

# Green Chemistry Nitrate Determination: An Alternative Nitrate Analysis Method

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A revolution is taking place in the chemical analysis of drinking water and wastewater. A new generation of analytical chemical analyzers has become available with the introduction of discrete robotic analyzers. These instruments are only now being certified for use with standard U.S. EPA methods. Some advantages of the systems, such as reduced reagent consumption and the use of robotics, permit easier adoption of enzymatic-based methods. For example, the most common nitrate analysis method involves the use of solid cadmium metal for the reduction of nitrate to nitrite. An alternative is the enzyme-catalyzed reduction of nitrate to nitrite, which is fully compatible with the pipet-driven liquid delivery system of discrete analyzers. This application

note describes the development of the enzyme-based nitrate reduction method for the AP300 automated ion analyzer (Lachat Instruments, Milwaukee, WI).

Nitrate levels in potable water and wastewater are set by U.S. EPA regulations to not exceed 10 ppm nitrate (N). Since nitrate is toxic to humans and livestock, nitrate analysis of water samples is one of the most frequently applied analytical methods. Acute nitrate toxicity can result in death when nitrite is formed internally from ingested nitrate. Nitrite binds to hemoglobin and inactivates its oxygen-carrying capacity, which may result in methemoglobinemia, or blue baby syndrome. Chronic nitrate toxicity may result in cancer, perhaps due to the formation of nitrosamines from nitrite. While animals generate nitrate and nitrite internally from nitric oxide, which is employed as a regulatory hormone to control the vascular system, nitrate toxicity only results when drinking water and food are high in nitrate. On the other hand, high nitrate levels in wastewater and runoff result in nitrate pollution, which has become a common problem in the world today. For example, the “dead zone” in the Gulf of Mexico near New Orleans is a product of nutrients transported by the Mississippi River from the large agricultural regions in the river’s watershed.

Methods for nitrate analysis in water approved by the U.S. EPA generally involve toxic agents such as cadmium and hydrazine. Cadmium is a toxic heavy metal, a regulated pollutant of water, and is found to damage the kidney, among other negative health effects. Cadmium is involved with a number of risks to humans and other animals, which can be found listed to some extent on the Material Safety Data Sheet (MSDS) for cadmium-based nitrate test kits. Hydrazine, a rocket fuel, is explosive and highly chemically reactive; it is clearly toxic to the environment. Both of these nitrate analysis methods present some health risks to the analytical chemist. Thus, an alternative nitrate analysis method utilizing green chemistry is highly desirable. Enzyme-based nitrate analysis is the green alternative method currently available.

An enzyme-based nitrate analysis method using nitrate reductase (NaR) from corn leaves was developed more than a decade ago by **The Nitrate Elimination Co., Inc. (NECi)** (Lake Linden, MI). More recently, the company introduced recombinant yeast NaR, which is called Superior Stock NaR or YNaR1.<sup>1</sup> YNaR1 is a highly stable form of the enzyme and, when used in the nitrate analysis method, has been shown to be as accurate and reproducible as the cadmium reduction method using real-world water samples.<sup>1</sup> The nitrate analysis method involves two steps (Figure 1). First, the

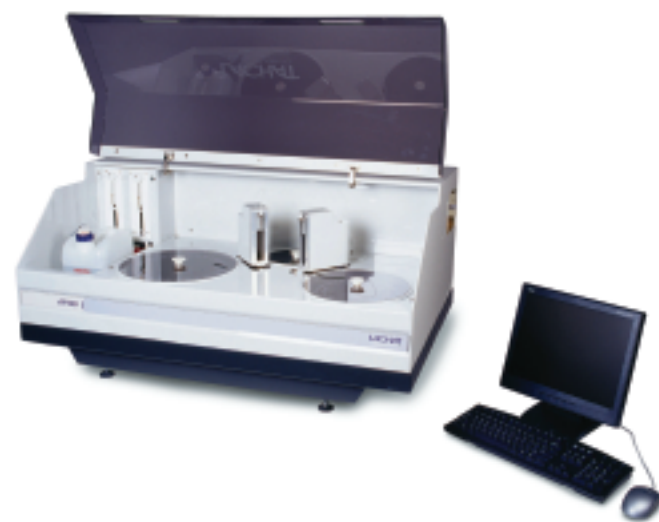


Figure 3 AP300 discrete analyzer.

nitrate is reduced to nitrite in a buffered system in a reaction catalyzed by NaR employing nicotinamide dinucleotide, reduced form (NADH) as the reductant. Next, the nitrite formed is reacted with the Griess reagents to generate a highly colored product that is quantified using a colorimeter or spectrophotometer. The second step is the same as in the cadmium reduction method. Thus, the enzyme reduction step simply replaces the toxic cadmium in the conversion of nitrate to nitrite. The key to development and commercialization of the green alternative method is the production of an inexpensive, analytical-grade NaR.

NECi production of NaR from corn leaves began in 1993. This NaR was first used to validate the enzyme-based nitrate analysis method in comparison to the cadmium reduction method.<sup>2</sup> However, NECi corn leaf NaR is difficult to produce in the large quantities needed for the development of the global nitrate analysis market. Under a grant from the National Institutes of Health (NIH) Small Business Innovative Research program, the company created a recombinant form of *Pichia angusta* NaR and expressed it in the methylotrophic yeast, *Pichia pastoris*.<sup>3</sup> *P. pastoris* is an ideal expression system for NaR since it does not utilize nitrate as a nutrient and does not make its own nitrate assimilation system, including NaR. Another advantage of *P. pastoris* is that it can be grown to very high density in a fermenter (Figure 2). The company optimized production of YNaR1 in a fermenter system equipped with a methanol sensor to provide better control of the agent inducing enzyme expression. After YNaR1 is extracted from the *P. pastoris* cells, the enzyme is purified to analytical grade in a single step using immobilized metal ion affinity chromatography. Production of YNaR1 is scalable, which makes it obvious that sufficient high-quality, inexpensive YNaR1 is being produced to meet current and future market demand for green nitrate analysis.

The nitrate analysis method utilizing YNaR1 has been implemented in the AP300 discrete analyzer (Figure 3).

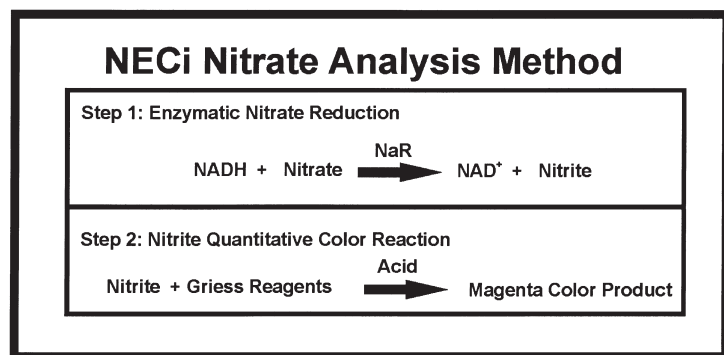


Figure 1 Steps involved in the enzymatic nitrate reduction method. Step 1 shows the enzyme-catalyzed reaction in which nitrate reductase (NaR) promotes the conversion of nitrate to nitrite with nicotinamide dinucleotide, reduced form (NADH) as the reductant. Step 2 shows the color formation from the reaction of nitrite with the Griess color reagents under acidic conditions. The amount of color product is quantified with a calibrated spectrophotometer. The second step is the same as in the certified cadmium reduction method.



Figure 2 Nitrate reductase producing *Pichia pastoris* cell line grown in a BioFlo 3000<sup>®</sup> 14-L fermenter (New Brunswick Scientific Co., Edison, NJ).

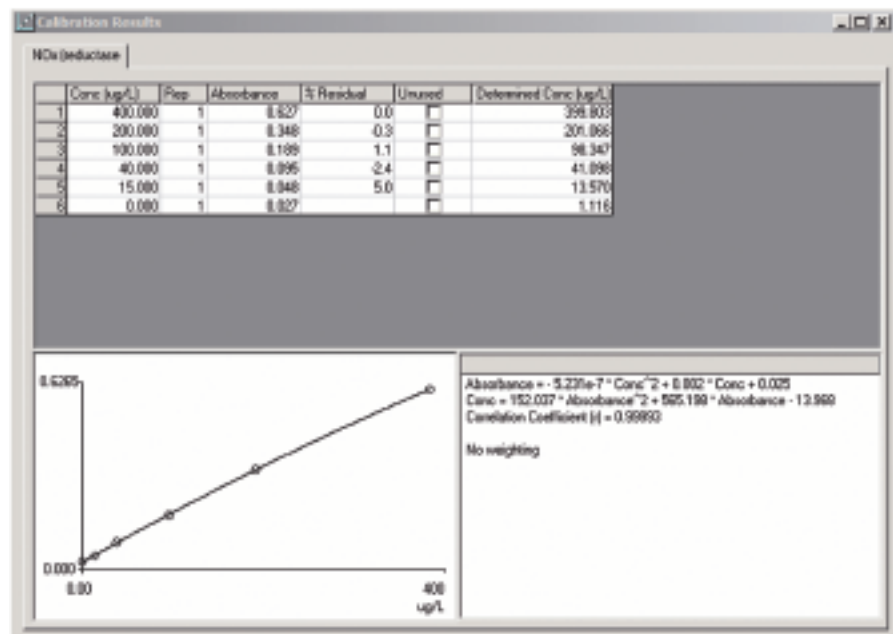


Figure 4 Calibration for the range of 15–400 µg NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup> – N/L.

The method is being called “enzyme-in-the-cup” since YNaR1 is dried in a stabilized form in the analyzer reaction cups. The analysis is carried out by automated addition to the enzyme-in-the-cup of a buffered solution containing NADH. After a brief stirring cycle, the water sample is added and the mixture is stirred again. The enzyme-catalyzed NADH-driven reduction of nitrate to nitrite is allowed to run to completion. Next, Griess color reagents are added and the color reaction runs to completion before it is automatically read by the AP300 colorimeter. The values are compared to a standard calibration curve previously generated, and the nitrate content of the samples is automatically calculated and reported in the AP300 computer system. (The method will soon be available.) Calibrations cov-

ering ranges as low as 15–400 µg NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup> – N/L have been achieved with this technology (Figure 4 and Table 1). Ten replicate analyses of a 200 µg NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup> – N/L resulted in a %RSD of 2.37%. A method detection limit (MDL) of 3.02 µg NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup> – N/L was obtained using a 10 µg NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup> – N/L standard.

## References

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**Table 1** Data obtained for MDL study using nitrate reductase and the AP300; MDL and minimum level using 10 µg NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup> – N/L standard

Replicate	Absorbance	Calculated concentration (µg/L)
1	0.0408	9.462
2	0.044	11.105
3	0.0407	9.411
4	0.042	10.078
5	0.041	9.565
6	0.0457	11.98
7	0.0426	10.386
Average		10.28386
Standard deviation		0.962468
MDL (3.14× the SD)		3.02
ML (3.18× the MDL)		9.6