



ENZYMATIC NITRATE ANALYSIS FOR EPA REGULATED LABORATORIES USING NITRATE REDUCTASE

A GUIDE TO UPDATING REGULATORY METHODS FOR CWA NITRATE REPORTING

NECi Superior Enzymes



September 2016



Overview

NECi Superior Enzymes' enzymatic nitrate quantification method has been recognized by the U.S. Environmental Protection Agency (EPA) as a qualified method for Clean Water Act (CWA) and Safe Drinking Water Act (SDWA) reporting.

NECi Superior Enzymes was notified through a letter of acceptance from the Office of Science and Technology Headquarters on April 4th, 2014 that nitrate reductase method for CWA nitrate reporting had been approved for nationwide use as an Alternate Test Procedure (ATP) under 40 CFR Part 136.

SUBJECT: Review of NECi Method N07-0003, Method for Nitrate Reductase Nitrate-Nitrogen Analysis (ATP Case No. N07-0003)

DATE: April 4, 2014

I have reviewed NECi Method N07-0003 (ATP Case No. N07-003), "Method for Nitrate Reductase Nitrate-Nitrogen Analysis", and the supporting validation data in ATP Case No. N07-0003. I determined that this method meets all requirements for measurement of nitrate and combined nitrate-nitrite in wastewater. That is, the performance of this method is substantially similar to methods listed at 40 CFR Part 136 for measurement of nitrate and combined nitrate-nitrite in wastewater.

I will recommend that this method be included in future regulatory actions in which EPA periodically adds to the list of approved methods at 40 CFR Part 136. However, this ATP review does not replace this notice and comment rulemaking process. In the interim a user may, on a facility-by-facility basis, seek approval from their regional authority for use of this method in measuring nitrate and combined nitrate-nitrite in Clean Water Act (CWA) programs.

If I can be of any additional assistance on this matter or others, please contact me at walker.lemuel@epa.gov.

Sincerely,

A handwritten signature in blue ink that reads "Lemuel Walker, Jr." in a cursive style.

Lemuel Walker, Jr.
CWA ATP Coordinator
Engineering and Analytical Support Branch
Engineering and Analysis Division
Office of Science and Technology

The approval of the ATP was a result of submitting an application to the Alternate Test Procedure program, which demonstrated the validity and quality of the method.

The application included:

- A detailed description of the nitrate reductase nitrate analysis method
- Other studies confirming applicability of the ATP for analysis of nitrate in wastewater discharges from representative facilities
- Comparability data for the performance of the proposed nitrate reductase ATP compared to the performance of the cadmium reduction reference method in the following variety of sample matrices:

Sample Type	NaR Reduction (mg N/L)	Cd Reduction (mg N/L)
Denver area treatment plant Influent wastewater	0.03	0.03
Denver area treatment plant Wastewater effluent #1	7.8	7.6
Denver Area treatment plant Wastewater effluent #2	0.23	0.26
Michigan paper mill waste stream effluent	0.04	0.03
Denver area metal finisher waste stream effluent	270.8	272.6
Denver area Commercial laundry waste stream effluent	4.8	4.8
Environmental Resources Associates #507 Hardness WasteWatR reference material	0.05	0.06
Michigan Confined Animal Feeding Operation (CAFO) effluent from tiled field	13.77	14.1
Low-nutrient seawater (collected offshore Hawaii)	0.027	0.030
ERA #608 Reference Standard	6.8	7.02
USGS PE N-116 (low nutrient-fortified river water)	0.45	0.48
USGS PE N-115 (high nutrient-fortified river water)	2.28	2.36



Clean Water Act Analytical Methods – Alternate Test Procedures

Information on the CWA-ATP Program can be found using the following link:

<https://www.epa.gov/cwa-methods/alternate-test-procedures>

To promote method innovation, EPA maintains a program that allows method developers to apply for EPA review of an alternative method to an existing approved method and potentially for EPA approval of that ATP. This ATP program is described for CWA applications at 40 CFR 136.4 and 136.5. Based on EPA's review, the performance of these ATPs is equally effective as other methods already approved for measurement.

Q: Is a method that is reviewed under the CWA ATP program an approved method?

A: No, first the method must be reviewed and found to be complete and supported by the documentation, then the method must be approved through rulemaking before the method may be added to 40 CFR Part 136 as an approved CWA method.

Q: Are there any exceptions to rulemaking before use of an alternate test procedure?

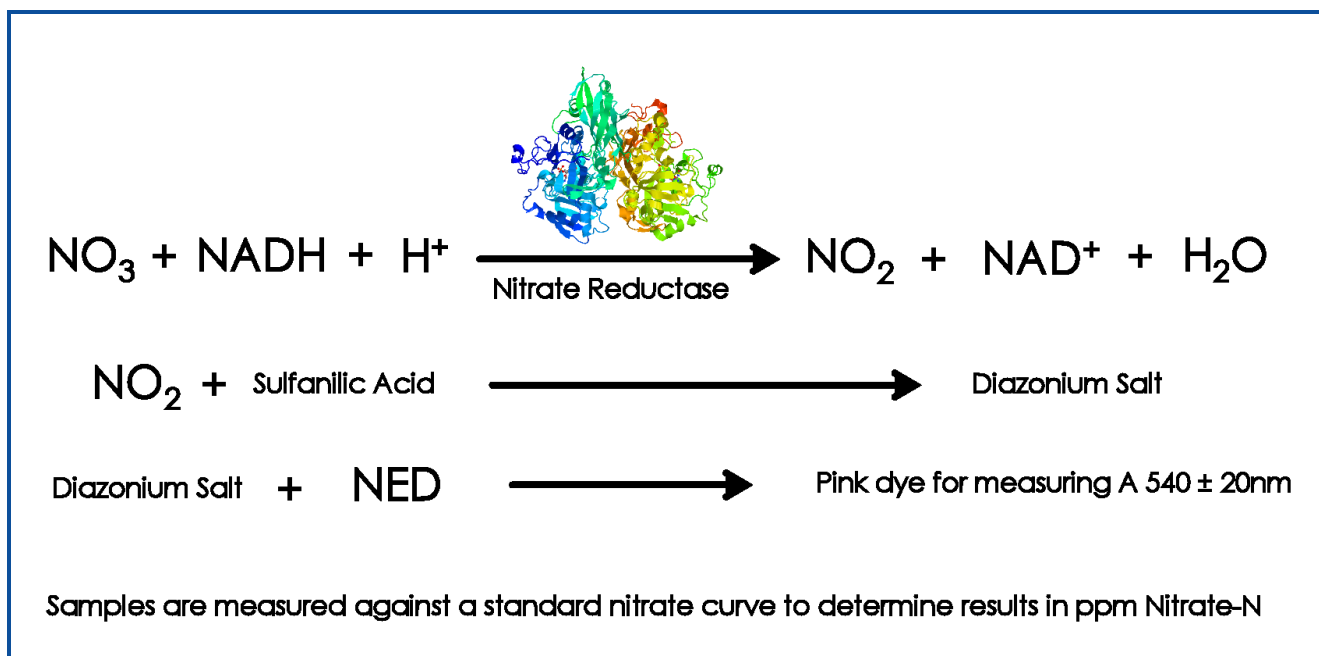
A: Yes, but only for wastewater methods. Approval for nationwide use requires a rulemaking process. In the interim, a facility may apply to an EPA Region for a limited-use ATP approval letter, i.e. for use at that facility. Generally it is not necessary for the limited use ATP applicant to submit data, or do a side-by-side comparison, if the method has already been reviewed for nationwide use under the CWA ATP program which requires multi-lab and comparability data and the review has resulted in a recommendation for inclusion in Part 136.

Once the MUR is codified, the ATP will then be referred to as an approved CFR Part 136 method. The MUR is officially codified as a Final Rule when signed by the EPA Administrator.

About the Method

The method involves the following steps:

- Enzymatic reduction of nitrate in a sample to nitrite using eukaryotic nitrate reductase;
- Diazotizing the nitrite originally in the sample plus the reduced nitrate with sulfanilamide followed by coupling with N-(1-naphthyl)ethylenediamine dihydrochloride under acidic conditions to form a highly colored azo dye;
- Colorimetric determination in which the absorbance of color at 546 nm is directly proportional to the concentration of the nitrite plus the reduced nitrate in the sample;
- Measurement of nitrite separately, if needed, by analysis of the sample while eliminating the reduction step;
- Subtraction of the nitrite value from that of the combined nitrate -nitrite value to measure nitrate separately if needed.



This reaction has been formatted to suit multiple analysis methods, including discrete analysis, flow through injection analysis, microplate analysis, test tube analysis, and easy on-site analysis. Total reaction is less than 1 mL, but can be adapted for larger volumes.



Excerpt from the U.S. EPA 2015 Methods Update Rule, pp 26-27:

“EPA proposes to add the USGS Methods I-2547-11 and I-2548-11 titled “Colorimetric Determination of Nitrate plus Nitrite in Water by Enzymatic Reduction, Automated Discrete Analyzer Methods,” to Table IB for the analytes nitrate, nitrite, and combined nitrate-nitrite. Method I-2548-11 is a low level (analytical range) version of Method I-2547-11. They are both included in the same method title. The method can be found in USGS Survey Techniques and Methods, Book 5, Chapter B8. The method is available for free from the USGS website. This method follows the same procedure as in **ATP Case No. N07-0003 - Nitrate Elimination Company Inc.’s (NECi) Method N07-0003, Revision 9.0, March 2014, “Method for Nitrate Reductase Nitrate-Nitrogen Analysis,”** which EPA also proposes to approve. Additional details on the ATP study and multi-laboratory validation can be found in Section E.1 below.

1. **The Nitrate Elimination Company Inc. (NECi) Method N07-0003**, “Nitrate Reductase Nitrate-Nitrogen Analysis,” Revision 9.0, dated March 2014 (**The Nitrate Elimination Company, Inc 2014a**). The analysis measures nitrate, nitrite, and combined nitrate-nitrite. **NECi Method N07-0003** is a “green” alternative to the other approved methods which use cadmium, a known carcinogen for the reduction of nitrate to nitrite prior to analyses. **NECi Method N07-003** uses automated discrete analysis and spectrophotometry to determine concentrations of nitrate and nitrite, combined or separately in wastewater.”

More about Enzymatic Nitrate Analysis

Enzymes are specific and selective, yielding accurate & reliable data even in complex mixtures. Nitrate reductase offers low detection limits, compatibility with a variety of sample types, and less interferences than the cadmium reduction method.

Minimal to no sample preparation is required, and no hazardous waste is generated from the enzymatic nitrate analysis method using nitrate reductase.

	Nitrate Reductase Reduction	Cadmium Reduction
Sulfate Interference	No	Yes
Hazardous Materials	No	Yes
Chloride Interference	No	Yes



Updating Nitrate Analysis to the Enzymatic Method

Each regional ATP Coordinator grants approval for use of the ATP at a facility on a case-by-case basis for compliance analysis until the ATP is approved by publication in a final rule in the Federal Register.

Contact your state region ATP coordinator. This may be the QA/QC Manager, EPA Regional Contact, or State Contact. Each state or region will have varying conditions that must be met before you can begin using the nitrate reductase method in your facility.

EPA REGION	ATP COORDINATOR	EMAIL CONTACT
1: ME, MA, RI, CT, NH, VT	Ann Jefferies*	Jefferies.ann@epa.gov
2: NY, NJ, PR, VI	Donna Ringel	Ringel.donna@epa.gov
3: PA, WV, VA, DE, MD, DC	Terry Simpson	Simpson.terry@epa.gov
4: KY, TN, NC, SC, MS, AL, GA, FL	Ray Terhune	Terhune.ray@epa.gov
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6: NM, OK, TX, AR, LA	David Stockton	Stockton.david@epa.gov
7: IA, NE, KS, MO	Bob Nichols	Nichols.Robert@epa.gov
8: MT, WY, ND, SD, UT, CO	Jeff Pritt	Pritt.jeff@epa.gov
9: CA, NV, AZ, HI	Roseanne Sakamoto	Sakamoto.Roseanne@epa.gov
10: WA, OR, ID, AK	Donald M. Brown	Brown.donaldm@epa.gov

Before MUR is codified, laboratories using discrete analyzer automated analysis may apply to their EPA region for a limited-use ATP approval letter for use at their facility only. **Generally,** it is not necessary for the limited-use ATP applicant to submit data, or do a side-by-side comparison since the nitrate reductase method has already been reviewed for nationwide use under the CWA ATP program, which requires multi-lab and comparability data, and has resulted in a recommendation for inclusion in Part 136. For example, the state of Maryland requires the following: Laboratories using any method other than automated discrete analysis must wait until the MUR is codified to implement this method into their laboratory.

Once MUR is codified, laboratories may implement the enzymatic method when using any discrete analyzer without any hesitation or approval process. Laboratories using anything other than automated discrete analyzer analysis (automated flow-through cell, manual test tube, microplate method, etc...) must contact their individual state assessor. The state assessor should contact the state regional ATP coordinator to begin the application process to request to use this method in CFR part 136.6. Laboratories may also contact the state regional ATP coordinator directly. This requires performance data, including MDL studies, spikes and blanks, usually 4 replicates, PT testing samples. This routine is commonplace for implementing any analytical method in regulated laboratories.

