Designation: D7781 – 23

Standard Test Method for Nitrite-Nitrate in Water by Nitrate Reductase[1]

This standard is issued under the fixed designation D7781; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method is applicable to the determination of nitrate plus nitrite (as nitrogen) in drinking water, surface, saline, wastewater, and ground waters. The applicable range of this test method is from 0.05 to 5 mg/L of nitrogen. The range may be extended upward by dilution of an appropriate aliquot. The 40 CFR Part 136 Method Detection Limit (MDL) is 0.02 mg/L.

1.2 It is the user's responsibility to ensure the validity of this test method for waters of untested matrices. The quality control criteria in Section 17 for method blanks, laboratory control samples, matrix spikes and matrix duplicates must be met.

1.3 The values stated in SI units are regarded as standard. No other units of measurement are included in this standard.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.5 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D0992 Method of Test for Nitrate Ion in Water (Withdrawn 1983)[3]

D1129 Terminology Relating to Water

D1141 Practice for Preparation of Substitute Ocean Water

D1193 Specification for Reagent Water

D1254 Method of Test for Nitrite Ion in Water (Withdrawn 1980)[3]

D3867 Test Methods for Nitrite-Nitrate in Water

D5810 Guide for Spiking into Aqueous Samples

D6146 Guide for Monitoring Aqueous Nutrients in Watersheds

3. Terminology

3.1 *Definitions*—For definitions of terms used in these test methods, refer to Terminology D1129.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *bispecific NaR, n*—nitrate reductase that can use either NADH or NADPH as its electron donor (cofactor).

3.2.2 *discrete analyzer, n*—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.

3.2.3 *Greiss reaction, n*—chemical formation of an azo dye by diazotization of nitrite ion with sulfanilamide and subsequent coupling with *N*-(1-naphthyl)ethylenediamine hydrochloride.

3.2.4 *NADH, n*—nicotinamide adenine dinucleotide, reduced form is a coenzyme found in all living cells.

3.2.5 *NADPH, n*—nicotinamide adenine dinucleotide phosphate, reduced form is a coenzyme found in all living cells; NADP+ is the oxidizing form and NADPH is the reducing form.

3.2.6 *nitrate reductase (NaR), n*—NADH:NaR (EC1.7.1.1 and CAS 9013-03-0) or bispecific NaR (EC 1.7.1.2 and CAS 9029-27-0) with 1 unit of enzyme activity defined as 1 micromol nitrite produced per minute at 30 °C, at pH 7 with NADH (refer to 3.2.4 and 10.2) as an electron donor.

4. Summary of Test Method

4.1 *Nitrite-Nitrate Nitrogen*—The sample is mixed with a buffered solution containing NAD(P)H: nitrate reductase (EC 1.7.1-3) and NADH or NADPH to reduce nitrate ion to nitrite ion. The combined nitrite-nitrate (expressed as mg/L NO_3+NO_2-N) is determined by diazotizing the total nitrite ion

[1] This test method is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.05 on Inorganic Constituents in Water.

Current edition approved April 1, 2023. Published April 2023. Originally approved in 2014. Last previous edition was approved in 2014 as D7781 – 14 which was withdrawn January 2023 and reinstated in April 2023. DOI: 10.1520/D7781-23.

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] The last approved version of this historical standard is referenced on www.astm.org.

with sulfanilamide and coupling with *N*-(1-naphthyl) ethylenediamine dihydrochloride to form a highly colored azo dye that is measured spectrophotometrically at about 540 nm.

4.2 *Nitrite Nitrogen*—The nitrite ion (expressed as mg/L NO_2-N) originally present in the sample can be determined separately by carrying out the procedure and omitting the reduction step.

4.3 *Nitrate Nitrogen*—The nitrate ion as nitrogen can be calculated as the difference between the combined nitrate plus nitrite (NO_3+NO_2-N) and the nitrite (NO_2-N):

$$mg/L\,NO_3 - N = mg/L(NO_3 + NO_2 - N) - mg/L(NO_2 - N) \quad (1)$$

5. Significance and Use

5.1 This test method replaces Methods D1254 (Nitrite) and D992 (Nitrate). The nitrite test method (Method D1254) used a reagent that is considered to be a potential carcinogen. The nitrate test method (Method D992) has been shown to have relatively large errors when used in wastewaters and also has greater manipulative difficulties than the test method described herein.

5.2 This test method can be used in place of Test Methods D3867 (Nitrite-Nitrate). Test Methods D3867 uses cadmium for the reduction of nitrate to nitrite. Cadmium is considered a toxic metal. Also, the heterogeneous cadmium reductant creates greater difficulty than the reduction described in this test method.

6. Interferences

6.1 Turbid samples should be filtered prior to analysis to eliminate particulate interference.

6.2 Sample color that absorbs at wavelengths between 520 and 540 nm interferes with the absorbance measurements. When color is suspect, analyze a sample blank, omitting the *N*-(1-naphthyl)ethylenediamine dihydrochloride from the color reagent.

NOTE 1—The instrumentation described in this standard may automatically correct for some turbidity and sample color. See the instrument manual for further information.

6.3 Certain ions may cause interferences. See Table 1.

7. Purity of Reagents

7.1 Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, when such specifications are available.[4] Other grades may be used, provided it is first ascertained that the reagent is of sufficient high purity to permit its use without lessening the accuracy of the determination.

[4] *ACS Reagent Chemicals, Specifications and Procedures for Reagents and Standard-Grade Reference Materials*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

TABLE 1 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

7.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D1193, Type I or Type II. Other reagent water types may be used, provided it is first ascertained that the water is of sufficiently high purity to permit its use without adversely affecting the bias and precision of these test methods.

8. Sampling and Sample Preservation

8.1 Collect the sample in accordance with Guide D6146.

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

TABLE 2 Example Concentrations of Calibration Standards

NO_3-N or NO_2-N, mg/L	mL of 10 mg/L Standard Solution/100 mL final volume
0.05	0.5
0.1	1.0
0.5	5.0
1.0	10
2.0	20
3.0	30
5.0	50

TABLE 3 Reduction Efficiency

NO_3-N or NO_2-N, mg/L	mL of 10 mg/L Standard Solution/100 mL final volume
Mean (%)	103
Standard Deviation	4.14
Lower Limit (%)	91
Upper Limit (%)	115

inhibiting reduction of nitrate to nitrite. Do attempt to remove residual chlorine.

9. Apparatus

9.1 Automated discrete analysis system (see 3.2.2).

10. Reagents

10.1 *Phosphate Buffer Solution*—Dissolve 3.75 g of potassium dihydrogen phosphate (KH2PO4), 0.01 g of disodium ethylenediaminetetraacetate dehydrate ($C_{10}H_{14}O_8N_2Na_2{\cdot}2H_2O$), and 1.4 g potassium hydroxide (KOH) in about 500 mL reagent water contained in a 1000 mL volumetric flask, dilute to the mark and mix. Transfer this solution to a screw-cap container and store at 2–6 °C. This solution is stable for 6 months.

10.2 *β-nicotinamide adenine dinucleotide, Reduced Form (NADH) Stock Solution (2 mg/mL)*—Dissolve 0.1 g NADH ($C_{21}H_{27}N_7O_{14}P_2$) in 25 mL of reagent water contained in a 50 mL volumetric flask, dilute to the mark and mix. Transfer 1-mL aliquots to 1.5 mL snap-cap colorless polypropylene vials and store at –20 °C. Stable for 1 month.

NOTE 5—NADH is a hygroscopic white powder that is freely soluble in water. The solids are stable if stored dry and protected from light. Neutral solutions are colorless and stable for 1 week if stored at 4 °C, but decompose rapidly under basic or acidic conditions.

10.3 *NADH Working Solution*—Thaw one 1-mL vial of NADH stock (refer to 10.2) and dilute to 10 mL with phosphate buffer (refer to 10.1). This reagent is stable for about 8 hours. Prepare sufficient NADH working solution for the number of samples and standards to be analyzed.

NOTE 6—NADH inhibits color formation in the Greiss reaction (refer to 3.2.3). The molar concentration of NADH in the reduction medium should be about twice that of the highest calibration standard.

10.4 *Sulfanilamide (SAN) Reagent (10 g/L)*—While stirring constantly add 300 mL of concentrated hydrochloric acid (HCl, 37 % w/v)) and 10 g of sulfanilamide (SAN, $C_6H_8N_2O_2S$) to about 500 mL reagent water contained in a 1000 mL volumetric flask, dilute to the mark and mix. This solution is stable for about six months when stored in a brown bottle at 20 °C.

10.5 *N-(1-naphthyl)ethylenediamine dihydrochloride (NED) Solution (1 g/L)*—dissolve 1 g NED ($C_{10}H_7NHCH_2CH_2NH_2{\cdot}2HCl$) in about 500 mL reagent water contained in a 1000 mL volumetric flask, dilute to the mark and mix. Transfer to a glass or amber screw-cap container. This solution is stable for 6 months at 20 °C.

10.6 *Nitrate Reductase (NaR)*—Follow the manufacturer's instructions for preparing a solution of 1 unit NaR (refer to 3.2.6) activity per mL of phosphate buffer (refer to 10.1). Dilute 3 units NaR to 20 mL with phosphate buffer. Store the solution at 2–6 °C, where it is stable for 8 hours. Prepare sufficient NaR for the total number of samples and standards to be analyzed.

NOTE 7—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.[5] 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.

10.7 *Nitrate Solution, Stock (1000 mg/L NO_3-N)*—Dry potassium nitrate (KNO_3) in an oven at 105 °C for 24 h. Dissolve 7.218 g in water in about 500 mL reagent water contained in a 1000 mL volumetric flask, dilute to the mark and mix. This solution is stable for up to 2 months with refrigeration. Alternatively, certified nitrate stock solutions are commercially available through chemical supply vendors and may be used.

10.8 *Nitrate Solution, Standard (10 mg/L NO_3-N)*—Dilute 10 mL of stock nitrate solution (10.7) to 1 L with water and store in a dark bottle. Prepare fresh as needed.

10.9 *Nitrite Solution, Stock (1000 mg/L NO_2-N)*—Place about 7 g of potassium nitrite (KNO_2) in a tared 125-mL beaker and dry for about 24 h to a constant weight in a desiccator containing a suitable desiccant. Adjust the weight of the dry potassium nitrite to 6.072 g. Add 50 mL of water to the beaker, stir until dissolved, and transfer quantitatively to a 1000-mL volumetric flask. Dilute to the mark with water store in a sterilized bottle under refrigeration. Prepare fresh as needed. Alternatively, certified nitrite stock solutions are commercially available through chemical supply vendors and may be used.

NOTE 8—Potassium nitrite is easily oxidized; use only dry, free flowing white, or yellowish white crystalline powder of this reagent.

10.10 *Nitrite Solution, Standard (10 mg/L NO_2-N)*—Dilute 10 mL of stock nitrite solution (10.9) to 1 L with water. This solution is unstable; prepare fresh as needed.

11. Hazards

11.1 All reagents and standards should be prepared in volumes consistent with laboratory use to minimize the generation of waste.

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

12. Calibration

12.1 Using the standard nitrate solution (10.8) prepare calibration standards by using the automated calibration function of the discrete analyzer (3.2.2). Table 2 specifies suggested calibration levels.

12.2 Prepare at least one calibration standard from the standard nitrite solution (10.10) at the same concentration as one of the nitrate standards to verify the efficiency of the reduction. Verify that reduction efficiency is between 90 and 115 % with each batch of enzyme. See Table 3.

NOTE 9—When the sample to be analyzed is saline water, use substitute ocean water (SOW) to prepare the standards (Practice D1141 or a commercially available synthetic seawater). Run a reagent water blank in addition to a SOW blank because the reagents used to prepare SOW frequently contain nitrite or nitrate, or both. Adjust this curve for the contaminant level in SOW.

NOTE 10—Most discrete analyzers generate calibration standards and calibration curves automatically. Follow the manufacturer's instructions for calibrating with individual calibration standards if an automatic calibration function is not available.

12.3 Develop the color and determine the absorbance of each standard as directed in the procedure (13.4.6).

12.4 Prepare a standard curve by plotting the absorbance (or optical density) of each processed calibration standard against its known concentrations. See Fig. 1 for an example of a calibration curve.

12.5 Verify the calibration each day, or before each use with a calibration verification solution (see 17.2.2).

13. Conditioning

13.1 *Removal of Interferences*—Remove interferences (Section 6) by the following procedures:

13.2 For turbidity removal, when suspended solids are present, filter the sample through a glass-fiber filter or a 0.45-µm filter. Centrifugation can be used as an option.

13.3 For correction for color interferences, if there is a possibility that the color of the sample might absorb in the photometric range from 530 ± 10 nm, determine the background absorbance.

NOTE 11—Many discrete analyzers automatically compensate for background absorbance and turbidity on each sample. Follow the manufacturer's instructions.

13.4 Prepare a method in the discrete analyzer software according to the following, or similar, conditions as recommended by the manufacturer:

13.4.1 Dispense 170 microliters of Nitrate Reductase NaR (10.6) plus 10 microliters of sample. Mix.

13.4.2 Add 15 microliters of NADH (10.3). Mix and measure the background absorbance (or optical density).

13.4.3 Incubate 600 seconds at 37 °C.

13.4.4 Add 25 microliters of SAN reagent (10.4). Mix and incubate 120 seconds at 37 °C.

13.4.5 Add 25 microliters of NED reagent (10.5). Mix and incubate 120 seconds at 37 °C.

13.4.6 Measure absorbance (or optical density) at 540 nm.

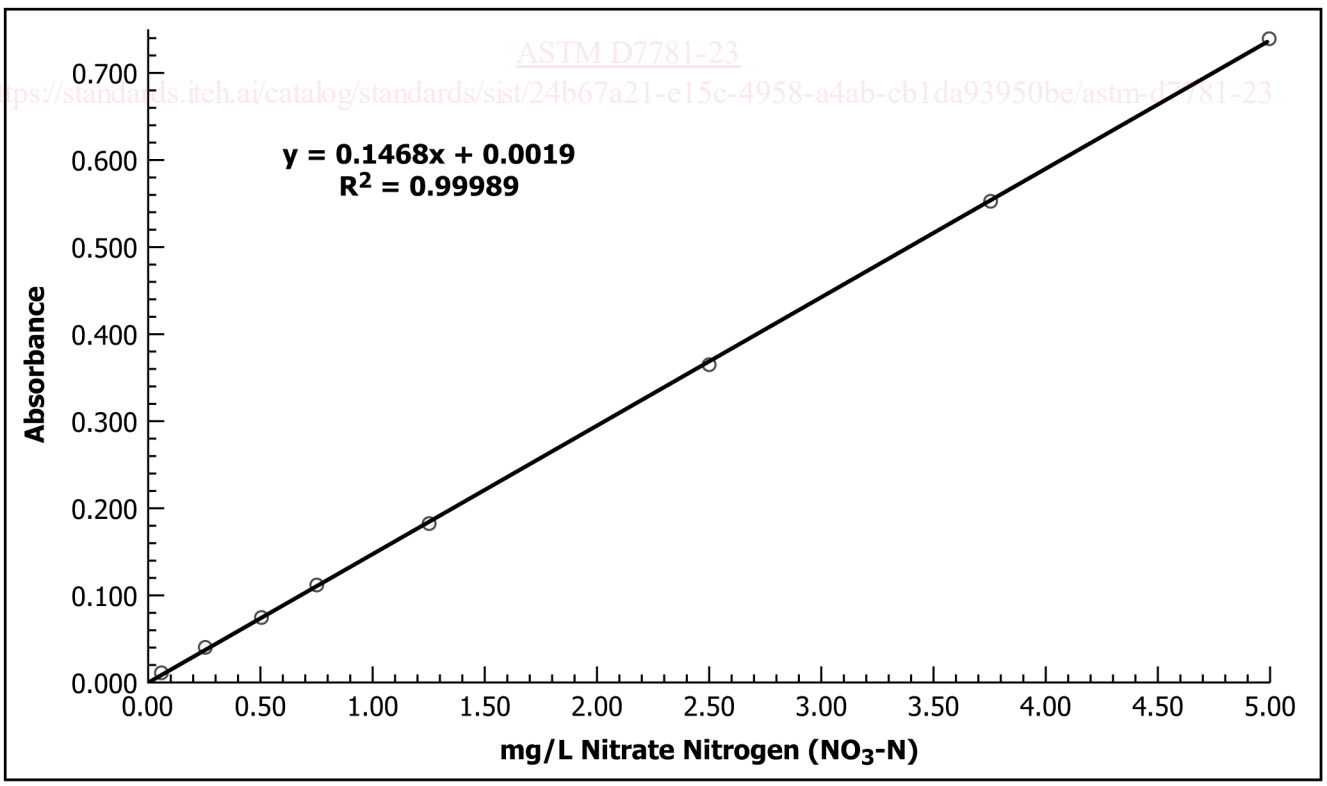

FIG. 1 Example Calibration Curve