Standard Operating Procedure for:

Nitrate + Nitrite

University of Missouri Limnology Laboratory

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1 Identification of the method

1.1 Measurement of nitrate+nitrite via in-line reduction followed by colorimetric flow injection analysis (APHA Method 4500-NO_3^- I).

2 Application matrix or matrices

2.1 This method is suitable for the analysis of environmental samples.

3 Detection limit

3.1 Method Detection Limit: 0.005 mg N/L

3.2 This Method Detection Limit was determined by taking the calculated concentrations of the lowest calibration standard from multiple analytical runs (using seven or more individual values taken from at least three analytical runs) and calculating a standard deviation from those concentrations. This standard deviation is then multiplied by the one-sided t-statistic at the 99% confidence level for the appropriate degrees of freedom (n – 1).

4 Scope of the method

4.1 This standard operating procedure is intended to provide MU Limnology technicians and analysts with guidance on the analysis of nitrate+nitrite with the Lachat Quikchem 8500. This document is not intended to replace individual training in this method by an experienced MU Limnology technician.

5 Summary of the method

5.1 Filtered water samples are drawn into the Lachat Quickchem flow injection system by a peristaltic pump. Dissolve nitrate is reduced to nitrate via an in-line copperized cadmium column. Nitrite reacts sequentially with sulfanilamide and N-(1-napthyl)ethyenediamene dihydrochloride to produce a soluble dye proportional to the original nitrate+nitrite concentration The resulting solution is measured at 520 nm and peak areas are converted to concentrations based on a 2nd order calibration curve.

5.2

- Operating Range: 0.005 to 1.0 mg N/L
- Sample Volume: 5 ml
- Sample Injection Volume: 1-2 ml
- Run Time: 1.5 hrs
- Samples per run: 40

6 Interferences

6.1 Nitrate is not a stable species in water. Samples must be filtered within 12 hours of collection and should be frozen immediately. Leaving samples thawed for extended periods may result in the transformation of nitrate in the sample.

6.2 Excess chlorine concentrations, oxygen, and unbuffered solutions may result in the degradation of the cadmium column.

6.3 While EDTA is used to reduce to reduce interference from metals, samples with unusually high concentrations of copper and iron may produce low results.

6.4 High sample turbidity, oil, and grease will all interfere with results.

7 Health and Safety

7.1 This method involves handling freshwater samples that may contain live microorganisms and therefore pose some threat of infection. Laboratory personnel who are routinely exposed to such water samples are encouraged to protect themselves from water borne illnesses by wearing clean disposable gloves and washing hands frequently.

7.2 Wear protective gloves, lab coats, and other appropriate PPE when handling all chemical substances used in this method. All technicians performing this method should review the MSDS for additional information and safety concerns regarding the chemical substances used throughout these procedures.

7.3 The following chemicals used in this method are considered especially hazardous and should be handled with extra care:

- Cadmium
- Sodium Hydroxide
- Phosphoric Acid
- Sulfanilamide

8 Personnel qualifications

8.1 This method is considered advanced. Lab personnel should be trained to the technician level in a number of other lab protocols before being trained in this method. They must also be familiar with all standard MU Limnology sampling handling and labeling procedures and appropriate SOPs. There is no operator designation for this method. The lowest level of certification is as a technician.

8.2 New technicians learning to operate this method should perform 4 runs before being certified.

- Run 1: The trainee should watch an experienced technician carry out all parts of a run (including reagent preparation, data analysis, etc.)
- Run 2: The trainee should carry out a run with close supervision.
- Run 3: The trainee should carry out a run independently with occasional check-ins.

- Run 4: The trainee should carry out a run full independently. An experienced technician should check the results of this run after its finished.
- If a trainee completes all 4 runs without significant issues (poor sample replication, bad calibration, drifting base lines), they may be certified as a technician.

9 Equipment and supplies

9.1 Sodium Hydroxide, Fisher Chemical, S318-500
9.2 Ammonium Chloride
9.3 Disodium Ethylenediamine Tetraacetic Acid Dihydrate, Fisher Chemical, S311-500
9.4 Copper Sulfate
9.5 O-Phosphoric Acid, 85%, Fisher Chemical A242-1
9.6 Sulfanilamide, Fisher Chemical, 04525-100
9.7 1.0 g N-(1-napthyl) ethylenediamine dihydrochloride, Acros Organics, 42399-0250
9.8 1000 mg N/L Nitrate Standard, RICCA, R5307900-120A
9.9 1000 mg N/L Nitrite Standard, RICCA, R5444900-500
9.10 Volumetric Glassware
9.11 Cadmium Column for Nitrate, Hach, 90237A
9.12 Whatman GF/F Glass Microfiber Filters, 0.7 □m pore size, 47mm, CAT No. 1825-047
9.13 47 mm Magnetic Filter Flask, PALL
9.14 Internal Vacuum System or Portable Vacuum Pump
9.15 1 ml and 10 ml micropipettes

10 Reagents and standards

10.1 Calibration Standards
10.2 Prepare standards as shown below in Table 1 in clean volumetric flasks. All stock solutions should be added quantitatively using a calibrated micropipette. Rinse all glassware three times with UPDI and then fill ~75 % of the way to the line. Then add appropriate stock solution, fill to the line with UPDI, cover with parafilm and invert three times to mix. Standards may be stored in plastic at 4 deg C for up to 100 hours after preparation.

10.3 Table 2: Preparation of Nitrate Standards

Standard Concentration (mg N/L)	1.0	0.5	0.25	0.1	0.05	0.025	0.01	0	1.0 nitrite
Total Standard Volume (ml)	100	250	500	1000	100	100	100	100	100
Volume 1000 mg N/L Nitrate Standard	0.1	0.125	0.125	0.1	-	-	-	-	-
Volume 1 mg N/L Nitrate Standard	-	-	-	-	5	2.5	1		-
Volume 1000 mg N/L Nitrite Standard	-	-	-	-	-	-	-	-	0.1

10.4 Sulfanilamide

Add ~600 ml of UPDI to 1L volumetric flask followed by 100ml of 85 % phosphoric acid, 40.0 g of sulfanilamide and 1.0 g of well crushed N-(1-naphthyl) ethylenediamine dihydrochloride. Magnetically stir until all reagents are dissolved, dilute the mark, and invert three times to mix. Store this reagent in an amber container at 4 deg C. This reagent may be kept for up to a month or when it begins to discolor (look for a faint red tint).

10.5 Ammonium Chloride Buffer

To a 1L volumetric flask add 600ml of UPDI followed by 85.0 g of ammonium chloride and 1.0 g of disodium ethylenediamine tetraacetic acid dihydrate and 4.7 g of sodium hydroxide. Magnetically stir until reagents are dissolved then add 4 drops of copper sulfate. Dilute to the mark and invert three times to mix. This reagent can be stored in plastic at room temperature for up to 3 months.

11 Quality Control

11.1 Reagent Coloring

Prior to beginning analysis, all reagents should be visually expected for discoloring. In particular, technicians should check for a slight red discoloration of the sulfanilamide reagent as this can be a sign that it is no longer usable. If discoloration is observed, reagents should be reprepared.

11.2 Baseline Check:

Before beginning the run, technicians should observe the instrument's baseline for 5 minutes and record it in the log. Technicians should ensure that the baseline remains stable in the range of 1V during this time period. Significant movement upwards or downwards in the baseline, or a baseline significantly above 1V may be a sign of reagent contamination. If the issue persists, regents should be remade.

11.3 Column Efficiency:

A 1 mg N/L nitrate standard and 1 mg N/L nitrite standard should be run prior to analysis of any samples. The column efficiency should be calculated immediately following the analysis of these standards. The run should be halted if the column efficiency is less than 80 %. The column should then be replaced. This process should be repeated at the end of the run as well.

11.4 Check Standards

For every 20 sample measurements (10 sets of 2 duplicates) a check standard must be analyzed. Check standards will be identical to the calibration standards used in the run. Check standards should be run in descending concentration starting from the 1 mg N/L standard down to the 0 mg N/L standard. The full range of standards used in the calibration must be run as check standards before ending a run, even if the total number of samples analyzed is less than 10*Number of standards.

11.5 Secondary Standards

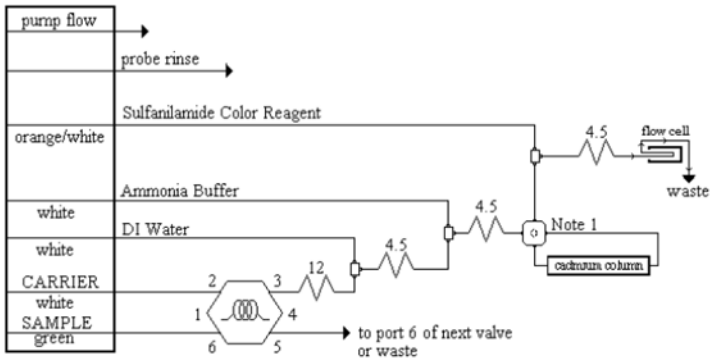
For every 20 sample measurements (10 sets of 2 duplicates) a secondary standard must be analyzed. Check standards should be identical to the calibration standards in concentration but prepared from a different stock solution. Secondary standards should be run in descending concentration starting from the 1 mg N/L standard down to the 0 mg N/L standard. The full range of standards must be run before ending a run, even if the total number of samples analyzed is less than 10*Number of standards.

12 Analysis

12.1 Set up.

- 30 minutes prior to analysis, all reagents and standards should be placed at room temperature and allowed to sit until they are no longer cold to the touch.
- Place samples in a room temperature water bath until completely thawed.
- Connect optical filters and reagent lines as shown below (Figure 1).
- Ensure that the nitrate column is in the “off” position.
- Place all reagent lines in the UPDI container and set hit “run” on the peristaltic pump.
- Let run for 10 minutes, then place reagent lines in their appropriate containers and let run for 5 minutes. Also place the rinse line is a separate container of UPDI.
- Turn the nitrate column to the “on” position.
- Open the nitrate template in the Omnion software and select “preview”.
- Enter standards and sample IDs into the Omnion software.
- Monitor the baseline for significant upward or downward drift for 10 minutes
- If there is no baseline drift, hit start.

17.3. NITRATE/NITRITE MANIFOLD DIAGRAM



Carrier: Helium degassed DI water
Manifold Tubing: 0.5 mm (0.022 in) i.d. This is 2.54 µL/cm.
AE Sample Loop: 7.5 cm x 0.5 mm i.d.
QC8000 Sample Loop: 13 cm x 0.5 mm i.d.
Interference Filter: 520 nm

12.2 Calibration and Standardization

- All standards should be measured twice.
- Omnion will automatically generate a calibration curve as standards are measured based on a second order calibration curve.
- Technicians should ensure that standards replicate well and that the curve generates an r^2 > 0.99. Technicians should also particularly check the low end of the calibration curve and ensure that there is not a significant deviation from the calibration curve by low standards.
- If any of these criteria are not met, halt and restart the run.

12.3 Sample analysis

- The first measured samples will be the 1 mg N/L nitrate standard and 1 mg N/L nitrite standard. Use the reported concentrations to calculate the column efficiency before allowing the run to proceed. If column efficiency is less than 80 %, do not proceed with the run.
- The Lachat may be left to operate unattended for the remainder of the run. However, the technician should check the instrument at least once between every check/secondary standard. Halt the run if a standard does the not meet QC criteria.
- Between check standards, technicians should check the concentrations of the newly analyzed samples for duplicates with poor replication and samples which exceed the high standard (1 mg N/L). These samples may be rerun or run with dilution respectively at the end of the run. Dilutions may be prepared in sample tubes using UPDI and a calibrated micropipette.

- Technicians should regularly monitor the flow injection system during the run and check for leaks or plugs.
- Technicians should regularly check that reagent containers are sufficiently full and that reagent lines are fully submerged.
- When sample analysis is finished, turn the nitrate column to "off" and transfer reagent lines to UPDI. Let run for 10 minutes, then remove from UPDI and allow the lines to pull air for another 10 minutes.
- If another run is being immediately performed, the previous steps may be ignored and the run can be started with the instrument as is.

13 Data acquisition, calculations, and reporting

13.1 At the completion of the run, the Omnion software will automatically export an Excel file with the run data.

13.2 This data includes determined concentrations. These concentrations are automatically calculated by the Omnion program using the second order calibration curve created at the start of the run.

13.3 Open the nitrate template and paste the run export into the tab labeled "Raw Export".

13.4 Open the calculations tab and enter appropriate sample information (site, date, dilution factor, etc.).

13.5 All calculations including averaging of duplicate measurement and calculation of QC parameters.

13.6 Check that all standards meet QC requirements. Standards which fail the QC requirements will be automatically indicated by the Excel sheet. If any standards do not meet the requirements, mark the preceding and following 10 samples as reruns.

13.7 Samples which fail to meet QC requirements will be indicated by the sheet and should be marked as reruns.

13.8 All sample results will be consolidated in the "summary" tab.

14 Computer hardware and software

14.1 Windows 7 Enterprise

14.2 Omnion Software 4.0, Lachat Instruments, Hach

15 Method performance

15.1 Desired Performance Criteria:

15.2 Method Detection Limit: 0.005 mg N/L

15.3 Precision: Duplicate CV <5 mg N/L or duplicate range < 0.02 mg N/L

15.4 Calibration $r^2 > 0.99$

16 Pollution prevention

16.1 All reagents and standards will be prepared in appropriate volumes so as to reduce waste.

16.2 All sample and reagents will be handled according to MU EHS policies in order to ensure proper disposal.

17 Data assessment and acceptable criteria for quality control measures and corrective actions for out-of-control or unacceptable data

17.1 Excel Cell References and Data Entry

Poor cell references or improperly entered data will lead to erroneous results. If a problem is noticed with a run, the data entry and the cell references should first be checked by a supervisor and pointed out to a technician if found.

17.2 r^2 Value

The mandatory r^2 for valid results is a minimum of 0.99. In the event that a technician proceeds with a run which has a calibration r^2 less than 0.99, all samples from this rerun should be rerun.

17.3 Column degradation

Columns nearing the end of their use, may degraded in efficiency during the course of the run. This will be reflected in a change in the column efficiency between the start and end of the run. If column efficiency declines by more than 5 % during the course of the run, samples should preferably be rerun. If reruns are not possible, sample concentrations may be corrected for efficiency decline as follows.

$$Corrected[S] = \left(\frac{E1}{E1 - (\left(\frac{E1 - E2}{N}\right) * S)} \right) * [S]$$

E1= Column Efficiency at the start of the run

E2= Column Efficiency at the end of the run

N= total number of samples and check standards in the run

S= sample position (i.e., a given samples numerical position in the run)

[S]= original sample concentration

17.4 Standard QC criteria

Both check and secondary standards must meet at least one of the following QC criteria:

1. The CV of standard's concentration and its known concentration is less than 5 %.
2. The absolute difference of the standard's concentration and its known concentration is less than 0.02 mg N/L.

If neither of these criteria are met the standard is not considered valid

17.5 Sample QC criteria

Sample duplicates must meet least one of the following QC criteria:

1. The CV of the two duplicates is less than 5 %.
2. The absolute difference of the two duplicates is less than 0.02 mg N/L.

If neither of these criteria are met, the sample result cannot be considered valid and should be rerun. If other nitrogen data was analyzed for the sample, nitrate results should also be compared to this data before data reports are finalized. Analysts should check that the following conditions are met.

1. ([NH4]+[NO3]) ≤ [TDN] ≤ [TN]

If the above conditions are not met, the issue should be investigated by analysts.

18 Waste management

18.1 All waste generated is considered hazardous.

18.2 All analyzed standards, and reagents should be treated as waste upon completion of the run.

18.3 Waste should be kept in an approved container with proper labeling.

18.4 Waste will not be held for longer than 6 months and MU Environmental Health and Safety (EHS) will be notified an appropriate time before this point so that waste can be collected and disposed of.

19 References

19.1 Standard Methods for the Examination of Water and Wastewater, 23rd Edition. 2017. Method 4500-NO_3^- I. American Public Health Association. Washington, DC.