QuikChem® Method 10-107-04-5-A

DETERMINATION OF NITRATE IN WATERS BY FLOW INJECTION ANALYSIS

NITRATE REDUCTASE

(LOW FLOW METHOD)

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QuikChem® Method 10-107-04-5-A

Nitrate in Waters Utilizing Nitrate Reductase Enzymatic Reduction (NECi Superior Stock)

High Range = 0.2 to 20.0 mg N/L as NO_3^-
Low Range = 0.02 to 5.0 mg N/L as NO_3^-

– Principle –

Nitrate (NO_3^-) is reduced virtually quantitatively to nitrite (NO_2^-) in the presence of the enzyme nitrate reductase (NaR) and β-nicotinamide adenine di-nucleotide, reduced form (NADH). NaR catalyzes the reduction of nitrate to nitrite with NADH as the reducing agent. The nitrite (reduced nitrate plus original nitrite) is then determined by diazotizing with sulfanilamide followed by coupling with N-(1-naphthyl)ethylenediamine dihydrochloride. The resulting water soluble dye has a magenta color which is measured at 540 nm.

– Interferences –

1. Low results would be obtained for samples that contain high concentrations of iron, copper or other metals. In this method, EDTA is added to the buffer to reduce this interference.
2. Humic acid compounds can interfere. To eliminate this interference lower the temperature of the enzymatic reduction reaction to 10ºC.
3. Anions such as SO_4^{2-}, PO_4^{2-}, Cl^-, Br^-, F^- will not interfere at levels up to 500 ppm.
4. NADH can interfere in the colorimetric portion of the method. HCl is used in the color reagent to eliminate this interference.

– Special Reagents –

1. NECi Superior Stock Nitrate Reductase, LA-ARK kit - Part number 800203-L (contains 1 vial of 3 units of YNaR enzyme, and NADH). This kit will make up the 50+ mLs of reagent to perform 50+ analyses. Other reagent option: YNaR1 Part number 800201 (1 unit of enzyme/vial). YNaR3 – Part number 800203 (3 units of enzyme/vial).

   Order from The Nitrate Elimination Company, Inc. 217 Calumet Street, Lake Linden, MI 49945. *Toll Free:* 1-888-NITRATE, *E-mail:* tech@nitrate.com, *Website:* www.nitrate.com.
2. NADH (β-Nicotinamide Adenine Dinucleotide) can also be purchased separately from Sigma, Part number N 8129 or equivalent).

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QuikChem® Method 10-107-04-5-A

DETERMINATION OF NITRATE IN WATERS BY NITRATE REDUCTASE USING FLOW INJECTION ANALYSIS

1. SCOPE AND APPLICATION

1.1. This method covers the determination of nitrate in drinking, surface, and wastewaters.

1.2. The applicable range is 0.2 to 20.0 mg N/L for the high range and 0.02 to 5 mg N/L for the low range. The statistically determined method detection limit is 0.023 mg N/L for the high range, and 0.009 mg N/L for the low range. The method throughput is 80 injections per hour.

2. SUMMARY OF METHOD

2.1. Nitrate (NO_3^-) is reduced virtually quantitatively to nitrite (NO_2^-) in the presence of the enzyme nitrate reductase (NaR) and β-nicotinamide adenine di-nucleotide, reduced form (NADH). NaR catalyzes the reduction of nitrate to nitrite with NADH as the reducing agent. The nitrite (reduced nitrate plus original nitrite) is then determined by diazotizing with sulfanilamide followed by coupling with N-(1-naphthyl)ethylenediamine dihydrochloride. The resulting water soluble dye has a magenta color which is measured at 540 nm.

3. DEFINITIONS

The definitions and purposes below are specific to this method, but have been conformed to common usage as much as possible.

3.1. ANALYTICAL BATCH -- The set of samples extracted/distilled/or digested at the same time to a maximum of 10 samples.

3.2. CALIBRATION BLANK (CB) -- A volume of reagent water in the same matrix as the calibration standards, but without the analyte.

3.3. CALIBRATION STANDARD (CAL) -- A solution prepared from the primary dilution standard solution or stock standard solutions. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.4. FIELD BLANK (FMB) -- An aliquot of reagent water or equivalent neutral reference material treated as a sample in all aspects, including exposure to a sample bottle holding time, preservatives, and all preanalysis treatments. The purpose is to determine if the field or sample transporting procedures and environments have contaminated the sample.

3.5. FIELD DUPLICATE (FD) -- Two samples taken at the same time and place under identical circumstances which are treated identically throughout field and laboratory procedures. Analysis of field duplicates indicates the precision associated with sample collection, preservation, and storage, as well as with laboratory procedures.

3.6. LABORATORY BLANK (LRB) -- An aliquot of reagent water or equivalent neutral reference material treated as a sample in all aspects, except that it is not taken to the

sampling site. The purpose is to determine if the if analytes or interferences are present in the laboratory environment, the reagents, or the apparatus.

3.7. LABORATORY CONTROL STANDARD (LCS) -- A solution prepared in the laboratory by dissolving a known amount of one or more pure compounds in a known amount of reagent water. It's purpose is to assure that the results produced by the laboratory remain within the acceptable limits for precision and accuracy. (This should not be confused with a calibrating standard).

3.8. LABORATORY DUPLICATE (LD) -- Two aliquots of the same environmental sample treated identically throughout a laboratory analytical procedure. Analysis of laboratory duplicates indicates precision associated with laboratory procedures but not with sample collection, preservation, or storage procedures.

3.9. QUALITY CONTROL CHECK SAMPLE (QCS) -- A sample containing analytes of interest at known concentrations (true values) of analytes. The QCS is obtained for a source external to the laboratory or is prepared from standards obtained from a different source than the calibration standards. The purpose is to check laboratory performance using test materials that have been prepared independently from the normal preparation process.

3.10. METHOD DETECTION LIMIT (MDL) -- The lowest level at which an analyte can be detected with 99 percent confidence that the analyte concentration is greater than zero.

4. INTERFERENCES

4.1. Low results would be obtained for samples that contain high concentrations of iron, copper or other metals. In this method, EDTA is added to the buffer to reduce this interference.

4.2. Humic acid compounds can interfere. To eliminate this interference lower the temperature of the enzymatic reduction reaction to 10ºC.

4.3. Anions such as SO_4^{2-}, PO_4^{2-}, Cl^-, Br^-, F^- will not interfere at levels up to 500 ppm.

4.4. NADH can interfere in the colorimetric portion of the method. HCl is used in the color reagent to eliminate this interference.

5. SAFETY

5.1. The toxicity or carcinogenicity of each reagent used in this method has not been fully established. Each chemical should be regarded as a potential health hazard and exposure should be as low as reasonably achievable. Cautions are included for known extremely hazardous materials.

5.2. Each laboratory is responsible for maintaining a current awareness file of the Occupational Health and Safety Act (OSHA) regulations regarding the safe handling of the chemicals specified in this method. A reference file of Material Safety Data sheets (MSDS) should be made available to all personnel involved in the chemical analysis. The preparation of a formal safety plan is also advisable.

5.3. The following chemicals have the potential to be highly toxic or hazardous, for detailed explanation consult the MSDS.

3.3.1. Hydrochloric acid

3.3.2. Potassium Hydroxide

6. EQUIPMENT AND SUPPLIES

6.1. Balance -- analytical, capable of accurately weighing to the nearest 0.0001 g.

6.2. Glassware -- Class A volumetric flasks and pipettes or plastic containers as required. Samples may be stored in plastic or glass.

6.3. Flow injection analysis equipment designed to deliver and react sample and reagents in the required order and ratios.

6.3.1. Sampler

6.3.2. Multichannel proportioning pump

6.3.3. Reaction unit or manifold

6.3.4. Colorimetric detector

6.3.5. Data system

6.3.6. NECi Superior Stock Nitrate Reductase, LA-ARK kit - Part number 800203-L (contains 1 vial of 3 units of YNaR enzyme, and NADH). This kit will make up the 50+ mLs of reagent to perform 50+ analyses. Other reagent option: YNaR1 Part number 800201 (1 unit of enzyme/vial). YNaR3 – Part number 800203 (3 units of enzyme/vial). Order from The Nitrate Elimination Company, Inc. 217 Calumet Street, Lake Linden, MI 49945. *Toll Free:* 1-888-NITRATE, *E-mail:* tech@nitrate.com, *Website:* www.nitrate.com.

6.3.7. NADH (β-Nicotinamide Adenine Dinucleotide) can also be purchased separately from Sigma, Part number N 8129 or equivalent).

7. REAGENTS AND STANDARDS

7.1. PREPARATION OF REAGENTS

Use deionized water (10 megohm) for all solutions.

Degassing with helium:

To prevent bubble formation, degas all solutions except the standards with helium. Use He at 140kPa (20 lb/in^2) through a helium degassing tube (Lachat Part No. 50100.) Bubble He through the solution for one minute.

Reagent 1. Ethylenediaminetetraacetic acid, disodium salt, dihydrate ($Na_2EDTA{\cdot}2H_2O$) 0.025 M

By Volume: In a **1 L** volumetric flask, add about **800 mL of DI water**, and dissolve **9.3 g of ($Na_2EDTA{\cdot}2H_2O$)**. Dilute to the mark with **DI water** and invert to mix.

By Weight: To a tared **1 L** container add **1000 g DI water,** and dissolve **9.3 g of ($Na_2EDTA{\cdot}2H_2O$)**. Invert to mix.

Reagent 2. Phosphate Buffer, pH = 7.5, 0.028 M (Carrier)

By Volume: To a **1 L** volumetric flask add **800 mL DI water**, **1.4 g potassium hydroxide** (KOH) and **3.75 g of potassium dihydrogen phosphate** (KH_2PO_4). Add **1 mL of disodium EDTA**, dissolve and dilute to the mark with **DI water**. Invert to mix.

Reagent 3. Sulfanilamide Color Reagent

By Volume: To a **1 L** volumetric flask, add about **600 mL DI water**. Then add **100 mL concentrated hydrochloric acid** (37% HCl), **10.0 g sulfanilamide** ($C_6H_8N_2O_2S$), and **1.0 g N-1-naphthylethylenediamine dihydrochloride** (NED). Shake to wet and stir to dissolve for 20 minutes. Dilute to the mark with **DI water**, and invert to mix. Store in a dark bottle and discard when dark pink.

7.2 ENZYMATIC REDUCTION REAGENTS

Reagent 4. Stock β-Nicotinamide Adenine Dinucleotide, (NADH) 2.82 mM, reduced form (Sigma N 8129 or equivalent).

By Volume: Dissolve 0.10 g of NADH in 30 mL of DI water in a 50-mL volumetric flask, dilute the solution to the mark with DI water. Dispense 4.0 mL aliquots of the stock NADH into 4 mL centrifuge tubes. Store the stock NADH in freezer at -20°; the stock will be stable for approximately 1 month in freezer.

Reagent 5. Working NaR-NADH Reagent

Remove (1) 3 units vial of NECi YNaR and one tube of NADH from the freezer and allow solutions to warm to room temperature (~20 minutes before reagent preparation). Reconstitute YNaR vial by adding 600 µL of phosphate buffer (reagent 2) to enzyme containing vial. Recap the vial and invert or vortex it several times over a 5-minute period. After the 5-minute period, transfer the dissolved enzyme into a 50-mL plastic centrifuge tube and add enough phosphate buffer to bring the volume to 50 mL, mix well. **Immediately before use**, add the 4 mLs of NADH solution to the 50-mL centrifuge tube and mix. Combining the YNaR and NADH accelerates the oxidation of NADH to NAD^+ leaving no reduced NADH available to the enzyme. If the combined YNaR/NADH solution has been sitting for over an hour and start experiencing lower recovery for NO_3, add another vial of NADH solution and the activity should return to near 100%.

If using YNaR1, Part number 800201 (1 unit of enzyme/vial), remove (3) of the 1-unit vials and add 600 µL of phosphate buffer (reagent 2) to each enzyme containing vial. Recap the vials and invert or vortex them several times over a 5-minute period. After the 5-minute period, transfer the dissolved enzyme into a 50-mL plastic centrifuge tube and add enough phosphate buffer to bring the volume to 50 mL. Then follow the procedure above to add the NADH solution immediately prior to use.

7.3. PREPARATION OF STANDARDS

By Volume: Four 1 L and seven 100 mL volumetric flasks.

By Weight: Four 1 L and seven 100 mL containers.

Standard 1. Stock Nitrate Standard 200.0 mg N/L as NO_3^-

In a **1 L** volumetric flask, dissolve **1.444 g potassium nitrate** (KNO_3) in about **600 mL DI water**. Dilute to the mark with **DI water** and invert to mix.

Standard 2. Stock Nitrite Standard 200.0 mg N/L as NO_2^-

In a **1L** volumetric flask ,dissolve **0.986 g sodium nitrite** ($NaNO_2$) or **1.214 g potassium nitrite** (KNO_2) in approximately **800 mL DI water**. Dilute to the mark and invert to mix. Prepare this standard and all nitrite standards fresh daily as the nitrite is not stable in solution.

Standard 3. Working Stock Standard 20.0 mg N/L as NO_3^-

By Volume: In a **1L** volumetric flask, add **100.0 mL Stock Standard 1.** Dilute to the mark with **DI water** and invert to mix. Make fresh weekly.

By Weight: To a tared **1 L** container, add about **100 g Stock Standard 1.** Measure the exact weight and divide by **0.1**. This will give you the total weight of the diluted solution to be made. Make up this solution to the total weight with **DI water**, using a disposable pipet for the last 10 g or so. Shake or stir before using. Make fresh weekly.

Standard 4. Working Stock Standard 20.0 mg N/L as NO_2^-

By Volume: In a **1L** volumetric flask, add **100.0 mL Stock Standard 2.** Dilute to the mark with **2 M KCl** (Reagent 4) and invert to mix.

By Weight: To a tared **1 L** container, add about **100 g Stock Standard 2.** Measure the exact weight and divide by **0.1**. This will give you the total weight of the diluted solution to be made. Make up this solution to the total weight with **DI water**, using a disposable pipet for the last 10 g or so. Shake before using.

Nitrate Standards – High Range

Working Standards (Prepare Daily)	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Concentration mg N/L as NO_3	20.0	10.0	5.00	2.00	1.00	0.50	0.20	0.00
By Volume								
Volume (mL) of **stock standard 3** diluted to 100 mL with **DI water**	100	50	25	10	5.0	2.5	1.0	---
By Weight								
Weight (g) of **stock standard 3** diluted to final weight (~100 g) divided by **factor** below with **DI water**	100	50	25	10	5.0	2.5	1.0	---
Division Factor Divide exact weight of the standard by this **factor** to give the final weight	---	0.5	0.25	0.1	0.05	0.025	0.01	---

Nitrate Standards – Low Range

Working Standards (Prepare Daily)	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
Concentration mg N/L as NO_3	5.00	2.00	1.00	0.50	0.20	0.10	0.05	0.02	0.00
By Volume									
Volume (mL) of **stock standard 3** diluted to 100 mL with **DI water**	25	10	5.0	2.5	---	---	---	---	---
Volume (mL) of **standard A** (5 mg N/L) with **DI water**	---	---	---	---	4.0	2.0	1.0	0.4	---
By Weight									
Weight (g) of **stock standard 3** diluted to final weight (~100 g) divided by **factor** below with **DI water**	25	10	5.0	2.5	---	---	---	---	---
Volume (mL) of **standard A** (5 mg N/L) with **DI water**	---	---	---	---	4.0	2.0	1.0	0.4	---
Division Factor Divide exact weight of the standard by this **factor** to give the final weight	0.25	0.1	0.05	0.025	0.04	0.02	0.01	0.004	---

Nitrite Standards - High Range*

Working Standards (Prepare Daily)	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Concentration mg N/L as NO_2	20.0	10.0	5.00	2.00	1.00	0.50	0.20	0.00
By Volume								
Volume (mL) of **stock standard 4** diluted to 100 mL with **DI water**	100	50	25	10	5.0	2.5	1.0	---
By Weight								
Weight (g) of **stock standard 4** diluted to final weight (~100 g) divided by **factor** below with **DI water**	100	50	25	10	5.0	2.5	1.0	---
Division Factor Divide exact weight of the standard by this **factor** to give the final weight	---	0.5	0.25	0.1	0.05	0.025	0.01	---

Nitrite Standards – Low Range*

Working Standards (Prepare Daily)	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
Concentration mg N/L as NO_2	5.00	2.00	1.00	0.50	0.20	0.10	0.05	0.02	0.00
By Volume									
Volume (mL) of **stock standard 4** diluted to 100 mL with **DI water**	25	10	5.0	2.5	---	---	---	---	---

Volume (mL) of **standard A** (5 mg N/L) with **DI water**	---	---	---	---	4.0	2.0	1.0	0.4	---

By Weight

Weight (g) of **stock standard 4** diluted to final weight (~100 g) divided by **factor** below with **DI water**	25	10	5.0	2.5	---	---	---	---	---
Volume (mL) of **standard A** (5 mg N/L) with **DI water**	---	---	---	---	4.0	2.0	1.0	0.4	---
Division Factor Divide exact weight of the standard by this **factor** to give the final weight	0.25	0.1	0.05	0.025	0.04	0.02	0.01	0.004	---

*Note: Nitrite standards should be used to test the enzymatic reduction efficiency. This should be done at least after the calibration and at the end of the run to ensure that the NO_3 value generated is correct.

Enzymatic Reduction Efficiency

The enzymatic reduction efficiency can be determined as follows:

- Calibrate with nitrate (NO_3) standards.
- Run a know concentration of nitrite (NO_2) standard
- Run a matching concentration of NO_3 standard
- The reduction efficiency is determined by the equation in Figure 4.
- If the efficiency is less than 90%, the working NaR-NADH reagent (reagent 7) should be reactivated with another 4-mL vial of the NADH solution or the NaR-NADH reagent should be remade.

$$E = \frac{[NO_3]}{[NO_2]} \times 100$$

where:

E = Reduction efficiency

$[NO_3]$ = concentration of nitrate standard

$[NO_2]$ = concentration of nitrite standard

Figure 4

8. SAMPLE COLLECTION, PRESERVATION AND STORAGE

8.1. Samples should be collected in hard or soft plastic or glass bottles. All bottles must be thoroughly cleaned and rinsed with reagent water. Volume collected should be sufficient to ensure a representative sample, allow for replicate analysis (if required), and minimize waste disposal.

8.2. Sample preservation and holding time requirements for drinking water samples are as follows:

8.2.1 For nitrate: Chill the sample to 4^{o}C and analyze within 48 hours, unless the sample is chlorinated. If the sample is chlorinated, chill the sample to 4^{O}C and analyze within 14 days.

8.2.2. For nitrite: Chill the sample to 4^{o}C and analyze within 48 hours.

8.2.3. For nitrate-nitrite (combined): Acidify to pH<2 with concentrated H_2SO_4 at the time of collection, and analyze within 28 days. Acid preserved samples can be analyzed directly without neutralization with this method.

8.3. For wastewater samples, requirements are nearly identical except that 1) nitrite samples must be analyzed with 48 hours whether or not chlorine is present, and 2) nitrate-nitrite samples must also be chilled to 4^{o}C.

8.4. In all cases, samples should be analyzed as soon as possible after collection.

9. QUALITY CONTROL

9.1. Each laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability, and the periodic analysis of laboratory reagent blanks, fortified blanks and other laboratory solutions as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data that are generated. An analytical batch shall be defined as environmental samples that are analyzed together with the same method and personnel, using the same lots of reagents, not to exceed the analysis of 20 environmental samples.

9.1.1. Analyses of matrix spike and matrix spike duplicate samples are required to demonstrate method accuracy and precision and to monitor matrix interferences (interferences caused by the sample matrix). The procedure and QC criteria for spiking are described in section 9.3.

9.1.2. Analyses of laboratory blanks are required to demonstrate freedom from contamination.

9.1.3. The laboratory shall, on an ongoing basis, demonstrate through calibration verification and analysis of the ongoing precision and recovery sample that the analysis system is in control.

9.1.4. The laboratory should maintain records to define the quality of data that is generated.

9.2. INITIAL DEMONSTRATION OF PERFORMANCE

9.2.1. Method Detection Limit (MDL) –To establish the ability to detect the analyte, the analyst shall determine the MDL per the procedure in 40 CFR 136, Appendix B

using the apparatus, reagents, and standards, that will be used in the practice of this method. An MDL less than or equal to the MDL in section 1.2 must be achieved prior to the practice of this method.

9.2.2. Initial Precision and Recovery – To establish the ability to generate acceptable precision results, the operator shall perform 10 replicates of a mid-range standard, according to the procedure beginning in Section 11.

9.2.2.1. Using the results of the replicates compute the average percent recovery (X) and the standard deviation (s) for the analyte. Use the following equation for the calculation of the standard deviation.

Where, n = Number of samples, x = concentration in each sample

9.2.2.2. Compare s and x results with the corresponding data in Section 17. If the results meet the acceptance criteria, system performance is acceptable and analysis of samples may begin. If however, s and x do not match the data in Section 17, system performance is unacceptable. In this event correct the problem, and repeat the test.

9.3. Matrix spikes- The laboratory must spike, in duplicate, a minimum of 5 percent of all samples (one sample in each batch of no more than twenty samples) from a given sampling site or if for compliance monitoring, from a given discharge. The two sample aliquots shall be spiked with the stock standard (section 7.2).

9.3.1. The concentration of the spike in the sample shall be determined as follows:

9.3.1.1. If, as in compliance monitoring, the concentration of the analyte in the sample is being checked against a regulatory concentration limit, the spiking level shall be at that limit or at 1 to 5 times higher than the background concentration of the sample (determined in Section 9.3.2), which ever is higher.

9.3.1.2. If the concentration of the analyte in a sample is not being checked against a limit, the spike shall be at the concentration of the precision and recovery standard used in Section 9.2.5 or at 1 to 5 times higher than the background concentration, whichever concentration is higher.

9.3.2. Analyze one sample aliquot out of each set of no more than twenty samples from each site or discharge according to the procedure beginning in Section 11 to determine the background concentration of (B) of the analyte.

9.3.2.1. If necessary, prepare a standard solution appropriate to produce a level in the sample at the regulatory compliance limit or at 1 to 5 times the background concentration (per Section 9.3.1).

9.3.2.2. Spike two additional sample aliquots with the spiking solution and analyze these aliquots to determine the concentration after spiking (A)

9.3.3. Calculate the percent recovery (P) of the analyte in each aliquot using the following equation.

Where, A = Measured concentration of analyte after spiking, B = measured background concentration of analyte, T = True concentration of the spike

9.3.4. The percent recovery of the analyte should meet current laboratory acceptance criteria.

9.3.4.1. If the results of the spike fail the acceptance criteria, and the recovery of the QC standard in the ongoing precision and recovery test of the analytical batch is within the current laboratory acceptance criteria, an interference is present. In this case, the results may not be reported for regulatory compliance purposes and the analyst must assess the potential cause for the interference. If the interference is attributable to sampling, the site or discharge should be resampled. If the interference is attributable to a method deficiency, the analyst must modify the method, repeat the test required in Section 9.1.2 and repeat the analysis of the sample and the matrix spike.

9.3.4.2. If the results of both the spike and ongoing precision and recovery test fail the acceptance criteria, the analytical system is judged to be out of control, and the problem shall be identified and corrected, and the sample reanalyzed.

9.3.5. Compute the relative percent difference (RPD) between two sample results using the following equation:

Where, D1 = Concentration of analyte in the sample, D2 = Concentration of analyte in the second (duplicate) sample.

9.3.6. The RPD for duplicates shall meet the current laboratory acceptance criteria. If the criteria are not met, the analytical system is judged to be out of control, and the problem must be immediately identified and corrected and the analytical batch reanalyzed.

9.4 Laboratory blanks – Laboratory reagent water blanks are analyzed to demonstrate freedom from contamination.

9.4.1. Analyze a laboratory reagent water blank initially (with the test in Section 9.2) and with each analytical batch of no more than twenty samples. The blank must be subjected to the same procedural steps as a sample.

9.4.2. If analyte is detected in the blank at a concentration greater than the Minimum Level (Section 1.6), analysis of the samples is halted until the source of contamination is eliminated and a blank shows no evidence of contamination. All samples must be associated with an uncontaminated method blank before the results may be reported for regulatory compliance purposes.

9.5. Calibration Verification – Verify calibration using the procedure described in Section 10

9.6. On-going Precision and Recovery (OPR) – With every analytical batch of no more than twenty samples, a midrange standard must be prepared using the procedure described in Section 11.

9.6.1. Compare the results with the current laboratory acceptance criteria. If the criteria are not met, the analytical system is judged to be out of control, and the problem must be immediately identified and corrected and the analytical batch reanalyzed.

9.7. Quality Control Samples (QCS) – It is suggested that the laboratory obtain and/or prepare a quality control sample using a source different from the source routinely used in section 9.7.1. The QCS is used to verify the concentrations of the calibration standards.

9.8. Depending on the specific program requirements, field replicates and field spikes of the analytes of interest into samples may be required to assess the precision and accuracy of the sampling and sample transporting techniques.

10. CALIBRATION AND STANDARDIZATION

10.1. Prepare reagents and standards as described in Section 7.

10.2. Set up manifold as shown in Section 17.

10.3. Input data system parameters as shown in Section 17.

10.4. Pump DI water through all reagent lines and check for leaks and smooth flow. Switch to reagents and allow the system to equilibrate until a stable baseline is achieved.

10.5. Place standards in the sampler. Input the information required by the data system.

10.6. Calibrate the instrument by injecting the standards. The data system will then associate the concentrations with the peak area for each standard to determine the calibration curve.

10.7. Verify calibration using a midrange calibration standard every ten samples or every analytical batch. Compute the percent recovery using the following equation:

Where, D = Determined concentration of analyte in the calibration standard, K = Actual concentration of the analyte in the calibration standard

10.8. If % recovery exceeds +/-10%, the analytical system is judged to be out of control, and the problem must be immediately identified and corrected and the analytical batch reanalyzed.

11. PROCEDURE

11.1 ENZYMATIC REDUCTION

11.1.1 **High Range Analysis -** Pipette 1 mL of the working NaR-NADH reagent (reagent 5) into the sample tube to analyze one sample replicate per sample tube. Pipette 2 mL of reagent 5 to analyze two replicates out of one sample tube. The ratio of sample to reagent 5 is a **20-fold dilution** (1000 µL NaR-NADH / 50 µL sample/standard). Therefore, add **50 µL sample/standard** to **1 mL of reagent 5 (NaR-NADH)** to analyze **one replicate**, and add **100 µL sample/standard** to **2 mL of reagent 5 (NaR-NADH)** to analyze **two replicates**. Once the sample/standard is added to the appropriate amount of the working NaR-NADH

reagent vortex the sample for ~ 5 seconds to ensure that the solution is completely mixed. Once the sample/standard has been added and mixed, **wait for at least 20 minutes to ensure that reaction has completed the enzymatic reduction.** Once the 20-minute reaction period has elapsed, the reduced samples can be stored at 4°C for up to 24 hours before analysis.

11.1.2. **Low Range Analysis -** Pipette 1 mL of the working NaR-NADH reagent (reagent 5) into the sample tube to analyze one sample replicate per sample tube. Pipette 2 mL of reagent 5 to analyze two replicates out of one sample tube. The ratio of sample to reagent 5 is a **10-fold dilution** (1000 µL NaR-NADH / 100 µL sample/standard). Therefore, add **100 µL sample/standard** to **1 mL of reagent 5 (NaR-NADH)** to analyze **one replicate**, and add **200 µL sample/standard** to **2 mL of reagent 5 (NaR-NADH)** to analyze **two replicates**. Once the sample/standard is added to the appropriate amount of the working NaR-NADH reagent vortex the sample for ~ 5 seconds to ensure that the solution is completely mixed. Once the sample/standard has been added and mixed, **wait for at least 20 minutes to ensure that reaction has completed the enzymatic reduction. You add twice as much sample/standard to NaR-NADH for the Low Range compared to the High Range analysis.** Once the 20-minute reaction period has elapsed, the reduced samples can be stored at 4°C for up to 24 hours before analysis.

The reason more sample/standard is added for the low range analysis is that to achieve a lower concentration range, more nitrate ions are needed for the enzymatic reduction nitrite to increase the color development for the analysis.

11.2 START UP PROCEDURE

11.2.1 Prepare reagents and standards as described in Section 7.

11.2.2 Set up manifold as shown in Section 17.

11.2.3 Input data system parameters as in Section 17.

11.2.4 Pump DI water through all reagent lines and check for leaks and smooth flow. Switch to reagents and allow the system to equilibrate until a stable baseline is achieved.

11.2.5 Place samples in the autosampler. Input the sample identification required by the data system. Follow the sample preparation listed in sections 11.1 and 11.2.

11.2.6. Troubleshooting Guide in the System Operation Manual. This guide is also available on request from Lachat Instruments.

12. DATA ANALYSIS AND CALCULATIONS

12.1. Calibration is done by injecting standards. The data system will then prepare a calibration curve by plotting peak area versus standard concentration. Sample concentration is calculated from the regression equation.

12.2. Report only those values that fall between the lowest and the highest calibration standards. Samples exceeding the highest standard should be diluted and reanalyzed.

12.3 Report sample results for nitrate/nitrite in mg N/L as NO_3^- or NO_2^- to two significant figures for samples above the MDL. Report results below the MDL as less than the detection limit.

13. METHOD PERFORMANCE

13.1. The method support data are presented in section 17. This data was generated according to a Lachat Work Instruction during development of the method.

13.2. Although Lachat Instrument publishes method performance data, including MDL, precision, accuracy and carryover studies, we cannot guarantee that each laboratory will be capable of meeting such performance. Individual laboratory and instrument conditions, as well as laboratory technique, play a major role in determining method performance. The support data serves as a guide of the potential method performance. Some labs may not be able to reach this level of performance for various reasons, while other labs may exceed it.

14. POLLUTION PREVENTION

14.1. Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The USEPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the United States Environmental Agency (USEPA) recommends recycling as the next best option.

14.2. The quantity of chemicals purchased should be based on expected usage during their shelf life and disposal cost of unused material. Actual reagent preparation volumes should reflect anticipated usage and reagent stability.

14.3. For information about pollution prevention that may be applicable to laboratories and research institutions, consult "Less is Better: Laboratory Chemical Management for Waste Reduction," available from the American Chemical Society's Department of Government Regulations and Science Policy, 1155 16th Street N. W., Washington D. C. 20036, (202) 872-4477.

15. WASTE MANAGEMENT

15.1. It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the hazardous waste identification rules and land disposal restrictions, and to protect the air, water and land by minimizing and controlling all releases from fume hoods and bench operation. Compliance with all sewage discharge permits and regulations is also required.

15.2. For further information on waste management consult the "Waste Management Manual for Laboratory Personnel", available from the American Chemical Society's Department of Government Regulations and Science Policy, 1155 16th Street N. W., Washington D. C. 20036, (202) 872-4477.

16. REFERENCES

16.1. U.S. Environmental Protection Agency, Methods for Chemical Analysis of Water and Wastes Method 353.2

16.2. Methods for Determination of Inorganic Substances in Water and Fluvial Sediments. Book 5. Chapter A1. U.S. Department of the Interior, U.S. Geological Survey.

16.3. Campbell, W.H.; Kinnunen-Skidmore, T.; Brodeur-Campbell, M.J.; Campbell, E.R. New and improved nitrate reductase for enzymatic nitrate analysis. *Am.Lab. News* **2004**, *22*(10), 12.

16.4. Patton CJ, AE Fischer, WH Campbell & ER Campbell (2002) Corn leaf nitrate reductase: A nontoxic alternative to cadmium for photometric nitrate determination in water samples by air-segmented continuous-flow analysis. Environmental Science and Technology, 36: 729-35.

16.5. Protocol for using Superior Stock Nitrate Reductase (YNaR1) and the NECi Enzymatic Nitrate Reduction Method in a Batch Mode These reagents are suitable for adapting to flow-injection analysis systems or for manual flow systems. Prepared by CJ Patton, US Geological Survey, Nov2003, and modified by NECi, 2005.

17. TABLE, DIAGRAMS, FLOWCHARTS, AND VALIDATION DATA

17.1. DATA SYSTEM PARAMETERS FOR THE QUIKCHEM 8000/8500

The timing values listed below are approximate and will need to be optimized using graphical events programming.

Sample throughput:	80 samples/h, 45 s/sample
Pump Speed:	35
Cycle Period:	45

Analyte Data:

Concentration Units:	mg N/L as NO_3
Inject to Peak Start:	15.7 s
Peak Base Width:	47.4 s
Chemistry:	Direct/Bipolar

Calibration Data:

High Range

Level	1	2	3	4	5	6	7	8
Concentration mg N/L	20.0	10.0	5.00	2.00	1.00	0.50	0.20	0.00

Low Range

Level	1	2	3	4	5	6	7	8	9
Concentration mg N/L	5.00	2.00	1.00	0.50	0.20	0.10	0.05	0.02	0.00

Calibration Fit Type:	2nd Order Polynomial
Weighting Method:	1/X
Force through zero:	No

Sampler Timing:*

Min. Probe in Wash Period:	5 s
Sample Period:	14 s

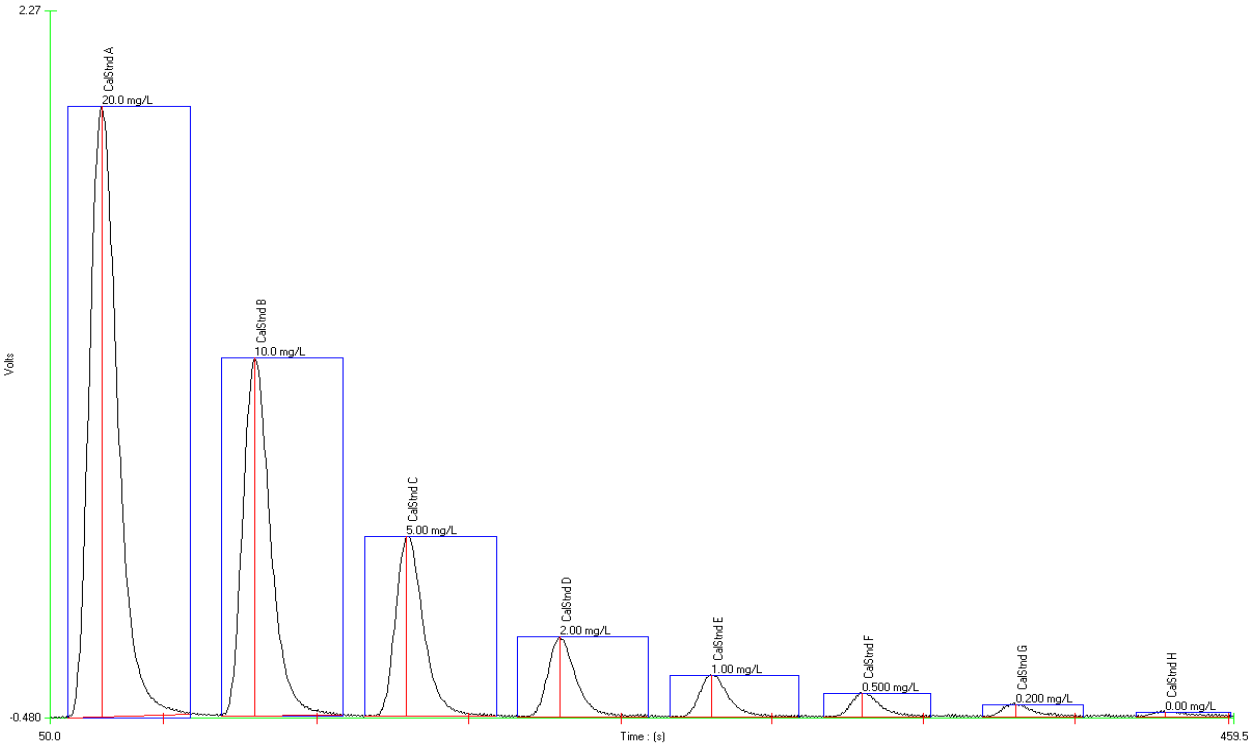
Valve Timing:*

Load Period:	9 s
Inject Period:	36 s

* Due to small sample volume the **timing is VERY critical for this method**. Since there is only ~ 1 mL of sample for one replicate and 2 mL for analyzing two sample replicates, the timing has needs to be optimized to ensure the sample gets loaded on the injection valve. To achieve the correct sample and valve timing run an universal dye sample to calculate the 'Time to Valve' timing; this timing should be ~ 20 seconds. Once this timing has been input into the software, place 1050 µL of universal dye into a sample tube to verify the 'Time to Valve', 'Sample Period', and 'Load Period' listed above are sufficient to inject a the sample slug onto the manifold. Once the 'Time to Valve' timing is calculated correctly, the times of 14 seconds for the 'Sample Period' and 9 seconds for the 'Load Period' should be correct, but timing modifications may be needed.

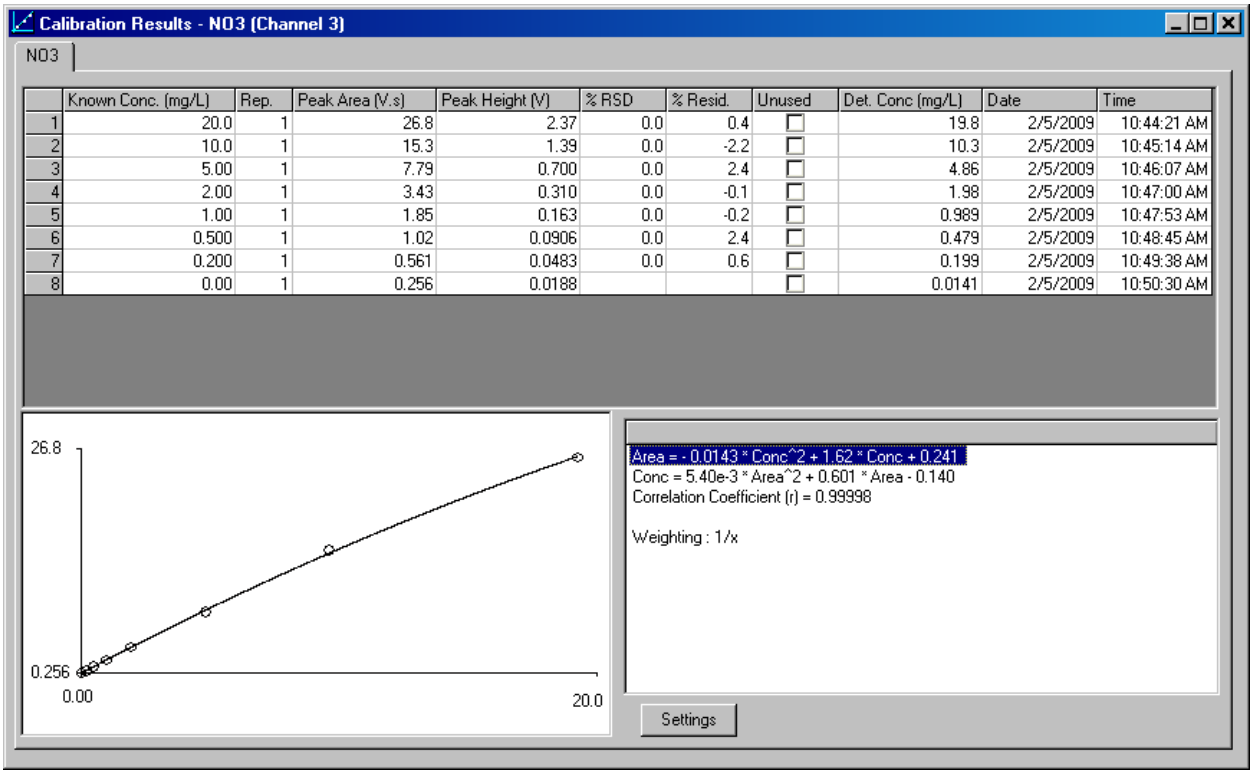
17.2. SUPPORT DATA FOR THE QUIKCHEM 8000/8500

Calibration Data for Nitrate – High Range

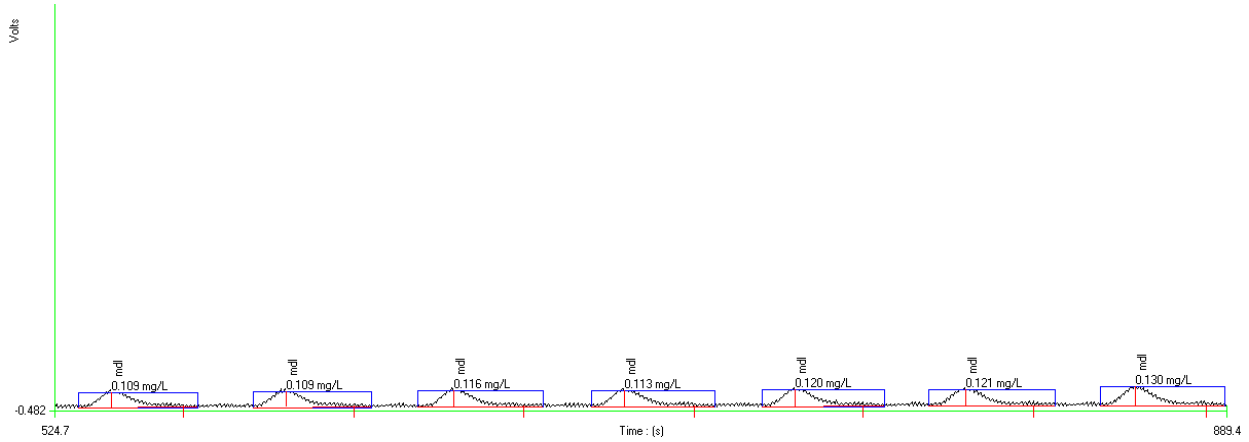


File Name: 2-5 HR cal mdl.omn
Acq. Date: 5 February 2009

Calibration Graph and Statistics



	Known Conc. (mg/L)	Rep.	Peak Area (V.s)	Peak Height (V)	% RSD	% Resid.	Unused	Det. Conc (mg/L)	Date	Time
1	20.0	1	26.8	2.37	0.0	0.4	☐	19.8	2/5/2009	10:44:21 AM
2	10.0	1	15.3	1.39	0.0	-2.2	☐	10.3	2/5/2009	10:45:14 AM
3	5.00	1	7.79	0.700	0.0	2.4	☐	4.86	2/5/2009	10:46:07 AM
4	2.00	1	3.43	0.310	0.0	-0.1	☐	1.98	2/5/2009	10:47:00 AM
5	1.00	1	1.85	0.163	0.0	-0.2	☐	0.989	2/5/2009	10:47:53 AM
6	0.500	1	1.02	0.0906	0.0	2.4	☐	0.479	2/5/2009	10:48:45 AM
7	0.200	1	0.561	0.0483	0.0	0.6	☐	0.199	2/5/2009	10:49:38 AM
8	0.00	1	0.256	0.0188			☐	0.0141	2/5/2009	10:50:30 AM



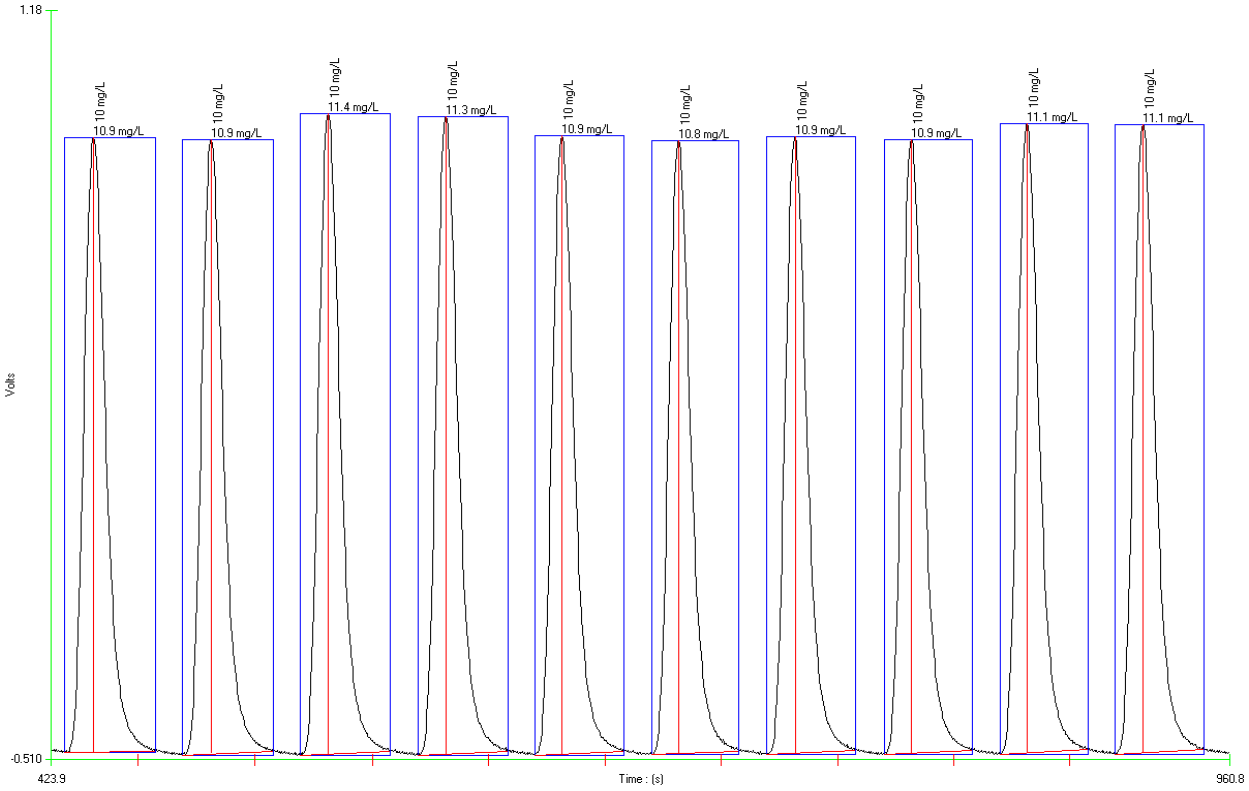
Method Detection Limit for nitrate using 0.1 mg N/L standard

MDL= 0.023 mg N/L

Standard Deviation (s) = 0.008 mg N/L, Mean (x) = 0.117 mg N/L, Known value = 0.10 mg N/L

File Name: 2-5 HR cal mdl.omn

Acq. Date: 5 February 2009



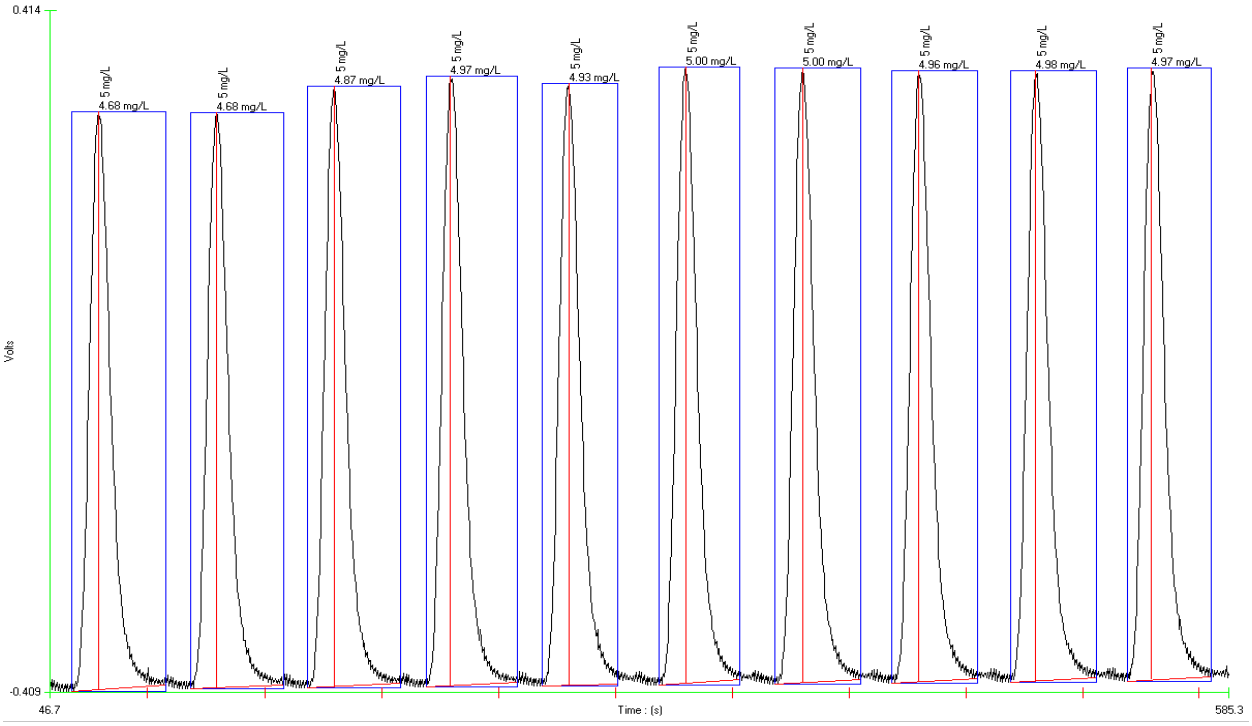
Precision data for nitrate using 10 mg N/L standard

% RSD = 1.80

Standard Deviation (s) = 0.20 mg N/L, Mean (x) = 11.02 mg N/L, Known value = 10 mg N/L

File Name: 2-5 HR prec effic.omn

Acq. Date: 5 February 2009



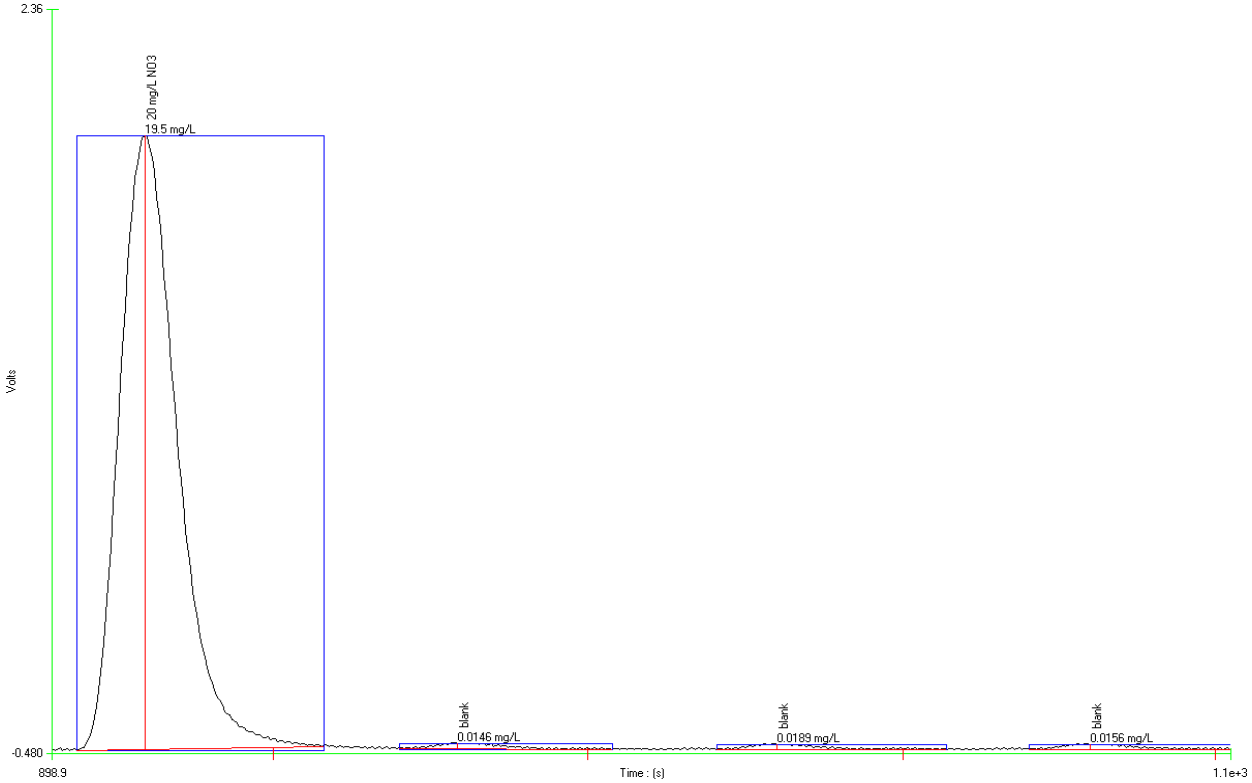
Precision data for nitrate using 5.0 mg N/L standard

% RSD = 2.53

Standard Deviation (s) = 0.124 mg N/L, Mean (x) = 4.90 mg N/L, Known value = 5.0 mg N/L

File Name: 2-5 HR prec preservation.omn

Acq. Date: 5 February 2009



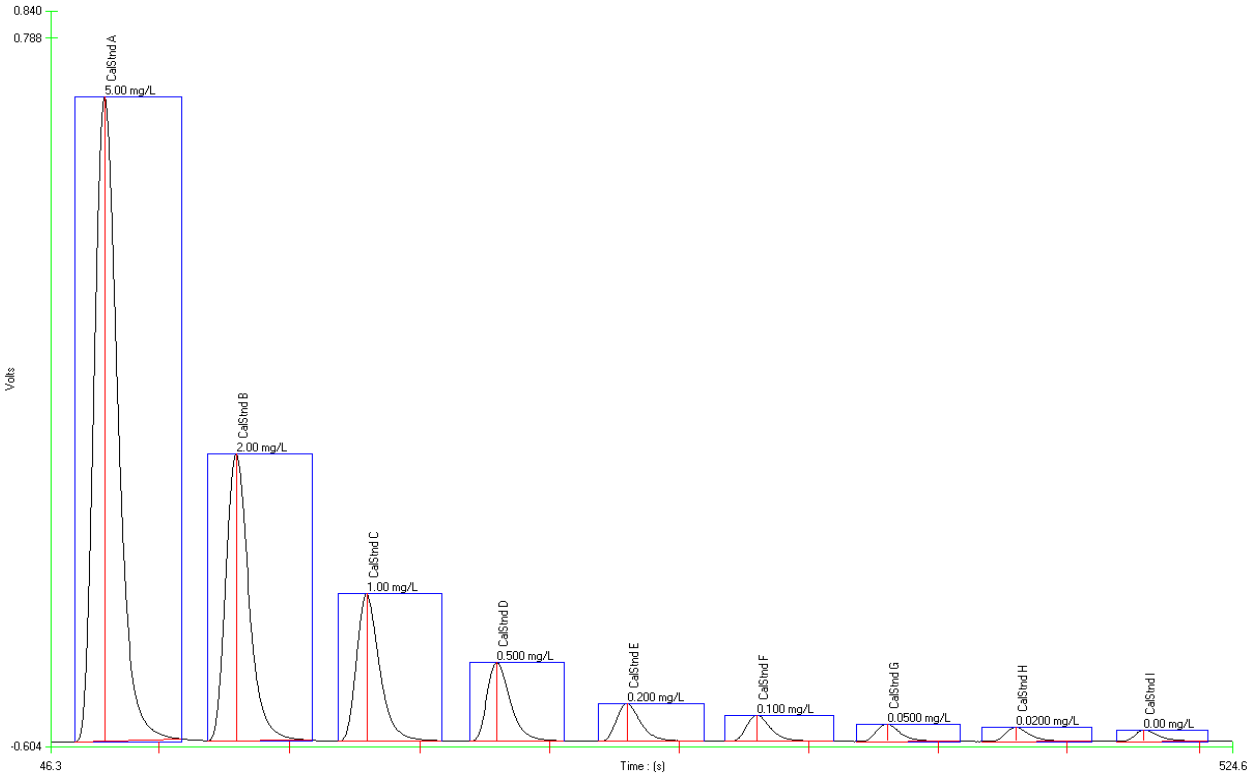
Carryover Study: 20 mg N/L standard followed by 3 blanks

Carryover Passed

File Name: 2-5 HR cal mdl.omn

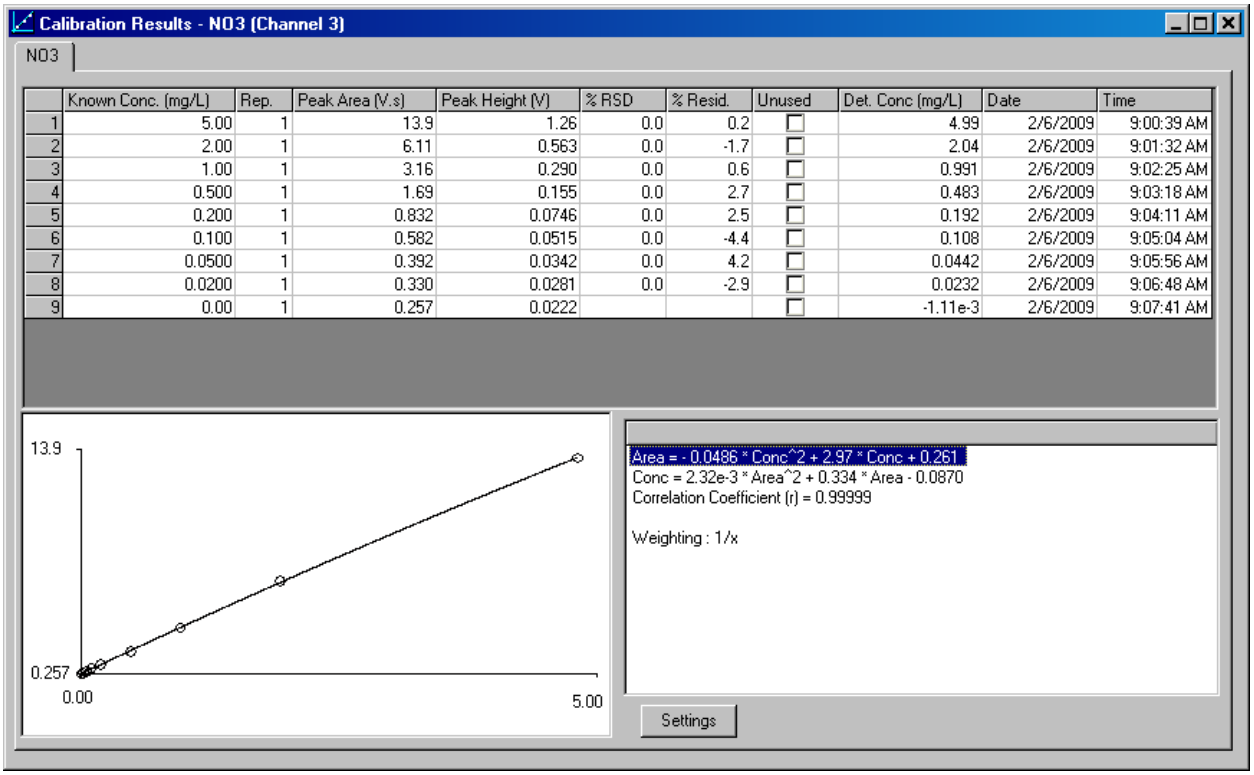
Acq. Date: 5 February 2009

Calibration Data for Nitrate – Low Range

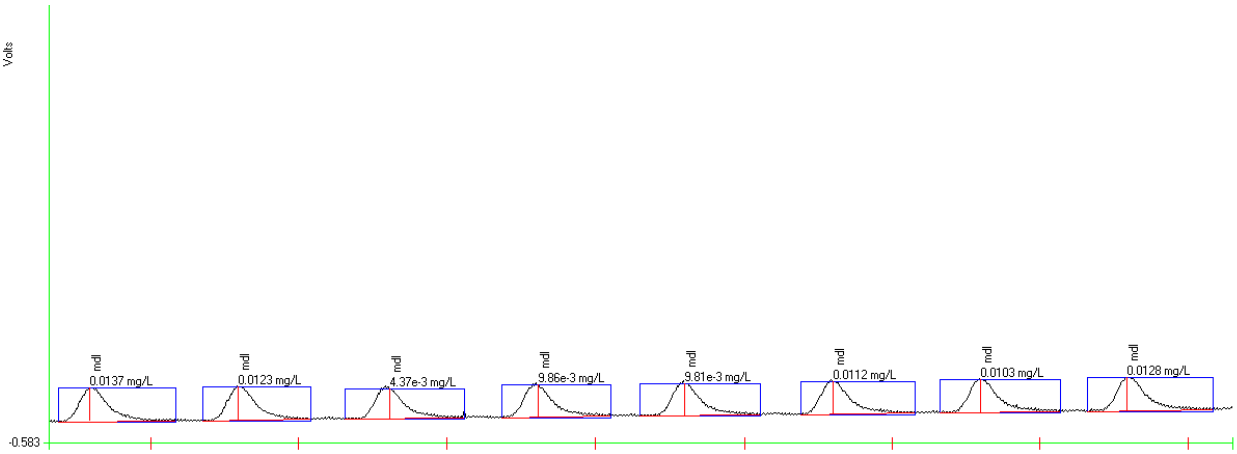


File Name: 2-6 HR cal.omn
Acq. Date: 6 February 2009

Calibration Graph and Statistics



	Known Conc. (mg/L)	Rep.	Peak Area (V.s)	Peak Height (V)	% RSD	% Resid.	Unused	Det. Conc (mg/L)	Date	Time
1	5.00	1	13.9	1.26	0.0	0.2	☐	4.99	2/6/2009	9:00:39 AM
2	2.00	1	6.11	0.563	0.0	-1.7	☐	2.04	2/6/2009	9:01:32 AM
3	1.00	1	3.16	0.290	0.0	0.6	☐	0.991	2/6/2009	9:02:25 AM
4	0.500	1	1.69	0.155	0.0	2.7	☐	0.483	2/6/2009	9:03:18 AM
5	0.200	1	0.832	0.0746	0.0	2.5	☐	0.192	2/6/2009	9:04:11 AM
6	0.100	1	0.582	0.0515	0.0	-4.4	☐	0.108	2/6/2009	9:05:04 AM
7	0.0500	1	0.392	0.0342	0.0	4.2	☐	0.0442	2/6/2009	9:05:56 AM
8	0.0200	1	0.330	0.0281	0.0	-2.9	☐	0.0232	2/6/2009	9:06:48 AM
9	0.00	1	0.257	0.0222			☐	-1.11e-3	2/6/2009	9:07:41 AM



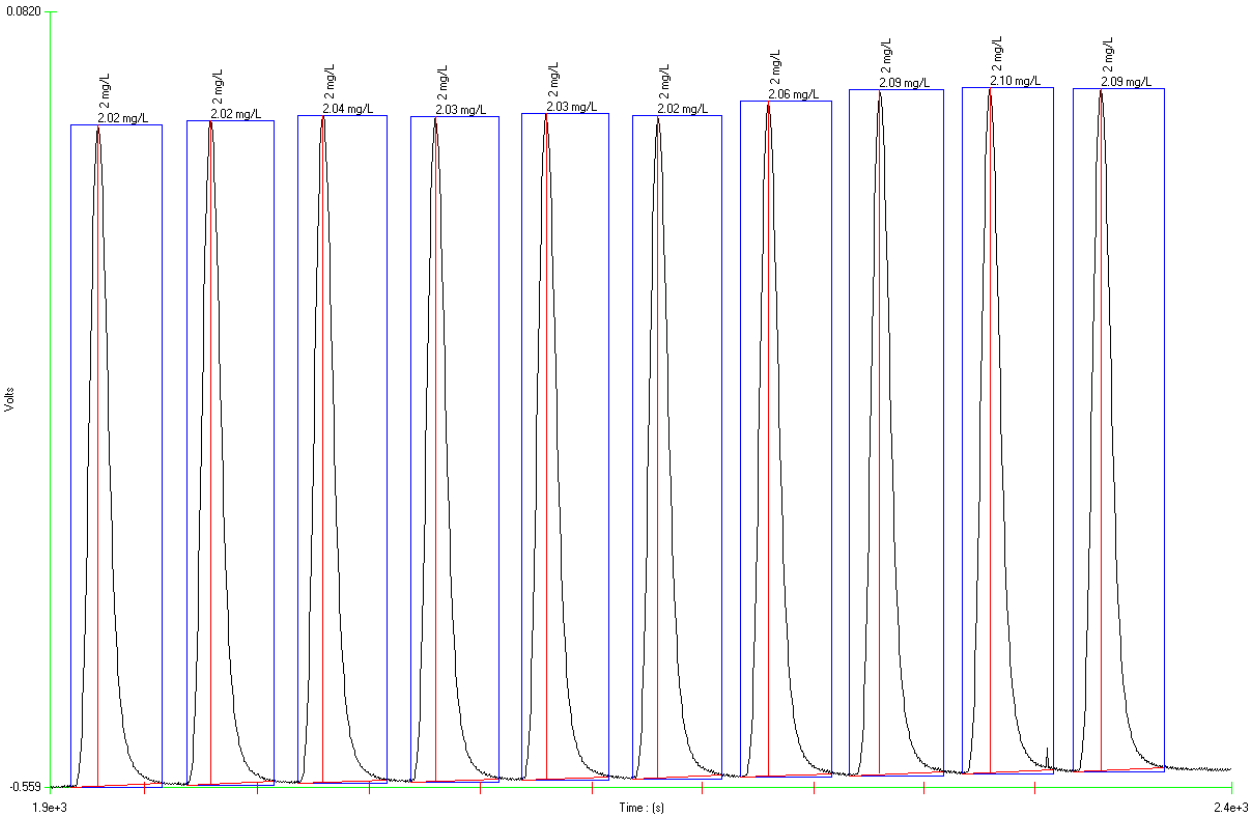
Method Detection Limit for nitrate using 0.01 mg N/L standard

MDL= 0.009 mg N/L

Standard Deviation (s) = 0.003 mg N/L, Mean (x) = 0.011 mg N/L, Known value = 0.010 mg N/L

File Name: 2-6 LR mdl.omn

Acq. Date: 6 February 2009



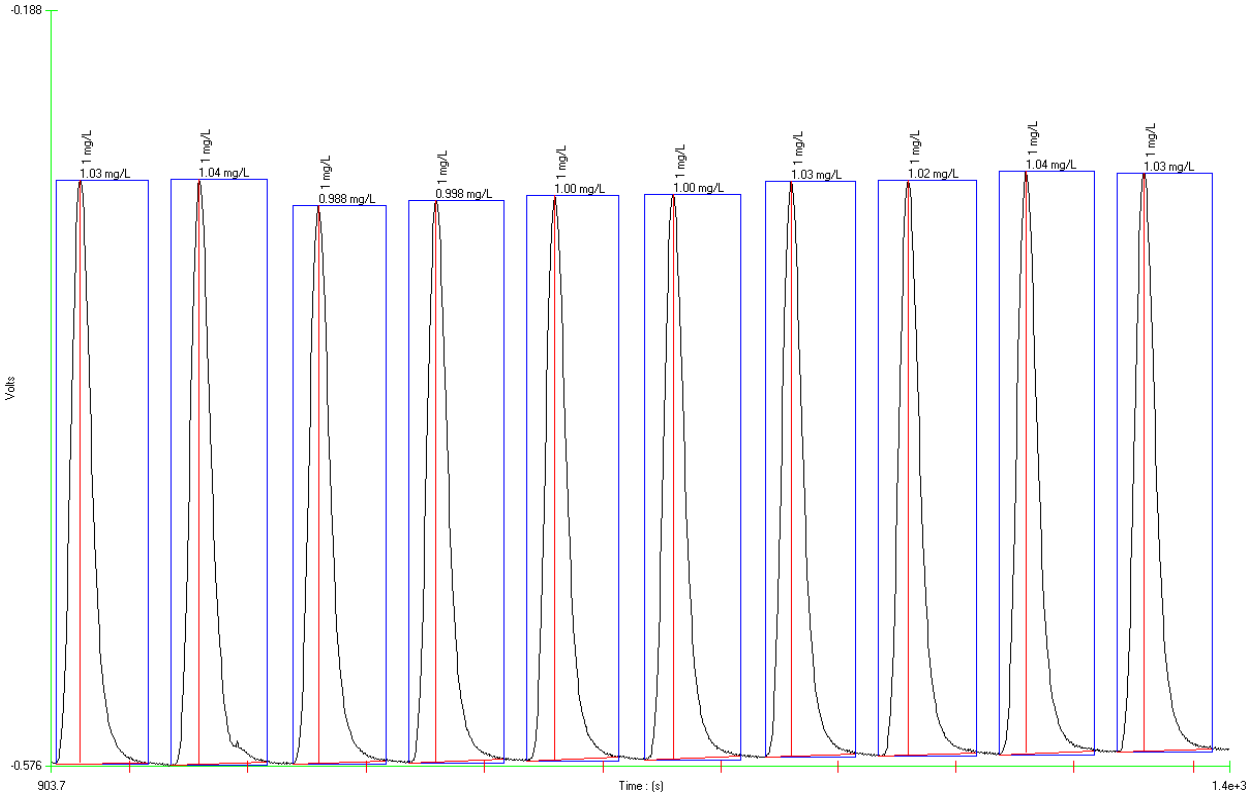
Precision data for nitrate using 2.0 mg N/L standard

% RSD = 1.58

Standard Deviation (s) = 0.032 mg N/L, Mean (x) = 2.05 mg N/L, Known value = 2.0 mg N/L

File Name: 2-6 LR support.omn

Acq. Date: 6 February 2009



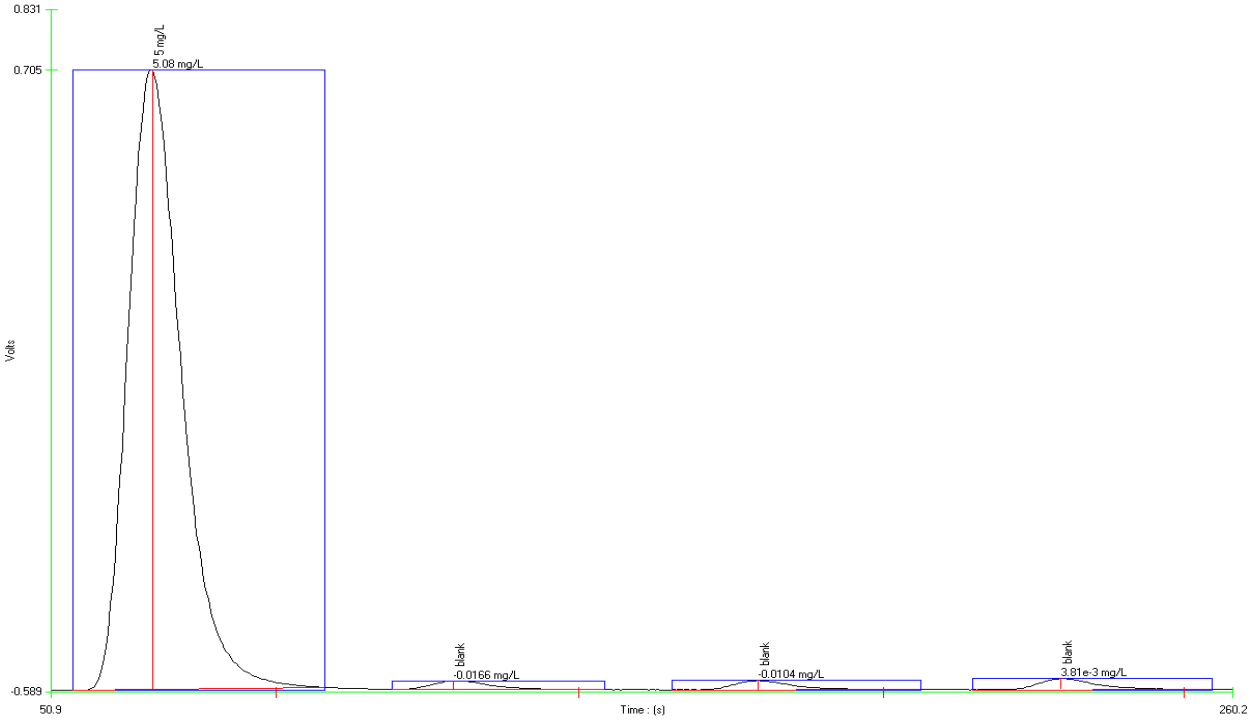
Precision data for nitrate using 1.0 mg N/L standard

% RSD = 1.90

Standard Deviation (s) = 0.019 mg N/L, Mean (x) = 1.02 mg N/L, Known value = 1.0 mg N/L

File Name: 2-6 LR support.omn

Acq. Date: 6 February 2009



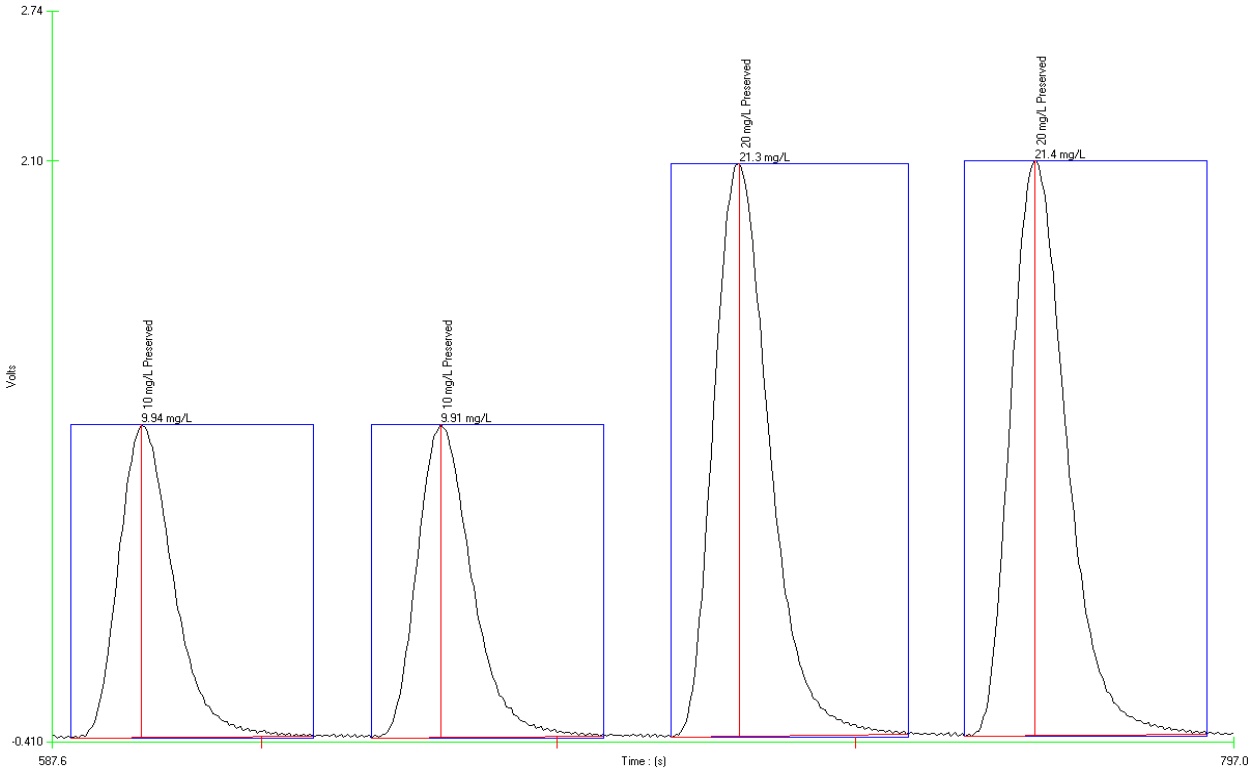
Carryover Study: 5 mg N/L standard followed by 3 blanks

Carryover Passed

File Name: 2-6 LR support.omn

Acq. Date: 6 February 2009

Acid Preserved Standards

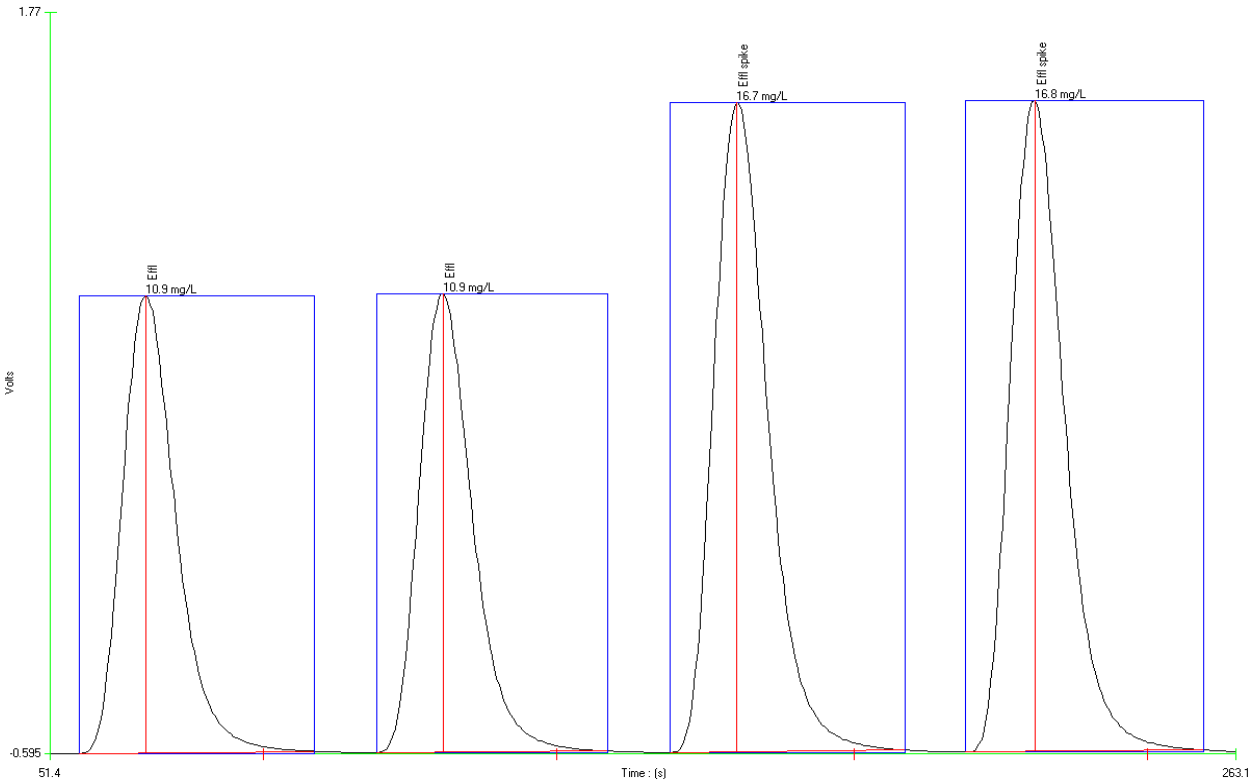


File Name: 2-5 HR prec preservation.omn
Acq. Date: 5 February 2009

Acid Preserved Standard	Avg Concentration	% Recovery
10 mg N/L	9.93	99.3%
20 mg N/L	21.35	106.8%

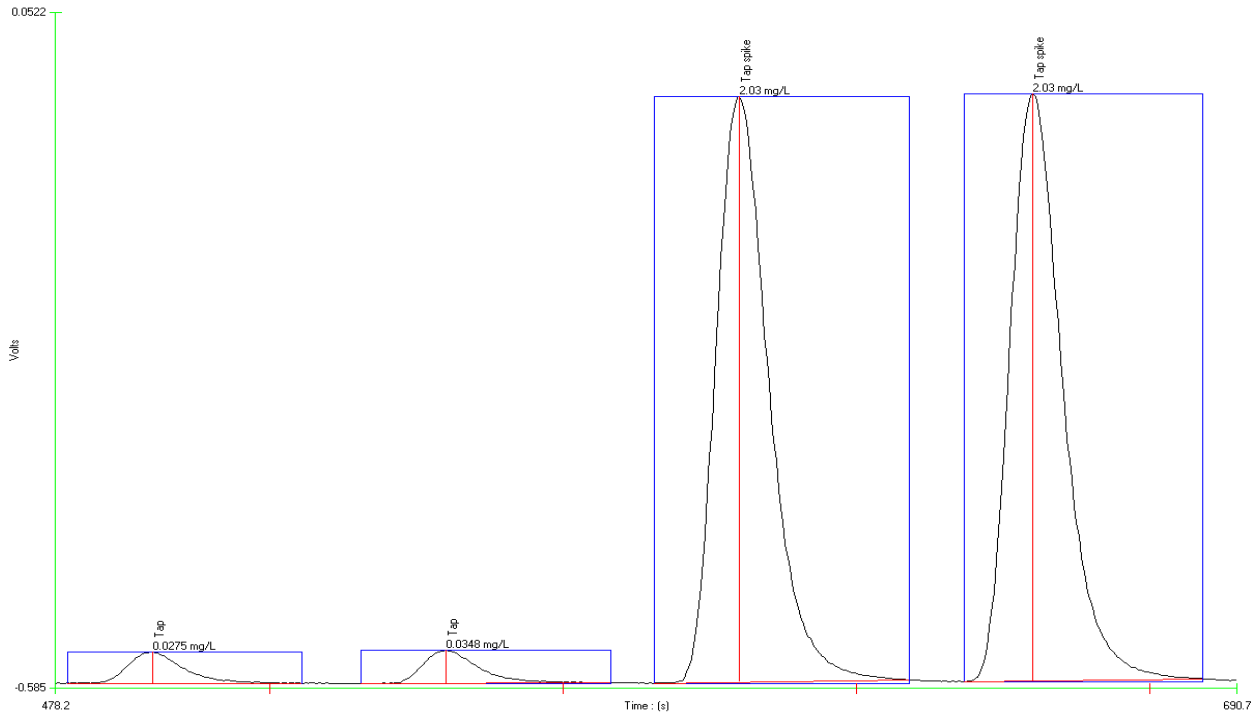
Analyzed preserved standards with calibration standards prepared in DI water. The preserved standards recovered at least 99%. Therefore, samples that have been acid preserved can be analyzed without a prior neutralization step. (Standard preservation of 2 mL concentrated H3SO4/L of sample).

Wastewater Spike



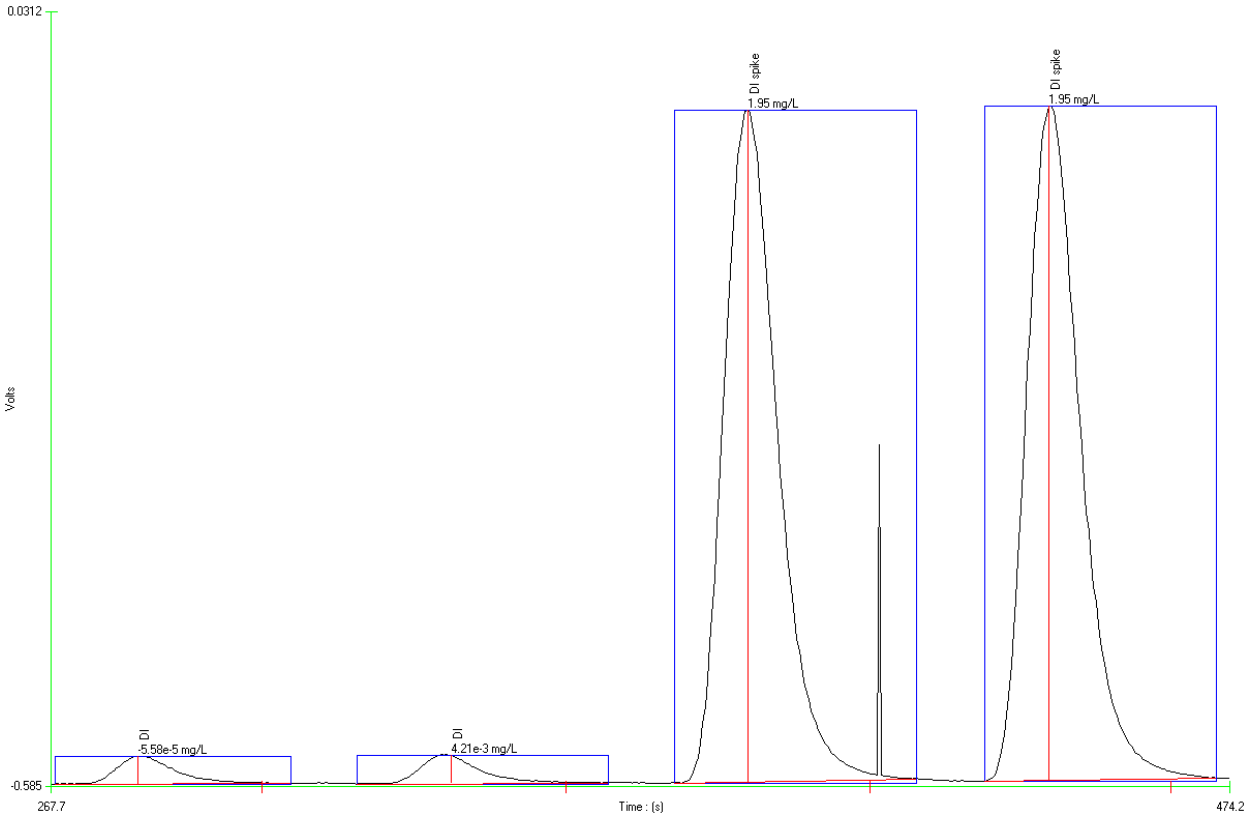
File Name: 2-5 HR spike mdl.omn
Acq. Date: 5 February 2009

Tap Water Spike



File Name: 2-6 LR support.omn
Acq. Date: 5 February 2009

Ultra-pure Water Spike



File Name: 2-6 LR support.omn
Acq. Date: 5 February 2009

Sample ID	Unspiked conc	Spiked conc	Spiked amount	% Recovery
Effluent	10.9 mg/L	16.75 mg/L	5 mg/L	117.0%
Tap Water	0.03 mg/L	2.03 mg/L	2 mg/L	99.94%
Ultra-pure Water	-0.017 mg/L	2.25 mg/L	2 mg/L	113.6%