**Nitrate via manual vanadium(III) reduction**

This method is a manual spectrophotometric procedure for determination of nitrate. A single reagent solution is prepared, which may be stored indefinitely for future use. At the time of analysis, reagent and sample are mixed directly in disposable cuvets, and absorbance read after color development. The method is believed to be valid for a wide range of matrix types, having been tested with the following: agricultural runoff, substitute ocean water, substitute wastewater, wetland drainage, milk serum, urine, and persulfate-digested samples.

This method is being presented to meet the need for a convenient yet sensitive and widely applicable procedure for determination of nitrate which does not require a large investment in equipment, lengthy preparation, or extensive training of personnel prior to analysis. It is based on published procedures (Doane TA and Horwath WR. 2003. Spectrophotometric determination of nitrate with a single reagent. Analytical Letters 36(12):2713-2722; Miranda KM, Espey MG, Wink DA. 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide-Biology and Chemistry 5(1):62-71.)

The reagent and analysis are easy to prepare and carry out, and involve less procedural considerations compared to existing photometric as well as non-photometric methods. A large number of samples may be processed at one time, with less sample requirement, waste generation, and preparation time compared to current standard methods. The procedure is equally convenient for determination of only a few samples, since the reagent may be stored and used as needed, with no additional set-up required prior to the moment of analysis.

***Principle***: Vanadium(III) in dilute acid solution effects the quantitative reduction of nitrate to nitrite and/or nitric oxide, both of which are captured by Griess reagents (sulfanilamide and N-(1-naphthyl)-ethylenediamine) to produce a red dye. The reagent solution, containing all of the above constituents, is mixed with the sample directly in cuvets. Absorbance is read at 540 nm following color development.

***Application/interferences***: The method is applicable to a wide range of sample types, including fresh and saline waters, acid and buffered solutions, and samples with high levels of dissolved organic matter. Samples containing high amounts of phosphate, sulfate, or dissolved organic matter (greater than about 100 ppm) may reduce the efficiency of reaction when very low amounts of nitrate (less than about 0.05 ppm N) must be determined; this is dealt with by simply using more reagent relative to sample. Other interferences include high (>100 ppm) concentrations of oxidizing agents such as Fe(III) or chlorine, as well as substances that interfere with the Griess reaction, including ascorbate and azide. Being a spectrophotometric procedure, samples must not be turbid

and should not show absorbance at 540 nm. Both of these characteristics may be corrected for, however, or the samples may be pretreated (e.g. by filtration).

***Apparatus***: Spectrophotometer for use at 540 nm.

***Concentration range***: Based on a 1 cm path length, the limit of detection in water samples is 0.002 ppm nitrate-N (0.14 micromolar nitrate). With sulfate, phosphate, and organic-rich matrices, the limit of detection is approximately 0.02 ppm N (1.44 micromolar nitrate). There is no set upper limit; sample and reagent volumes may be adjusted to accommodate high sample concentrations.

***Reagent preparation***: (Quantities of chemicals need not be exact.)
Pour approximately 200 ml 0.5 M HCl into a bottle. Place this on a balance and directly weigh approximately 0.5 g vanadium(III) chloride into the bottle (so as to avoid it sticking to spatulas, weigh dishes, etc.). Depending on the supplier, it should all dissolve with brief gentle shaking; if there are still undissolved particles present, filter through a >2 micron syringe filter. Add about 0.2 g sulfanilamide and 0.01 g N-(1-naphthyl)ethylenediamine dihydrochloride and dissolve.

Note on vanadium(III) chloride. Vanadium chloride tends to give off corrosive fumes, especially when exposed to moist air. Once opened, minimize the absorption of water vapor into the $VCl_3$ bottle by storing it over a small amount of desiccant (e.g. anhydrous calcium sulfate) in a sealed container. If necessary during reagent preparation, tare the bottle with HCl and dispense the $VCl_3$ in a hood; return to the balance to check the weight. Once dissolved, fumes will no longer be given off. Other than the above, no excessive precautions need to be observed; vanadium(III) chloride is not classified as harmful to the environment or toxic (supplier MSDS data).

***Procedure***: The following proportions of sample and reagent are optimized to give the maximum precision in each concentration range. They were determined such that a standard with the upper concentration in each range gives an absorbance that is as high as possible but not so high as to begin deviating from linearity. These are guidelines and may be adjusted by the analyst.

- For sample concentrations up to 20 ppm N, use 20 microliters sample and 1000 microliters reagent. The working lower limit (limit of quantitation) is approximately 0.1 ppm N.
- For up to 10 ppm N, use 45 microliters sample and 1000 microliters reagent. The working lower limit in this case is approximately 0.075 ppm N.
- For greatest sensitivity, use 500 microliters sample and 500 microliters reagent. The limit of quantitation is 0.007 ppm N with a linear calibration through zero obtainable up to about 0.75 ppm N.

- For measuring low (<1 ppm) concentrations in samples rich in sulfate, phosphate, or organic matter, use 100 microliters sample and 800 microliters reagent. The limit of quantitation in this case is 0.04 ppm N.

Chose a sample to reagent ratio that is appropriate for the expected range of concentrations in the sample batch. If the samples in a batch vary greatly in concentration, the low level samples might be analyzed separately from the high samples for greater accuracy. (If sample concentrations in a new batch are entirely unknown, their concentration range may be quickly estimated by screening several representative samples. Mix equal parts sample and reagent. Do the same for several standards. Heat the samples briefly in an oven or under hot water. Compare the colors to decide on an optimal concentration range.)

Pipet the samples into semimicro cuvets. Then pipet reagent into all cuvets. Cap the cuvets with cuvet caps, and invert them gently to mix. The samples may be left for color to develop at room temperature. It is maximum after approximately 6-10 hours. This is a slow reaction, but should not be an inconvenience; it is often convenient to mix one day and read absorbance the next day, especially when analyzing many samples at one time. Furthermore, the color is stable for at least 48 hours following addition of reagent, so there is some freedom as to when absorbance is read.

Read absorbance at 540 nm against a reagent blank (i.e. water). Analyze standards together with the samples to provide a calibration for calculating sample concentrations.

***Notes***:

1. This procedure measures nitrate plus any nitrite present as well. If nitrite is significant it may be quantified separately. When significant amounts of nitrite are present, it is important that the color be allowed to develop completely, since the color reaction with nitrite is complete within minutes, and the results will be affected if the conversion of nitrate as well is not complete.
2. If desired, heating will accelerate color development. For example, the samples may be mixed in small test tubes and heated at approximately 100$^{o}$C for 10-15 minutes to fully develop color. Increasing the concentration of $VCl_3$ in the reagent will also accelerate color development.
3. The reagent will keep up to about a week if refrigerated, or indefinitely if stored frozen. It may be convenient to prepare a large batch of reagent and freeze several small bottles from this, so that only the amount needed at any one time is thawed.
4. Vanadium(III) is sensitive to air and light; it is easily oxidized if left at room temperature in the presence of air for much longer than the time reasonably required for processing a set of samples (i.e. leaving uncapped for several days). The reagent becomes less effective as this happens, i.e. a lower absorbance reading is obtained for a given sample as compared to unchanged reagent.

***Evaluation of method*** *(*data from Doane TA and Horwath WR. 2003. Analytical Letters 36(12):2713-2722.)

1. A set of 10 soil extracts, ranging from 1-10 ppm N, was analyzed by the proposed procedure and by automated cadmium reduction/Griess reaction (EPA method 353.2). Average difference between results was 3.4%.
2. Two groundwater samples were determined a total of five times on five different days, by the same analyst using the same equipment and same standards. A different batch of reagent was used for each analysis. Sample 1 averaged 0.54 ppm N with a relative standard deviation of 5.5%; sample 2 averaged 8.07 ppm with a RSD of 1.5%.
3. The suitability of the procedure for unknown, potentially complex matrices was evaluated by determining recovery of small and large spikes added to a variety of sample types. Samples included milk serum, plant extracts, compost extract, eutrophic river water, wine, and urine. Recovery of a small spike ranged from 94-106%; recovery of a large spike ranged from 95-106%.

***Further evaluation of method***

The proposed procedure was again evaluated, using procedures for reporting and analyzing validation data as outlined in the EPA *Protocol for Approval of New Methods for Inorganic and Organic Analytes in Wastewater and Drinking Water*, Section 9.3.1. These results are presented below.

1. Determination of method detection limit (MDL) and minimum level/limit of quantitation (ML)

Seven replicate samples in reagent grade water, spiked to contain 0.006 ppm N, were analyzed. A low range calibration (500 microliters each sample and reagent) was used to determine the concentrations of these seven samples. This calibration was:

| concentration, ppm $NO_3$-N | absorbance |
|---|---|
| 0 | 0 |
| 0.01 | 0.016 |
| 0.05 | 0.080 |
| 0.1 | 0.161 |

$r^2 = 1$

The following absorbances and calculated concentrations were obtained for the seven replicate samples:

| absorbance | concentration |
|---|---|
| 0.009 | 0.0057 ppm N |

| | |
|---|---|
| 0.010 | 0.0063 |
| 0.010 | 0.0063 |
| 0.009 | 0.0057 |
| 0.009 | 0.0057 |
| 0.009 | 0.0057 |
| 0.012 | 0.0075 |

The MDL, calculated as 3.14 times the standard deviation of these measurements, is 0.002 ppm N. The ML, calculated as 3.18 * MDL, is 0.007 ppm N.

2. Initial precision and recovery (IPR)

*Reference matrix (water)*

IPR for the reference matrix was determined using a concentration equal to the compliance level for nitrate (‘*high level*”). An additional evaluation was performed at a low level.

*a. High level*

Four aliquots of reagent grade water were spiked with nitrate to achieve a concentration of 10 ppm N, the current regulatory compliance level. These were carried through the analytical process.

The following calibration (using 45 microliters sample, 1000 microliters reagent) was used to determine the concentrations of these four samples:

| concentration, ppm $NO_3$-N | absorbance |
|---|---|
| 1 | 0.141 |
| 2 | 0.282 |
| 5 | 0.646 |
| 10 | 1.234 |

$r^2 = 0.9995$

The following absorbances, calculated concentrations, and percent recoveries were obtained for the four replicate samples:

| absorbance | concentration | recovery |
|---|---|---|
| 1.230 | 9.93 ppm N | 99.3 % |
| 1.235 | 99.7 | 99.7 |
| 1.233 | 99.5 | 99.5 |
| 1.240 | 10.01 | 100.0 |

Average percent recovery was 99.6%, with a standard deviation of 0.35. The relative standard deviation (RSD) is 0.35.

*b. Low level*

The same reference matrix IPR procedure was carried out with four aliquots of reagent water spiked to contain 0.02 ppm N, in order to evaluate IPR at concentrations of nitrate close to the ML. These aliquots were carried through the analytical process.

The following calibration was used to determine the concentrations of these four samples:

| concentration, ppm $NO_3$-N | absorbance |
|---|---|
| 0 | 0 |
| 0.01 | 0.016 |
| 0.05 | 0.086 |
| 0.1 | 0.168 |

$r^2 = 0.9998$

The following absorbances, calculated concentrations, and percent recoveries were obtained for the four replicate samples:

| absorbance | concentration | recovery |
|---|---|---|
| 0.033 | 0.0195 ppm N | 98 % |
| 0.034 | 0.0201 | 101 |
| 0.033 | 0.0195 | 98 |
| 0.035 | 0.0207 | 104 |

Average percent recovery was 100%, with a standard deviation of 2.84. The relative standard deviation (RSD) of these results is 2.84.

*c. Sample matrix (agricultural runoff)*

Four aliquots of a representative field sample, containing 1.15 ppm $NO_3$-N, were spiked with nitrate to give an expected final concentration of 9.81. These were carried through the analytical process. The calibration used to determine these samples was the same as that in '*a. High level*' above.

The following absorbances, calculated concentrations, and percent recoveries were obtained for the original sample and the four replicate spiked samples:

| absorbance | concentration | recovery |
|---|---|---|

| | | |
|---|---|---|
| 0.172 | 1.15 | - |
| | | |
| 1.219 | 9.84 ppm N | 100.3 % |
| 1.215 | 9.80 | 99.9 |
| 1.226 | 9.89 | 100.8 |
| 1.223 | 9.87 | 100.6 |

The average percent recovery was 100.4%, with a standard deviation of 0.4.

3. Additional matrices

The method was further evaluated in other matrices with a range of background concentrations. A sample was either prepared or collected, its nitrate concentration determined, and the concentration of a spiked sample (MS) and a duplicate spiked sample (MSD) determined. The spike consisted of a small volume (<5% of the sample volume) of an appropriate nitrate standard. The samples were spiked such that, in most cases, the final concentration of nitrate was one to five times the background concentration. In each case, the recovery of nitrate and the relative percent difference (RPD) between the MS and MSD were determined.

*a. Substitute Ocean Water, ASTM Standard D 1141-98 (2003)*

The calibration used to analyze these samples was the same as that given above for method detection limit determination.

The following absorbances were obtained:

| | |
|---|---|
| Unaltered sample | 0.015 |
| MS | 0.047 |
| MSD | 0.045 |

giving the following results:

| | |
|---|---|
| background concentration | 0.009 ppm N |
| MS concentration | 0.029 ppm N |
| MSD concentration | 0.028 ppm N |

The expected concentration of the spiked samples was 0.028 ppm N, therefore:

| | |
|---|---|
| MS recovery | 104 % |
| MSD recovery | 100 % |
| | |
| RPD between MS and MSD concentration | 3.9 % |

*b. Substitute Wastewater, ASTM Standard D 5905-98 (2003)*

The calibration used to analyze these samples was the following:

| standard concentration, ppm $NO_3$-N | absorbance |
|---|---|
| 0.1 | 0.015 |
| 0.5 | 0.069 |
| 1 | 0.140 |
| 2 | 0.275 |
| 5 | 0.647 |

$r^2 = 0.9993$

The following absorbances were obtained:

| | |
|---|---|
| Unaltered sample | 0.048 |
| MS | 0.143 |
| MSD | 0.147 |

giving the following results:

| | |
|---|---|
| background concentration | 0.31 ppm N |
| MS concentration | 1.05 ppm N |
| MSD concentration | 1.08 ppm N |

The expected concentration of the spiked samples was 1.07 ppm N, therefore:

| | |
|---|---|
| MS recovery | 98 % |
| MSD recovery | 101 % |

RPD between MS and MSD concentration 2.9 %

*c. Wetland drainage*

The calibration used to analyze these samples was the same as that used for substitute wastewater.

The following absorbances were obtained:

| | |
|---|---|
| Unaltered sample | 0.095 |
| MS | 0.576 |

| | |
|---|---|
| MSD | 0.583 |

giving the following results:

| | |
|---|---|
| background concentration | 0.68 ppm N |
| MS concentration | 4.42 ppm N |
| MSD concentration | 4.47 ppm N |

The expected concentration of the spiked samples was 4.5 ppm N, therefore:

| | |
|---|---|
| MS recovery | 98 % |
| MSD recovery | 99 % |

| | |
|---|---|
| RPD between MS and MSD concentration | 1.2 % |

*d. Milk serum*

The calibration used to analyze these samples was the same as that used for substitute wastewater.

The following absorbances were obtained:

| | |
|---|---|
| Unaltered sample | 0.170 |
| MS | 0.635 |
| MSD | 0.623 |

giving the following results:

| | |
|---|---|
| background concentration | 1.26 ppm N |
| MS concentration | 4.88 ppm N |
| MSD concentration | 4.78 ppm N |

The expected concentration of the spiked samples was 5.06 ppm N, therefore:

| | |
|---|---|
| MS recovery | 96 % |
| MSD recovery | 95 % |

| | |
|---|---|
| RPD between MS and MSD concentration | 1.9 % |

*e. Urine*

The calibration used to analyze these samples was the following (using 20 microliters sample and 1000 microliters reagent):

| standard concentration, ppm $NO_3$-N | absorbance |
|---|---|
| 5 | 0.328 |
| 10 | 0.628 |
| 15 | 0.922 |
| 20 | 1.170 |

$r^2 = 0.9981$

The following absorbances were obtained:

| | |
|---|---|
| Unaltered sample | 0.754 |
| MS | 1.187 |
| MSD | 1.197 |

giving the following results:

| | |
|---|---|
| background concentration | 12.4 ppm N |
| MS concentration | 20.0 ppm N |
| MSD concentration | 20.2 ppm N |

The expected concentration of the spiked samples was 21.2 ppm N, therefore:

| | |
|---|---|
| MS recovery | 94 % |
| MSD recovery | 95 % |

| | |
|---|---|
| RPD between MS and MSD concentration | 1.0 % |

*f. Sample processed for analysis of total dissolved nitrogen by persulfate digest follwed by nitrate determination (Method 4500-N C., Standard Methods, APHA/AWWA/WEF, 20th ed.).* The sample was spiked following digestion.

The calibration used to analyze these samples was the following:

| standard concentration, ppm $NO_3$-N | absorbance |
|---|---|
| 0.1 | 0.014 |
| 0.5 | 0.070 |
| 1 | 0.141 |
| 5 | 0.643 |

$r^2 = 0.9997$

The following absorbances were obtained:

| | |
|---|---|
| Unaltered sample | 0.092 |
| MS | 0.145 |
| MSD | 0.148 |

giving the following results:

| | |
|---|---|
| background concentration | 0.68 ppm N |
| MS concentration | 1.09 ppm N |
| MSD concentration | 1.11 ppm N |

The expected concentration of the spiked samples was 1.06 ppm N, therefore:

| | |
|---|---|
| MS recovery | 102 % |
| MSD recovery | 105 % |

| | |
|---|---|
| RPD between MS and MSD concentration | 2.1 % |