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Corn Leaf Nitrate Reductase—A Nontoxic Alternative to Cadmium for Photometric Nitrate Determinations in Water Samples by Air-Segmented Continuous-Flow Analysis

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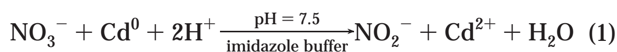
Development, characterization, and operational details of an enzymatic, air-segmented continuous-flow analytical method for colorimetric determination of nitrate + nitrite in natural-water samples is described. This method is similar to U.S. Environmental Protection Agency method 353.2 and U.S. Geological Survey method I-2545-90 except that nitrate is reduced to nitrite by soluble nitrate reductase (NaR, EC 1.6.6.1) purified from corn leaves rather than a packed-bed cadmium reactor. A three-channel, air-segmented continuous-flow analyzer—configured for simultaneous determination of nitrite (0.020–1.000 mg-N/L) and nitrate + nitrite (0.05–5.00 mg-N/L) by the nitrate reductase and cadmium reduction methods—was used to characterize analytical performance of the enzymatic reduction method. At a sampling rate of 90 h^{-1}, sample interaction was less than 1% for all three methods. Method detection limits were 0.001 mg of NO_2^- -N/L for nitrite, 0.003 mg of NO_3^-+ NO_2^- -N/L for nitrate + nitrite by the cadmium-reduction method, and 0.006 mg of NO_3^-+ NO_2^- -N/L for nitrate + nitrite by the enzymatic-reduction method. Reduction of nitrate to nitrite by both methods was greater than 95% complete over the entire calibration range. The difference between the means of nitrate + nitrite concentrations in 124 natural-water samples determined simultaneously by the two methods was not significantly different from zero at the $p = 0.05$ level.

Introduction

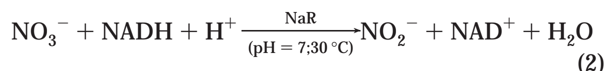
Nitrite in natural-water samples typically is determined colorimetrically using Griess reaction (*1*) reagents—usually sulfanilamide and *N*-(1-naphthyl)ethylenediamine (*2*). Equally specific and sensitive colorimetric reagents for nitrate determinations are not available. For this reason, nitrate is frequently reduced to nitrite prior to analytical determinations. Although zinc (*3*), hydrazine (*4*), bacterial nitrate reductase forms (EC 1.9.6.1) (*5*), and ultraviolet radiation (*6*) have been used for this purpose, cadmium—reactor types include packed bed (*7, 8*), wire-in-tube (*9–11*), and open tubular (*12–14*)—generally is the preferred reagent because it provides near-quantitative reduction of nitrate to nitrite with negligible reduction of nitrite to lower oxidation species (*8*) (see eq 1).



$$NO_3^- + Cd^0 + 2H^+ \xrightarrow[\text{imidazole buffer}]{pH = 7.5} NO_2^- + Cd^{2+} + H_2O \quad (1)$$

Growing awareness of cadmium's toxicity, however, makes its continued use a concern to analysts and regulators alike. Costs associated with processing cadmium-containing waste streams and disposing of them properly also are increasing. These factors motivated us to explore more environmentally benign reducing reagents for routine nitrate determinations in water.

Nitrate reductase purified from corn leaves (NADH:NaR, EC 1.6.6.1), designated *NaR* throughout this paper, irreversibly catalyzes reduction of nitrate to nitrite with high specificity (*15, 16*) (see eq 2).



$$NO_3^- + NADH + H^+ \xrightarrow[(pH = 7;30\ ^\circ C)]{NaR} NO_2^- + NAD^+ + H_2O \quad (2)$$

Unlike bacterial nitrate reductase forms, which are oxygen sensitive and require a toxic cofactor, NaR is fully functional in air-saturated solutions. The cofactor for NaR, the reduced form of β-nicotinamide adenine dinucleotide (NADH), is nontoxic and costs much less than the β-nicotinamide adenine dinucleotide phosphate (NADPH) cofactor required by fungal nitrate reductase forms (*17*) (EC 1.6.6.2 and 1.6.6.3).

Campbell and others (*18*) described the benefits of replacing cadmium with NaR in field-test kits for determination of nitrate in water. In this study we demonstrate that NaR is an ideal candidate to replace cadmium as the reagent of choice for routine determination of nitrate in natural-water samples by automated, continuous-flow methods such as U.S. Environmental Protection Agency (USEPA) method 353.2 (*19*) and U.S. Geological Survey (USGS) method I-2545-90 (*20*). We also describe the air-segmented continuous-flow analyzer used to implement the NaR-reduction method and compare its performance with that of a conventional, automated cadmium-reduction (CdR) method for a variety of synthetic and natural-water samples.

Experimental Section

Instrumentation. A three-channel, air-segmented continuous-flow analyzer (Alpkem RFA-300) was used for simultaneous determination of nitrite and nitrate + nitrite by the CdR and NaR reduction methods. This analyzer consisted of a model 301 sampler, a model 302 peristaltic pump, a model 313 analytical cartridge base equipped with three analytical cartridges, a model 314 power module, and three model 305A photometers. The sampling rate was 90 h^{-1} (sample time = 30 s; wash time = 10 s) for all three channels. *Dwell times*—time between aspiration of a sample and its arrival at the detector—were about 2, 2.5, and 15.5 min for the nitrite, CdR nitrate + nitrite, and NaR nitrate + nitrite analytical cartridges, respectively. Analytical cartridge diagrams for each method are shown in Figures 1–3. Analytical cartridges were

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assembled at the USGS National Water Quality Laboratory (NWQL) from commercially available, 1-mm inside diameter (i.d.) components. Photometer flow cells (10-mm path length × 0.5-mm i.d.) had internal volumes of about 2 μL. See Figures 1−3 and accompanying captions for analytical cartridge component part numbers (P/N) and additional details. The pump was equipped with an electronic *air-bar*, which ensured reproducible proportioning of the analytical stream by intrasample air bubbles. Sample dispersion (*carryover*) was minimized by *pecked* sample introduction and *bubble-through-the-flow-cell* detection. Details of these essential instrumentation features can be found elsewhere (*21*).

Photometric data were acquired and processed using FASPac software (Astoria-Pacific, Clackamas, OR). This software runs under Microsoft Windows on a PC platform and includes a model 350 interface box that controls the sampler and digitizes analogue photometer outputs with 16-bit resolution.

Excel (Microsoft, Redmond, WA) and Origin (OriginLab Corp., Northampton, MA) software were used for data manipulation, statistical analysis, and scientific graphing.

Reagents and Calibrants. ASTM type I deionized water (DI water) was used exclusively in this work. Class A volumetric flasks and EDP Plus pipets (Rainin Instruments, Emeryville, CA) equipped with a variety of liquid ends were used for solution preparation. Reagents and calibrants were prepared as follows. Phosphate buffer stock solution was prepared by dissolving 0.01 g of the disodium ethylenediamine tetraacetic acid (EDTA) in 1 L of 0.025 M potassium dihydrogen phosphate (KH_2PO_4). Phosphate buffer (pH 7.5) was prepared by combining 83 mL of phosphate buffer stock with 17 mL of 0.1 M KOH. This buffer was used to prepare NaR and NADH reagents. Freeze-dried NaR (two vials, each containing 0.5 unit of NaR—product NaR-1, Nitrate Elimination Company, Inc., Lake Linden, MI) were reconstituted according to the manufacturer's instructions. The reconstituted NaR was diluted to a volume of 20 mL with phosphate buffer and immediately cooled to about 4 °C in a water−ice bath. The NaR reagent was maintained at this temperature during use. Freeze-dried, desiccated NaR as supplied by NECi is stable for 6 months at 4 °C and 12 months at −20 °C. NADH was prepared by dissolving 0.002 g of β-nicotinamide adenine dinucleotide (reduced form) in 10 mL of pH 7.5 phosphate buffer. The 20-mL volume of NaR and 10-mL volume of NADH are sufficient for about 2 h (180 nitrate determinations) of analyzer operation. At 4 °C, the useful lifetimes of reconstituted NaR and NADH solutions are about 8 h. [Note: a mixed enzyme reagent containing NADH (0.003 g), NaR (0.5 unit), and Brij-35 surfactant (50 μL) diluted to a volume of 30 mL with pH 7.5 phosphate buffer was used in preliminary experiments.] Diluent for the NaR nitrate method and the nitrite method contained 1 mL of Brij-35 surfactant (30% w/v solution) per liter of DI water.

Packed-bed cadmium reactors (PBCdR) used for the CdR method were prepared by slurry packing 40−60 mesh, copperized cadmium granules into PTFE Teflon tubing (5 cm long × 1.6-mm i.d.). Cadmium granules were retained by insertion of hydrophilic plastic frits (40-μm nominal pore size) at the ends of the PBCdR. More details about PBCdR preparation can be found elsewhere (*20*). Imidazole buffer (0.1 M, pH 7.5) for the CdR nitrate method contained 6.8 g of imidazole, 2.5 mL of concentrated (~12 M) hydrochloric acid, and 0.5 mL of 2% (w/v) copper sulfate ($CuSO_4 \cdot 5H_2O$) diluted to a volume of 1 L with DI water. Add 0.1 mL of Brij-35 surfactant per 100 mL of imidazole buffer immediately before use. Sulfanilamide (SAN) reagent contained 100 mL of concentrated (~12 M) HCl and 10 g of SAN diluted to a volume of 1 L with DI water. N-1-Naphthyl ethylenediamine dihydrochloride (NED) reagent contained 0.5 g of NED diluted to a volume of 1 L with DI water.

Primary nitrate calibrant (1 mL ≡ 0.500 mg NO_3^- -N) contained 3.6088 g of potassium nitrate per liter of DI water. Primary nitrite calibrant (1 mL ≡ 0.100 mg NO_2^- -N) contained 0.4926 g of sodium nitrite per liter of DI water. Mixed calibrants and spike solutions were prepared by appropriate dilution of the primary calibrants. Mixed calibrants used routinely during this work contained 80% nitrate-nitrogen and 20% nitrite-nitrogen—for example, the calibrant with a nominal concentration of 3.75 mg of NO_3^- -N + NO_2^- -N/L contained 3.0 mg of NO_3^- -N/L and 0.75 mg of NO_2^- -N/L. The analyzer was calibrated (six mixed calibrants and a blank) at the beginning of each 90-sample run comprised of samples, calibrants, and reference solutions. Typically blank and continuing calibration verification (CCV) solutions were determined at 20-sample intervals during each run.

Results and Discussion

The main objective of this work was to demonstrate that replacing toxic cadmium with NaR in standard, air-segmented continuous-flow methods for determination of nitrate + nitrite in water samples—USEPA 353.2 and USGS I-2545-90, for example—is not only feasible but practical. Feasibility assessment criteria for the NaR method were as follows: (1) achieve quantitative reduction of nitrate to nitrite over the entire calibration range, so that equivalent concentrations of nitrate and nitrite result in equivalent colorimetric indicator reaction response; and (2) obtain analytical results for nitrate + nitrite determinations in environmental water samples that are statistically equivalent to those determined at present by accepted CdR methods.

Practicality assessment criteria for the NaR method were as follows: (1) automation level comparable to CdR methods—that is, pour samples and walk away; (2) sampling rate comparable to CdR methods—typically 40−100 h^{-1}; (3) analyzer waste stream volume equal to or less than CdR methods; and (4) operational costs equivalent to CdR methods.

NaR has been fully characterized in previous work (*15*−*17*). Its cofactor is NADH. NaR is not inhibited by oxygen (*15*), and its reactivity is maximum at pH ~ 7 and 30 °C. Nitrate is the only high efficiency substrate for NaR. NaR catalyzes reduction of chlorate, bromate, and iodate with much less efficiency. Products of these alternate reductions neither react nor interfere in the Griess-reagent indicator reaction used in this work. Cyanide and azide are moderate inhibitors of the nitrate reducing function, but mM concentrations are needed to block enzyme activity in the presence of nitrate. Heavy metals (Zn, Cu, Hg, Pb, Ag, Au, etc.) inhibit NaR, but adding EDTA to the buffered enzyme reagent mitigates such interference. Nitrite is not a substrate for NaR and acts only as a weak inhibitor.

In accordance with USGS protocols, all natural-water samples included in this study were membrane filtered (0.45 μm nominal pore size) at collection sites to remove biota that might otherwise destabilize dissolved nutrient concentrations during 30-day, refrigerated storage (*22*). The USEPA also recommends filtering samples prior to nitrate analysis by their method 353.2 (*19*). Nonetheless, nitrate analysis of low-turbidity, whole-water samples by CdR- and NaR-reduction methods also should be feasible. Also note that air-segmented continuous-flow analysis (CFA) was chosen in preference to flow-injection analysis (FIA) for this work because it is the technique of choice when long reaction times (greater than 1 min), low flow rates (0.5−0.7 mL min^{-1}, typically), and high sampling rates (80−120 samples h^{-1}) are required (*21, 23*). These CFA characteristics substantially enhanced analytical sensitivity, throughput, and reagent conservation (particularly of soluble NaR) and reduced wastestream volume in the NaR-reduction nitrate method and the other methods described here.

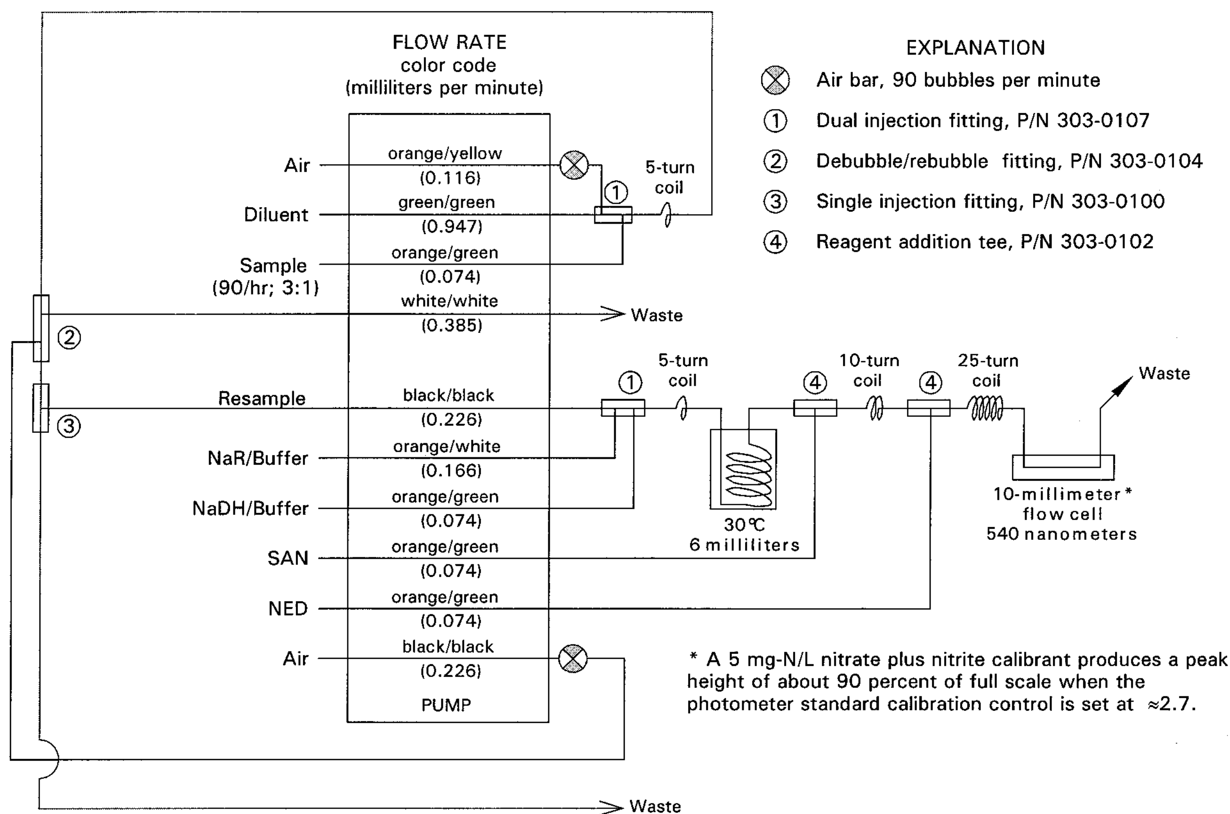

FIGURE 1. Analytical cartridge diagram for determination of nitrate plus nitrite by the soluble corn-leaf nitrate reductase (NaR) method.

In preliminary experiments, feasibility criteria were explored using a simpler analytical cartridge than the one shown in Figure 1. Here, the sample stream (37 μL min^{-1}) combined with an air-segmented (45 bubbles min^{-1}) mixed enzyme reagent stream (226 μL min^{-1}) that contained NADH, NaR, and Brij-35 surfactant dissolved in pH 7.5 phosphate buffer. After mixing in a 5-turn coil, this air-segmented analytical stream flowed into one or two 2-mL time-delay coils with thermostats set at 30 °C. At the flow rates stated, each 2-mL coil provided about 6 min of reaction time. Addition and mixing of colorimetric reagents and photometric detection were exactly as shown in Figure 1.

During initial reagent optimization experiments, it became apparent that the NADH cofactor inhibited chromophore formation in the Griess-reagent indicator reaction.

Table 1 lists results from a series of experiments in which the effect of NADH on the slope of nitrite calibration curves in the absence of NaR was investigated. Here the molar ratio of NADH in relation to the concentration of the highest nitrite calibrant—that is, 5 mg NO_2^- -N/L or ≈ 357 μM—was varied in the range of 0–22. Data in Table 1 reveal a steady decrease in indicator reaction sensitivity as the NADH concentration increases. When NaR was included in the buffered NADH reagent, similar trends were observed in the slopes of nitrate calibration curves, except that the sensitivity was zero in the absence of NADH, because without it, NaR does not reduce nitrate to nitrite. In accordance with these experiments, a 2-fold molar excess of NADH relative to the upper nitrate concentration level was chosen as the best compromise between an adequate excess of cofactor on one hand and unacceptably high inhibition of the indicator reaction on the other.

In the next round of optimization experiments, pairs of nitrate and nitrite solutions with nominal concentrations of 5.0, 2.5, and 1.0 mg-N/L were determined simultaneously by the CdR method (Figure 2) and the preliminary NaR method using the optimized mixed enzyme reagent. Data in Table 2 demonstrate that with these reagent concentrations and two, 2-mL reaction coils, the NaR method quantitatively reduced nitrate to nitrite over the entire calibration range and produced results comparable to those obtained with the cadmium reduction method. Complete reduction of nitrate to nitrite by the NaR method was not achieved when the experiment was repeated with a single 2-mL reaction coil (see Table 2). When the concentration of NaR in the mixed enzyme reagent was doubled, quantitative reduction of nitrate to nitrite was achieved using one 2-mL reaction coil, but this approach was not pursued because it would have increased per-test costs associated with the NaR reduction method.

The next phase of NaR method development involved refinement of the analytical cartridge design. For the air-segmented continuous-flow analyzer used in this work (1-

TABLE 1. Griess Indicator Reaction Sensitivity Measured in Relation to NADH Concentration

reagent identifier	molar ratio[a] $[NADH]/[NO_2^-]$	calibration slope[b]	relative sensitivity
S6	0.0	0.826	1.000
S1	2.4	0.624	0.755
S2	7.2	0.421	0.510
S3	12.0	0.308	0.373
S4	17.0	0.245	0.296
S5	21.7	0.226	0.274

[a] Tabulated ratios include dilution factor (DF) corrections for dilution of the sample by the reagent [DF = 0.037/(0.037+0.226) = 0.141] and dilution of reagent by the sample (DF = 0.859). For example, the molar concentration of the 5 mg of NO_2^- -N/L calibrant is 357 μM, which after dilution by the reagent stream is about 50 μM (357 μM × 0.141). Likewise, the molar concentration of the NADH in reagent solution S1 is 141 μM. After it merges with the sample stream its concentration is about 121 μM (141 μM × 0.859). [b] Based on linear regression of peak heights (absorbance) for nitrite calibrants with nominal concentrations of 5.0, 2.5, and 1.0 mg of NO_2^- -N/L.

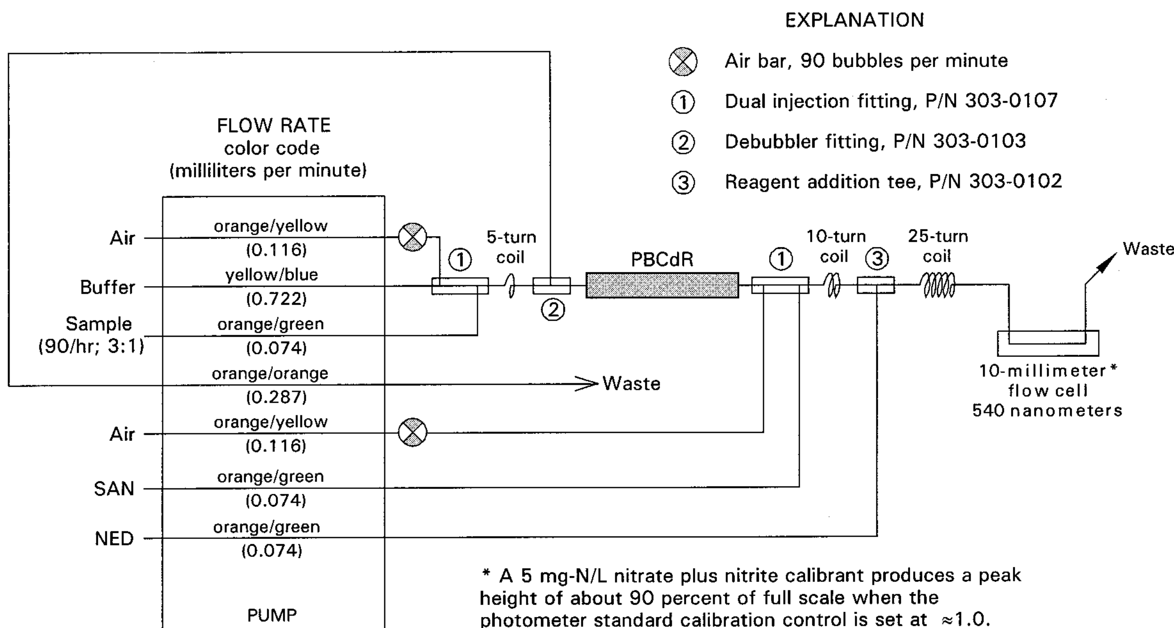

FIGURE 2. Analytical cartridge diagram for determination of nitrate plus nitrite by the packed bed cadmium reactor (CdR) method.

TABLE 2. Data Demonstrating Greater than 95% Complete Reduction of Nitrate to Nitrite by the CdR and Preliminary NaR Methods

	concentration of nitrate or nitrite in mg-N/L					
	CdR (found)		NaR[a] (found)		NaR[b] (found)	
nominal	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-
5.00	5.00	5.00	5.00	5.01	3.30	5.02
2.50	2.53	2.47	2.53	2.46	1.81	2.46
1.00	1.01	0.99	0.99	1.02	0.80	1.03

[a] Two 2-mL reaction coils. Reaction time prior to addition of colorimetric reagents was about 12 min. [b] One 2-mL reaction coil. Reaction time prior to addition of colorimetric reagents was about 6 min.

mm internal diameter conduits), optimum air segmentation frequency (n) and flow rate (F) are about 90 bubbles min^{-1} and 0.5–0.7 mL min^{-1}, respectively (*21*). Benefits of using less than optimum values for n and F (45 bubbles min^{-1} and 0.263 mL min^{-1}) in preliminary experiments—reduced NaR requirement, extended residence time in reaction coils, reduced waste stream volume—were offset by increased sample interaction and poor hydraulic stability that degraded precision of analytical results. Dilution of samples prior to addition of NaR, therefore, was explored as a means to achieve a more favorable balance among reagent conservation, waste reduction, and analytical performance.

Coverly (*24*) provides operational details and performance characteristics of parallel-plate dialyzers and *dilution loops*, both of which are used routinely to achieve 15- to 150-fold dilution factors in CFA applications. Dilution by continuous-flow dialysis is straightforward. The extent of dilution is governed by the length and depth of dialyzer channels, donor and recipient streamflow rates, temperature, and the type and thickness of the dialysis membrane. The main disadvantage of dilution by dialysis is temporal variation in analyte transport rates that can be caused by temperature fluctuations, ionic strength differences between donor and recipient streams, and membrane fouling. Dilution loops are auxiliary analytical cartridges (see the upper section of Figure 1) in which samples are proportioned into an air-segmented diluent stream, mixed, and returned to the inlet side of the pump. A small portion of each diluted sample is aspirated into the primary analytical cartridge (lower section of Figure 1), while the remainder flows to waste. Dilution loops are less affected by temperature fluctuations and sample-to-sample ionic strength variation than dialyzers and were easy to implement with the CFA hardware used for this work. Dispersion that otherwise would have occurred when diluted samples made a second pass through the pump was reduced substantially by maintaining air-segmented flow within the resample pump tube (*24*). This result was achieved by debubbling and immediately rebubbling the dilution loop stream just before it reached the resample fitting (see fittings 2 and 3 in Figure 1). The volume of these newly injected air bubbles was about three times greater than normal to ensure that the resample pump tube would aspirate a portion of each that was sufficiently large to fully occlude the 1-mm i.d. conduits of the primary analytical cartridge. The withdrawal legs of the waste and resample pump tubes connected to fittings 2 and 3 were shortened to about 3 cm. Slight adjustment (± 3 mm) of the length of the resample pump tube might be necessary if resampled air bubbles shear as they pass under the pump rollers. Results reported in the remainder of this paper were obtained with the dilution loop analytical cartridge shown in Figure 1. For some types of CFA analyzers, substituting a dialyzer for the dilution loop in the NaR-reduction method analytical cartridge might be easier to implement and should yield comparable results.

Figure 4 shows peaks obtained for nitrate + nitrite calibrants in the concentration range of 0.25–5.00 mg-N/L by the CdR (a) and NaR (b) methods operating at a sampling rate of 90 samples h^{-1}. Insets in this figure reveal the outstanding linearity of calibration functions obtained with both methods. Slopes of the calibration functions for both methods are about the same because the variable gain output settings of both photometers—see, *standard calibration control* information in Figures 1 and 2—were adjusted to obtain peak heights of about 90% of full scale for the highest concentration calibrant. Actually, full-scale (5 V) absorbance was about 1.0 and 0.4 for the CdR and NaR methods, respectively. This sensitivity difference between the CdR and NaR methods resulted from the larger sample dilution factor designed into the NaR method analytical cartridge to minimize enzyme consumption. Sample interaction (% I), as measured by the interaction test patterns (*25*) shown in Figure 5, was less than 1% for both methods. The 2.5 and

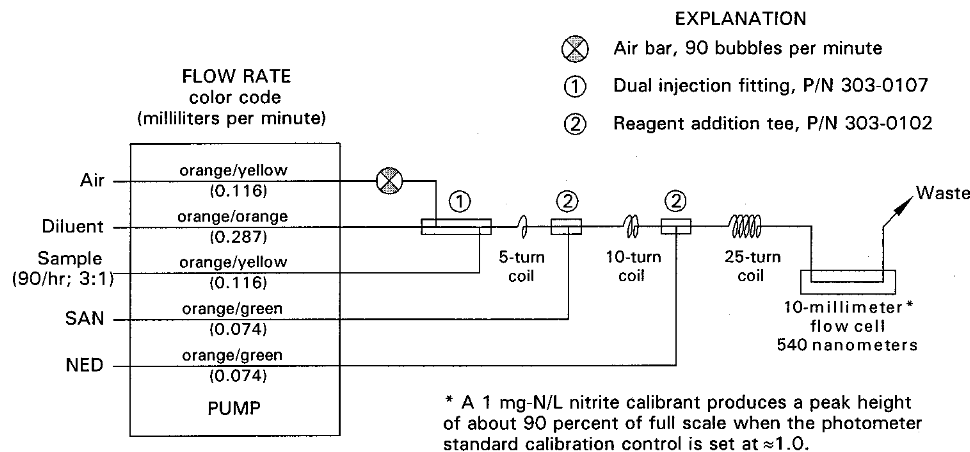

FIGURE 3. Analytical cartridge diagram for determination of nitrite.

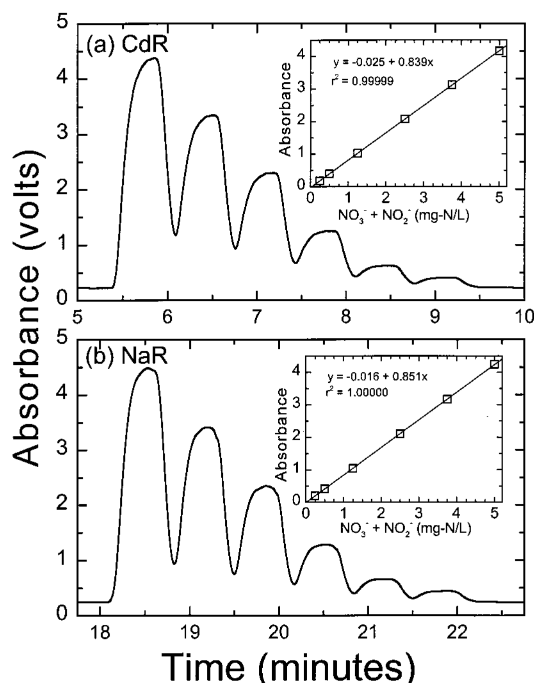

FIGURE 4. Recorded peaks and calibration plots for nitrate + nitrite determined simultaneously by the CdR-reduction (a) and NaR-reduction (b) methods. Calibrants contain 80% nitrate-nitrogen and 20% nitrite-nitrogen—for example, calibrant 2, which has a nominal concentration of 3.75 mg-N/L, contains 3.0 mg of NO_3^- -N/L and 0.75 mg of NO_2^- -N/L. Sampling rate is 90 h^{-1} (sample time = 30 s, wash time = 10 s). Reduction of nitrate to nitrite by (a) packed bed cadmium reactor (dwell time = 165 s) and (b) soluble NaR (dwell time = 930 s).

15.5 min dwell times for the CdR and NaR methods are also discernible in Figure 5. Interestingly, % I was less for the NaR method than for the CdR method despite the 13-min longer dwell time of the former. This fact indicates that analytical stream dispersion in the dilution loop of the NaR analytical cartridge is less than that resulting from unsegmented flow through the PBCdR of the CdR analytical cartridge.

A natural-water sample with low nitrate and nitrite concentrations was used to estimate method detection limits (MDL) for the nitrite (0.001 mg-N/L), CdR nitrate + nitrite (0.003 mg-N/L), and NaR nitrate + nitrite (0.006-N/L) methods. These values were calculated according to the USEPA method (*26*) as indicated in Table 3. The difference between MDLs for the two nitrate + nitrite methods is consistent with the approximately 2-fold higher dilution factor of the NaR method. MDLs for both methods exceed requirements for the 0.05−5.00 mg-N/L reporting range of USGS method I-2545-90. Tables 4 and 5 provide spike recovery results for seven natural-water samples with ambient nitrate concentrations in the range of 0.02−4.4 mg NO_3^- -N/L. These samples were spiked at a level of 0.25 mg of NO_3^- -N/L by adding 50 μL of a 1+9 dilution of the stock nitrate

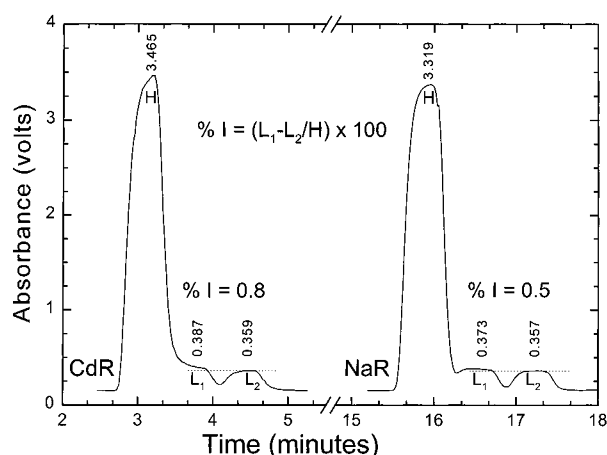

FIGURE 5. Interaction test patterns used to assess sample interaction (carryover) of the CdR and NaR methods. The dwell times for the CdR (2.5 min) and NaR (15.5 min) methods are also evident because time (*x*-axis) is indexed from aspiration of the first sample—the peak designated *H* in this figure.

TABLE 3. Data and Calculations Used To Estimate Method Detection Limits (MDL) for Nitrite and Nitrate + Nitrite by the CdR and NaR Methods

	concentration found (mg-N/L)		
NWQL sample ID	NO_2^-	$NO_3^- + NO_2^-$ CdR reduction	$NO_3^- + NO_2^-$ NaR reduction
S00160070	0.0035	0.070	0.075
S00160070	0.0034	0.069	0.071
S00160070	0.0027	0.067	0.073
S00160070	0.0030	0.069	0.070
S00160070	0.0029	0.067	0.072
S00160070	0.0026	0.069	0.073
S00160070	0.0025	0.068	0.075
S00160070	0.0027	0.069	0.072
average	0.0029	0.069	0.073
standard deviation	0.0004	0.001	0.002
number of values	8	8	8
degrees of freedom	7	7	7
t-value (one-sided, 99%)	2.998	2.998	2.998
MDL	0.0012	0.003	0.006

TABLE 4. Spike Recovery Data for CdR Method

	nitrate concentration (mg NO_2^- + NO_3^- -N/L)				
NWQL ID	unspiked	spiked	found	added	recovery (%)
S002200228	0.016	0.253	0.237	0.250	95
S002200226	0.089	0.336	0.247	0.250	99
S002200195	0.225	0.475	0.250	0.250	100
S002200065	0.677	0.926	0.249	0.250	100
S002200186	0.986	1.221	0.234	0.250	94
S002200016	1.703	1.959	0.256	0.250	103
S002200017	4.400	4.690	0.290	0.250	116
			av recovery:		101

TABLE 5. Spike Recovery Data for NaR Method

	nitrate concentration (mg NO_2^- - + NO_3^- -N/L)				
NWQL ID	unspiked	spiked	found	added	recovery (%)
S002200228	−0.001	0.241	0.243	0.250	97
S002200226	0.074	0.330	0.256	0.250	102
S002200195	0.208	0.477	0.269	0.250	108
S002200065	0.662	0.953	0.291	0.250	117
S002200186	0.977	1.221	0.244	0.250	98
S002200016	1.726	2.009	0.282	0.250	113
S002200017	4.276	4.472	0.196	0.250	78
			av recovery:		102

TABLE 6. Between-Run Nitrate + Nitrite Concentration Differences in Natural-Water Samples Used for Spike Recovery Experiments

	CdR-reduction method			NaR-reduction method		
NWQL ID	run 1	run 2	difference	run 1	run 2	difference
S002200228	0.003	0.016	−0.013	0.008	−0.001	0.009
S002200226	0.081	0.089	−0.008	0.083	0.074	0.009
S002200195	0.226	0.225	0.001	0.215	0.208	0.006
S002200065	0.696	0.677	0.019	0.657	0.662	−0.005
S002200186	0.958	0.986	−0.028	0.979	0.977	0.002
S002200016	1.714	1.703	0.011	1.735	1.726	0.009
S002200017	4.293	4.400	−0.107	4.314	4.276	0.038

TABLE 7. Concentrations Measured for 1.00 mg of NO_2^-N/L and 1.00 mg of NO_3^-N/L Standards that Were Analyzed Consecutively at 15-Sample Intervals during a Typical Run To Check for Quantitative Reduction of Nitrate to Nitrite[a]

	CdR reduction method			NaR reduction method		
cup number	NO_2^- mg-N/L	NO_3^- mg-N/L	reduction (%)	NO_2^- mg-N/L	NO_3^- mg-N/L	reduction (%)
13, 14	0.995	0.997	100.2	0.996	1.009	101.3
34, 35	0.987	0.975	98.8	0.992	0.994	100.2
51, 52	0.972	0.977	100.5	0.978	0.968	99.0
70, 71	1.023	1.005	98.2	0.989	0.991	100.2

[a] Percent reduction = $(1 - ((C_{NO_2^-} - C_{NO_3^-})/C_{NO_2^-})) \times 100$.

calibrant to 10-mL volumes of each sample. Average nitrate recovery was 101% for the CdR method and 102% for the NaR method. Nitrite concentrations in these samples were less than 0.01 mg of NO_2^- -N/L. Results for between-run duplication of nitrate concentrations for samples used for the spike recovery experiments are listed in Table 6. Differences between duplicate results were typically about 0.01 mg of NO_3^- -N/L. Table 7 provides concentrations determined for paired nitrite and nitrate solutions (each with a nominal concentration of 1.00 mg -N/L) that were determined at approximate intervals of 20 samples to assess completeness of nitrate reduction during the course of each run. Reduction of nitrate to nitrite by the CdR and NaR methods was consistently greater than 95% complete.

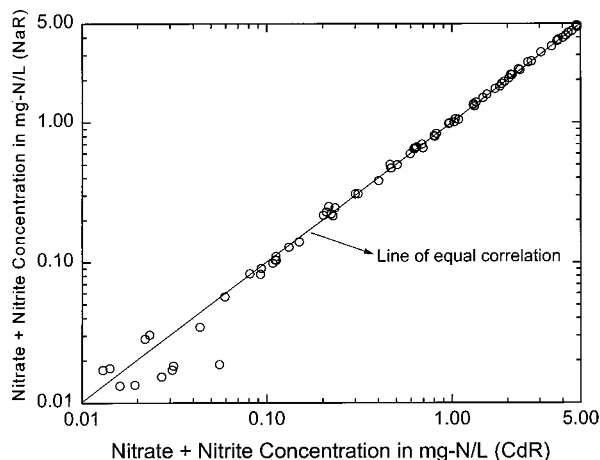

FIGURE 6. Nitrate + nitrite concentrations determined simultaneously by the CdR and NaR methods for 124 natural-water samples received at the USGS National Water Quality Laboratory for analysis. Concentrations greater than 5 mg-N/L (2 points) and less than 0.01 mg-N/L (21 points) that were included in statistical analyses are not shown.

In concluding work, a subset of 124 natural-water samples received at the USGS NWQL from August 3−9, 2000, were determined for nitrate + nitrite by the CdR and NaR methods on August 9, 10, and 11, 2000. Linear regression of CdR method data (x) in relation to NaR method data (y) resulted in a slope of 1.005 ± 0.002 and a y-intercept of 0.001 ± 0.003. The correlation coefficient r^2 was 1.00 ± 0.03 and highly significant ($p < 0.0001$). The difference between means of nitrate + nitrite concentrations determined by the CdR and NaR methods was −0.004 mg-N/L, which on the basis of a paired t-test (degrees of freedom = 123; t-value = −1.457; p-value = 0.1477) was not significantly different from zero at the $p = 0.05$ level. A subset of these data (101 paired results) with nitrate + nitrite concentrations in the range of 0.01−5.00 mg-N/L plotted on a log−log scale scattered about the line of equal correlation is shown in Figure 6. Representative, digitally acquired peak recordings excerpted from a portion of one of the NaR (a) and CdR (b) method comparison runs on August 11, 2000, are shown in Figure 7.

Data presented in this section demonstrate conclusively that replacement of toxic cadmium with more environmentally benign NaR in time-honored nitrate determination methods, such as USEPA 353.2 and USGS I-2549-90, is feasible. Reduction of nitrate to nitrite is near quantitative with either method. Likewise, difference between means of nitrate concentrations in environmental water samples determined by the CdR and NaR methods were not significantly different from zero at the $p = 0.05$ level. Practical operation criteria for the NaR method were also achieved. The level of automation for the CdR and NaR methods is equivalent, and analytical rates for both (90 h^{-1}) are excellent. The NaR method produces 36 mL of waste h^{-1}. This estimated volume does not include the waste stream from the dilution loop, which contains only DI water, natural water, and surfactant, and should pose no disposal hazard. The CdR method produces about twice as much cadmium-containing waste (74 mL h^{-1} of operation).

Comparing operation costs of the two methods is less clear. A kilogram of granular cadmium costs about $750 and would last for several years, even in a facility as large as the USGS NWQL where about 25 000 samples are analyzed annually for nitrate. An annual cost for cadmium of about $250, however, does not include time that analysts must spend preparing PBCdRs for use, the expense of CdR method

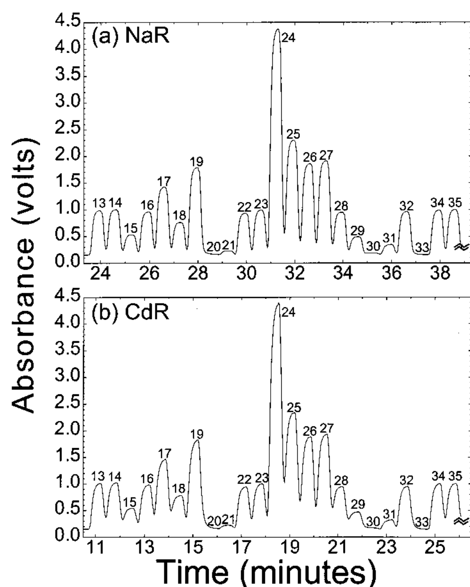

FIGURE 7. Peaks recorded for nitrate + nitrite concentrations determined simultaneously by the NaR (a) and CdR (b) methods. Environmental samples (peaks 15−29), reference samples (peaks 30−32), and a blank (peak 33) are bracketed by reduction check standards with nominal concentrations of 1 mg of NO_3^- -N or NO_2^- -N/L (peaks 13 and 34, NO_3^-, peaks 14 and 35, NO_2^-).

waste disposal, and the intangible costs of potential exposure of laboratory personnel to this toxic metal. The NaR nitrate method described here requires 1 unit of enzyme per 180 determinations. The theoretical annual NaR requirement at the NWQL, therefore, would be about 140 units (25 000/180). A more realistic estimate of about 175 units per year to account for waste would bring annual costs for NaR to about $5000−20 times greater than the annual cost estimated for cadmium. It is debatable where the actual cost of using cadmium lies along the continuum from price per kilogram, to disposal costs, to the intangible costs associated with handling biohazards. If new production methods for NaR could reduce its cost by a factor of 5 or 10, however, it seems likely that CdR methods would be quickly abandoned in favor of more environmentally benign NaR methods. Cheaper or reusable NaR in the form of immobilized enzyme reactors would also open up a wide range of new analytical applications, which we continue to explore.

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