
9	3.04*	3.00	98.7
10	2.50	2.60	104

* Did not follow instructions and used a 3.04 mg/L NO3-N standard

** First analysis not within 95 - 105% so repeated

The new standard requires that calibration verification standards (CCV) fall within 90 - 110% of their true value. Many EPA QA programs require that a second source calibration verification (ICV) be analyzed and fall within 95 - 105% of the true value. Data in Table 3 verifies that ICV criteria of EPA methods and the CCV criteria of the new ASTM standard were met. These second source standards were prepared from a stock standard that differs from the standard used to calibrate the instruments. The closeness of the results to the true value demonstrates the accuracy of the calibration curves generated by each instrument.

Reduction Efficiency

Each laboratory was then asked to analyze a 2.5 mg/L NO2-N standard so that reduction efficiency of the enzyme could be calculated. Reduction efficiency is calculated as follows:

% RE = (mg/L NO3-N / mg/L NO2-N) x 100

Table 7 presents the results of reduction efficiency testing from all the labs. Based on the results in Table 4, a 95% confidence interval suggests that reduction efficiency should be between 95 and 115%, however, 95% is higher than the 90% lower limit used in the EPA cadmium reduction method (335.2) so the lower efficiency limit of the standard was adjusted to the 99% confidence level of 91%; ASTM D7781 reduction efficiency limits were set at 91 - 115%. According to these limits, laboratory 4 in Table 7 exceeded the limit.

Table 7 Reduction Efficiency

Lab	Mg/L NO2-N (Found)	Mg/L NO3-N (found)	% Efficiency
1	2.46	2.51	102
2	2.45	2.48	101
3	2.38	2.55	107
4	2.22	2.59	117
5	2.48	2.62	106
6	2.46	2.56	104
7	2.42	2.51	104
8	2.43	2.49	102
9	3.04*	3.00	98.7
10	2.48	2.60	105

Initial Demonstration of Laboratory Capability (IDC)

Section 17.3.1 of the new ASTM D7781 standard requires laboratories to demonstrate they are capable of obtaining results of sufficient accuracy and precision if they have not run the test before, a new analysts is running the test, or if maintenance was performed on the instrument. IDC is carried out by analysis of four replicates of an Independent Reference Material containing Nitrate Nitrogen at a concentration near the mid point of the method calibration curve. The mean and standard deviation of the four replicates are calculated and compared to the certified values provided by the chemical supplier.

Part of the inter-laboratory study purpose was to verify that the acceptance limits calculated from recovery data obtained at each individual laboratory during the inter-laboratory trial would be narrow enough to meet the acceptance criteria of a commercially available certified standard. Thus, each laboratory was asked to analyze a 2.5 mg/L NO3-N standard in quadruplicate so that average recovery and % Relative Standard Deviation (RSD) could be calculated. Table 8 summarizes the results from the IDC performed at each lab.

Table 8 Initial Demonstration of Capability

Lab	% Recovery	%RSD
1	101	0.71
2	102	0.54
3	96.2	2.23
4	106	0.73
5	102	0.78
6	103	0.92
7	101	1.13
8	98.4	0.66
9	101	0.95
10	99.3	2.45
average	101	1.23
Standard Deviation	2.66	0.67

Lower Limit (99% CI)	92.9	0
Upper Limit (99% CI)	109	3

The 93 - 109% recovery with a maximum RSD of 3% demonstrates that the method and the different discrete analyzers with different analysts at 10 different laboratories all obtain accurate and precise results on mid-range standards in a clean matrix. The data also shows that in a properly run laboratory the method should obtain acceptable results from certified reference materials.

Determination of the Method Detection Limit (MDL)

The ASTM Standard does not require the determination of detection limits, however, since the standard is intended for EPA reporting the determination of detection limit is required. The inter-laboratory trial asked each laboratory to determine detection limits by analyzing the lowest standard (0.05 mg/L NO3-N) seven times. The standard deviation of the seven replicates is calculated and multiplied by 3.14 to estimate the MDL.

Seven of the 10 laboratories submitted MDL data collected in accordance with 40 CFR Part 136 Appendix B as described above. The average detection limit of these 7 laboratories was 0.02 mg/L.

A better indication of detection limit is made by plotting concentration of the inter laboratory study samples versus the % RSD. Figure 4 plots the single laboratory results while Figure 5 plots the multiple laboratory results.

Figure 4 Single Laboratory Precision

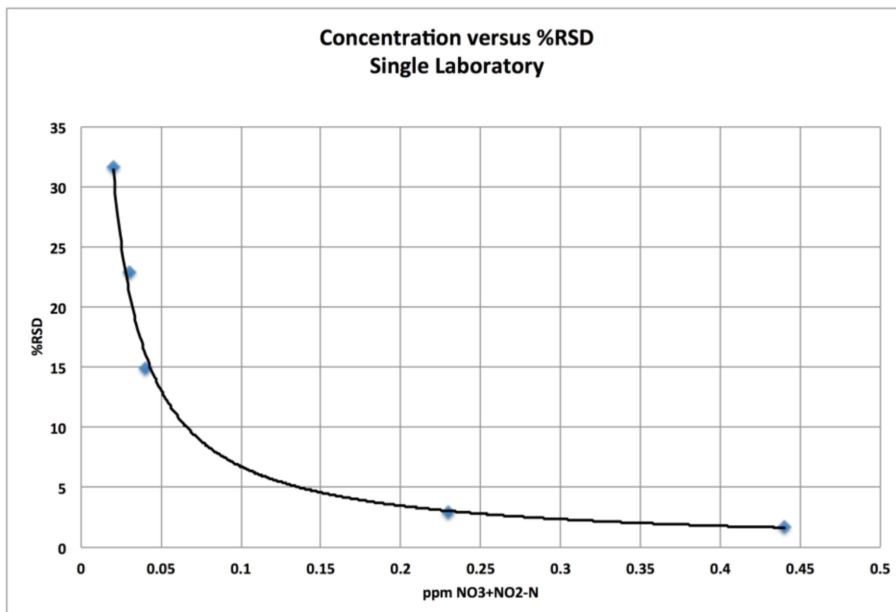
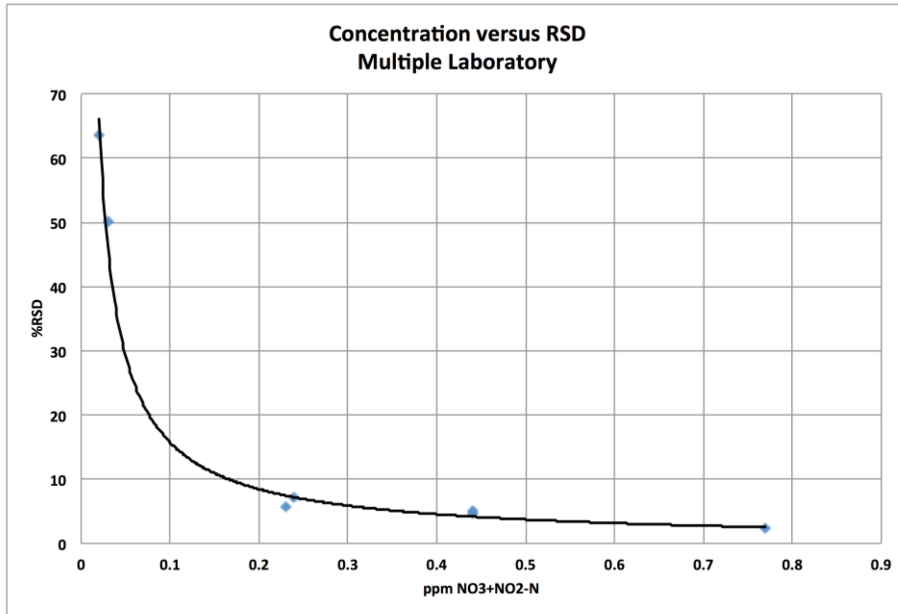


Figure 5 Multiple Laboratory Precision



Precision

A multiple laboratory validation study was conducted to verify that this new ASTM Standard, DD7781 Test Methods For Nitrite-Nitrate in Water by Nitrate Reductase, is sensitive, rugged, and sufficiently user friendly for routine use at EPA certified drinking water laboratories and at laboratories reporting results for Clean Water Act compliance.

Drinking Water Validation

The multiple laboratory validation for drinking water consisted of 5 laboratories and 5 matrices of 7 replicates of each matrix for a total of 35 determinations per matrix. The matrices consisted of each laboratory's own tap water, a high TDS (> 500 ppm) source water, a high TOC (~ 2 ppm) source water, an Environmental Resource Associates (ERA) Water Supply #698 QC sample, and USGS N-116 Nutrient Fortified River Water. While the study coordinators knew the "true value" of the ERA and the USGS samples they were unknown to the laboratories performing the test (laboratories 1&2 are the study coordinator). Results of this study are found in Table 9.

Table 9 Multiple Laboratory Results for Drinking Water

Sample Matrix	**Mean (mg/L)**	**Standard Deviation**	**No. of Laboratories**	**No. of Results**	**Multi-laboratory %RSD**
High TDS (500 ppm)	0.77	0.02	5	35	2.28%
High TOC (2 ppm)	1.12	0.02	5	35	1.36%
ERA #698 WS	6.59	0.17	4	28	2.51%
USGS N116	0.44	0.02	6	42	5.09%

The individual laboratory tap water samples are not included in Table 6 because each water sample had a different nitrate concentration. One laboratory did not report results for the ERA sample since it was above the 5 mg/L maximum standard in the calibration curve. One of the wastewater laboratories analyzed the USGS N116 sample but did not report results for the drinking water samples. Since this laboratory's results were statistically equivalent to the other 5 labs the results were included.

The "true value" of the ERA QC standard was 6.80 mg/L NO3-N. The average recovery of the 4 participating laboratories was 6.59 mg/L NO3-N or 96.9% recovery. The "true value" of the USGS N116 sample was 0.44 mg/L NO3-N. The average recovery of the 6 participating laboratories was 0.44 mg/L NO3-N or 100% recovery. The multiple laboratory precision was generally less than 5% with the USGS N116 having a multiple laboratory RSD of 5.09%. As shown in Figure 4 and 5, the %RSD is expected to rise with lower concentrations.

Wastewater Validation

The multiple laboratory validation for waste water consisted of 10 laboratories and 9 matrices of 3 replicates of each matrix for a total of 30 determinations per matrix. The matrices consisted of real world samples collected from industries with wastewater effluents or discharges expected to contain nitrate, a seawater sample, and a high hardness standard from ERA. Results of this study are found in Table 10.

Table 10 Multiple Laboratory Results for Waste Water

Sample Matrix	**Mean (mg/L)**	**Standard Deviation**	**No. of Laboratories**	**No. of Results**	**Single Operato r %RSD**	**Multiple Operator % RSD**
WW treatment plant influent	0.03	0.0131	10	30	22.9%	41.9%
wastewater treatment plant effluent #1	7.73	0.3181	10	30	1.03%	4.12%
wastewater treatment plant effluent #2	0.23	0.0126	10	30	2.92%	5.46%
Paper Mill waste stream effluent	0.04	0.0156	10	30	14.9%	41.7%
metal finisher wastewater effluent	273	10.234	8	24	24.3%	3.75%
commercial laundry wastewater effluent	4.90	0.2123	10	30	13.3%	4.34%
ERA #507 Hardness	0.02	0.0144	10	30	36.8%	61.5%
Confined Animal Feeding Operation (CAFO) effluent	13.9	0.4623	10	30	12.6%	3.33%
Low Nutrient Seawater	0.02	0.0112	10	30	31.7%	62.1%

As expected, the single and multiple laboratory precision (%RSD) are high for samples containing the lower concentrations. While each of these samples contained nitrate above the calculated MDL, the samples with high %RSD were all below the concentration of the lowest calibration standard. Two laboratories did

not report results for the metal finisher wastewater because of its extremely high concentration.

Bias

Recovery of nitrate in samples can be affected by matrix components. While the true value of an unknown sample can never be known, potential negative bias can be detected through the analysis of a laboratory fortified matrix, or matrix spike. Negative interferences due to humic acids and/or trace metals was discussed in the Interference section of this report. The inter-laboratory study examined matrix effects of each sample by requiring each laboratory to conduct a series of matrix spikes. It is important to note that the purpose of this inter-laboratory study was to evaluate the ability of this new ASTM method to obtain equivalent results regardless of laboratory, analyst, or discrete analyzer. The data presented in Tables 9 & 10 demonstrated that this standard ASTM D7781 did just that; the results for drinking water and wastewater in unknown samples analyzed on different discrete analyzers by different analysts were essentially equivalent. We must also note that the operation of discrete analyzers is completely automatic including preparation of the calibration curve from a single stock, analysis and calculation of results, to dilution and re-analysis of off-scale samples. The only part of this validation study that was not fully automatic was the fortification of laboratory samples. Hence, while the variability of sample results shown in Tables 9 &10 is exclusively due to the variability of the method and the discrete analyzer, the variability in the recovery of spiked samples combines the method/discrete analyzer with operator error.

Preparation of Laboratory Fortified Matrices

Laboratories were given the following instructions:

1) Dispense, in duplicate, 10 micro liters of the 50-mg/L NO3-N spiking solution into a 2 milliliter auto-sampler vial.
2) Add 990 micro liters of sample to the vial
3) Mix by aspirating and dispensing back and forth using a 1000 microliter digital pipet.

Drinking Water Spike and Spike Duplicate Recoveries

Four laboratories reported acceptable spike sample results on all of the drinking water matrices. One laboratory did not report spike results for the sample containing TOC. While the inter-laboratory study plan asked analysts to spike each replicate, only one of the acceptable spike recoveries is included in this validation package. This is because a laboratory will not normally spike a single matrix so many times so we felt that the data was not necessary. The laboratories were instructed to report all results whether they felt the spikes passed or failed. This is because there were no acceptance criteria provided to the laboratories. Drinking water methods typically require spike recoveries between 90 - 110%. Therefore, we included 0.5 ppm spike sample results that were sufficiently above the nitrate concentration of the sample to obtain an adequate recovery. Table 11 is a summary of the recovery on the drinking water matrices.

Table 11 Overall Spike and Spike Duplicate Data for Drinking Water

Matrix	Average Spike Recovery (%)	Average RPD (%)
High TDS (500 ppm)	101	2.20
High TOC (2 ppm)	99.5	3.25
ERA #698 WS	98.4	3.99

Spike and spike duplicate data demonstrate that it is possible to obtain adequate spike recovery and precision using this method on drinking water methods. The ASTM standard includes acceptance criteria requiring that spike recovery and precision be within defined or the sample analysis must be repeated. These limits are consistent with other drinking water nitrate methods. Table 12 is the table from the method.

\

Table 12 Drinking Water Quality Control Acceptance Criteria

QC Sample	% Recovery	% RPD
CCV	90 - 110	N/A
LCS	90 - 110	N/A
MS/MSD	90 - 110	≤ 10 %

Wastewater Spike and Spike Duplicate Recoveries

Ten laboratories reported spikes and spike duplicates for all of the wastewater matrices. Seven of the laboratories spiked, in duplicate, all of the matrices while two labs spiked all but the metal finisher effluent and the commercial laundry effluent. One laboratory only spiked two of the matrices. Spike recoveries and %RPD were tabulated and average recovery was calculated across all labs (multi-laboratory recovery and precision). Also, the average recovery and RPD of all matrices analyzed by each laboratory was calculated (single laboratory recovery and precision). The multiple laboratory recovery averaged 97% with a 99% CI range of 69.7 - 125% Recovery. The single laboratory recovery averaged 96.2% with a 99% CI range of 69 - 123%. The multiple laboratory RPD averaged 6.29% with a 99% CI range of 0 - 21.9%. The single laboratory RPD averaged 6.5% with a 99% CI range of 0 - 28.7%. A summary of the multiple laboratory spike and RPD results are given in Table 13.

Table 13 Overall Spike and Spike Duplicate Data for WasteWater

Matrix	Average Spike Recovery (%)	Average RPD (%)
WW treatment plant influent	94.1	3.7
wastewater treatment plant effluent #1	99.7	8.1
wastewater treatment plant effluent #2	97.9	2.7
Paper Mill waste stream effluent	102	1.9
metal finisher wastewater effluent	98.0	15.3
commercial laundry wastewater effluent	101	3.2
ERA #507 Hardness	95.2	5.9
Confined Animal Feeding Operation (CAFO) effluent	91.5	16.6
Low Nutrient Seawater	93.5	1.6

Spike and RPD limits were set based on the results averaged across all the laboratories (multiple laboratory results). These acceptance limits are broader than those applied to drinking water, but wastewater matrices have greater variability and are more complex. According to the standard, if a wastewater spike or duplicate does not meet the acceptance criteria provided in Table 14 the data should either be flagged or repeated.

Table 14 Wastewater Quality Control Acceptance Criteria

QC Sample	% Recovery	% RPD
CCV/IPR	90 - 110	≤ 10 %
LCS/LCSD	85 - 115	≤ 15 %
MS/MSD	70 - 125	≤ 25 %

Participating laboratories

Table 15 lists the laboratories, in no particular order, that participated in this multiple laboratory validation study.

Lab Name	Contact
USGS / NWQL Analyzer 1	Charles Patton
USGS / NWQL Analyzer 2	Charles Patton
OI Analytical	Libby Badgett
ThermoFisher DA #1	Steven White
ThermoFisher DA #2	Stephen White
ThermoFisher DA #3	Stephen White
University of Maryland/Solomons	Jerry Frank
Klamath Tribes	Kris Fischer
Geochemical Testing	Tim Boergstresser
Westco Scientific	Bill Georgian
Astoria Pacific	Winston Pavitt