

# Determination of Nitrate in Natural Waters by Vanadium Reduction and the Griess Assay: Reassessment and Optimization

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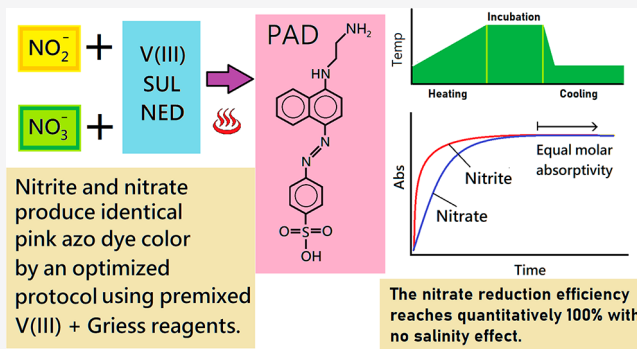
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**ABSTRACT:** An accurate, precise, simple, and efficient spectrophotometric method has been developed for the determination of nitrate concentrations in natural waters by using premixed vanadium and the Griess reagents. We have systematically reassessed and optimized several key parameters influencing nitrate to nitrite reduction and pink azo dye formation, which include the order of reagent addition, reagent concentrations, heating temperatures, and cooling procedures. Samples added with the premixed reagents are heated gradually in a 50 °C water bath for 25–30 min followed by a quick cooling step to fix the final yield. Both nitrite and nitrate produce a stable and equal molar absorptivity of  $\sim 50000 \text{ M}^{-1} \text{ cm}^{-1}$  under the optimized condition, without showing a significant salt effect. With a detection limit of  $0.2 \mu\text{M}$  and a precision of 1% (relative standard deviation) for nitrate at  $5\text{--}50 \mu\text{M}$ , this simple and reliable protocol provides an alternative to the traditional Cd–Cu reduction method for routine nitrate analysis in natural waters and other aqueous samples.

**KEYWORDS:** vanadium reduction, nitrite, nitrate, Griess reaction, pink azo dye



## INTRODUCTION

The concentrations of nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ) are fundamental parameters in oceanography, limnology, and many other environmental and agricultural disciplines, so fast and accurate analysis of the concentrations is crucial for the studies or routine monitoring. The determination of nitrite in aqueous samples is customarily based on the classic Griess assay, in which nitrite first reacts with sulfanilamide (SUL hereafter) to form a diazonium ion (DAZ hereafter) that further reacts with a coupling agent to form a dense pink-colored compound.<sup>1</sup> The most frequently used coupling agent is *N*-1-naphthylethylenediamine (NED hereafter), and the resultant pink azo dye (PAD hereafter) is measured spectrophotometrically at 543 nm.<sup>2</sup> Nitrate can be determined by the same reaction after a prior reduction to nitrite. The most widely adopted reduction procedure is to allow the sample to pass through a copper-coated cadmium column.<sup>3,4</sup> Despite concerns about its toxicity, the preparation and optimization of a Cd–Cu reduction column are highly time-consuming and the reduction efficiency may suffer from inconsistency for various reasons. Frequent reconditioning of the Cd–Cu column is inevitable and tedious. As a consequence, many other protocols have been proposed.<sup>5</sup> Among them, the vanadium(III) reduction method seems to have the potential to replace the Cd–Cu reduction column for nitrate analysis.

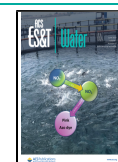
The vanadium reduction protocol was first introduced by Cox<sup>6</sup> and later described by Braman and Hendrix,<sup>7</sup> who used the V(III) ion to reduce both nitrate and nitrite to nitric oxide (NO) under heated conditions, and the concentrations were measured by chemiluminescence. The study of Miranda et al.<sup>8</sup> made a simple but significant modification by adding V(III) and the premixed Griess reagents together to trap nitrite by SUL and NED to form pink azo dye (instead of nitric oxide), so that the combined concentration of nitrite and nitrate (also noted as  $\text{NO}_x$ ) can be quantified by spectrophotometry (see Figure 1). Since then, this simple premixed protocol has been widely adopted by numerous studies.<sup>9–20</sup> The protocol can be carried out manually<sup>12,13</sup> on automated microplates<sup>14</sup> and flow injection analysis (FIA)<sup>15,16,19</sup> or sequential injection analysis (SIA).<sup>18</sup> Although these methods all use similar chemical principles, the reagent strengths and heating conditions vary significantly so that molar absorptivities reported by those studies ranged from 1000 to  $46000 \text{ M}^{-1} \text{ cm}^{-1}$  (Table 1). In addition, two obvious problems exist. First, the absorbance of

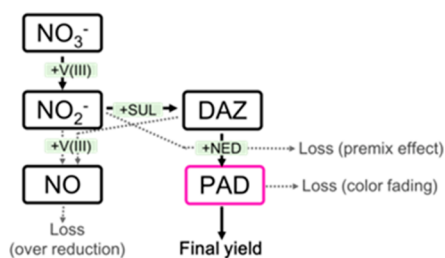
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**Figure 1.** Flowchart showing the potential reaction pathways of PAD formation for nitrite and nitrate measurement. Nitrate is reduced to nitrite by V(III) under heated conditions, and nitrite is trapped by a pre-added SUL/NED mixture to form DAZ and PAD (see solid arrows). The potential pathways for losses may be attributed to the reactions shown as dashed lines.

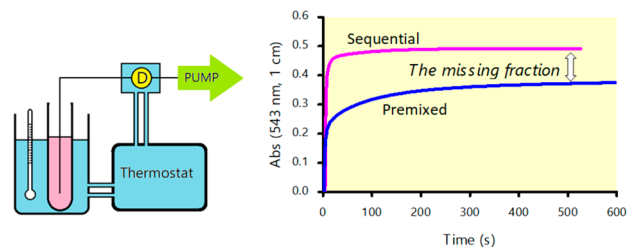
PAD formation using the premixed protocol is significantly lower than that using the sequential addition protocol, and the cause is unknown. Second, PAD is prone to fade right after the heating step, and the fading trends between the original nitrite and nitrite reduced from nitrate are probably not synchronized;<sup>11–13</sup> thus, the reduction efficiency can hardly be evaluated precisely. It appears that reassessment and optimization of reaction conditions for the premixed protocol are needed.

In this study, we have systematically investigated the effects of reagent strengths, heating, and matrix effects to understand

the reaction kinetics of PAD formation under the premixed reagents. An optimized operational procedure for nitrate analysis is proposed.

## EXPERIMENTAL SECTION

**Apparatus.** A simple device was designed to monitor PAD formation at various temperatures (Figure 2, left). A beaker or



**Figure 2.** Device for monitoring PAD formation at the designated temperature (left). The sample vessel is placed in a thermostat bath, and after reagents are added, the liquid is taken up by a peristaltic pump. The liquid flow may be stopped or continue to flow through a 1 cm flow cuvette with a 71  $\mu\text{L}$  capacity, which is also connected to a thermostat jacket in a spectrophotometer (D). Typical PAD formation curves (right) for a 10  $\mu\text{M}$  nitrite sample at room temperature using sequential reagent addition and premixed reagent addition. The gap between the two curves is the missing fraction. The final concentrations of SUL, NED, and  $\text{H}^+$  are 4.3, 0.43, and 67 mM, respectively.

**Table 1.** Comparison of Reagent Strengths of Various Protocols Involving Vanadium and Premixed Griess Reactions

protocol	analyte	temp (°C)	incubation time (min)	$[\text{V}^{3+}]$ (mM)	$[\text{SUL}]$ (mM)	$[\text{NED}]$ (mM)	$[\text{H}^+]_{\text{add}}$ (mM)	$[\text{SUL}]/[\text{NED}]$ ratio	$\epsilon(\text{est.})^a$ ( $\text{M}^{-1} \text{cm}^{-1}$ )	DL ( $\mu\text{M}$ )
21	$\text{NO}_2^-$ (Seq) <sup>c</sup>	room	5	—	4.3	0.43	67	10	53000	—
8	$\text{NO}_2^- + \text{NO}_3^-$	37	30–45	17	19.4	0.64	433	30.1	27000	0.5
9	$\text{NO}_2^- + \text{NO}_3^-$	room	840–960	2.5–5.5	1.2–2.5	0.04–0.08	49–109	30.1	40000	0.72
10	$\text{NO}_2^-$	room	—	—	5.2	0.17	27	30.1	—	—
	$\text{NO}_2^- + \text{NO}_3^-$	25	30–45	10	23.2	0.77	380	30.1	—	0.5
14	$\text{NO}_2^-$	room	15	—	5.8	0.39	270 <sup>b</sup>	15.1	—	—
	$\text{NO}_2^- + \text{NO}_3^-$	room	45	10.2	11.6	0.77	740 <sup>b</sup>	15.1	1000	0.5
12	$\text{NO}_2^-$	room	60	—	5.3	0.18	55	30.1	44000	—
	$\text{NO}_2^- + \text{NO}_3^-$	45	60	16.5	7.5	0.25	403	30.1	35000	0.4
13	$\text{NO}_2^-$	room	20	—	1.4	0.09	29	15.1	46000	—
	$\text{NO}_2^- + \text{NO}_3^-$	60	25	11.6	1.3	0.08	571	15.1	37000	0.05
11	$\text{NO}_2^-$	room	10–15	—	16.1	0.43	867	37.6	45000	—
	$\text{NO}_2^- + \text{NO}_3^-$	60	40–45	2.1	16.1	0.43	867	37.6	30000	7.14
15	$\text{NO}_2^- + \text{NO}_3^-$	60	flow	0.3	1.2	0.08	144	15.1	13350	0.1
18	$\text{NO}_2^-$	room	—	—	2.7	0.11	56	25.1	35000	0.02
	$\text{NO}_2^- + \text{NO}_3^-$	70	1.5	6.4	1.7	0.1	336	18.1	25000	0.14
17	$\text{NO}_2^- + \text{NO}_3^-$	60	45	17.5	4.15	0.14	360	30.1	23800	0.55
20	$\text{NO}_2^- + \text{NO}_4^-$	80	7	4.54	2.07	0.068	257	30.1	27500	0.15
this study	$\text{NO}_2^-$ (Seq) <sup>c</sup>	room	3	—	30.3	0.5	104	60.2	53000	0.02
	$\text{NO}_2^- + \text{NO}_3^-$	50	30	10.2	34.8	0.58	180	60.2	50000	0.2

<sup>a</sup>Molar absorptivities were estimated graphically. <sup>b</sup>Phosphoric acid was used instead of HCl. <sup>c</sup>(Seq), by a sequential process.

test tube was used as the reaction vessel and immersed in a thermostatically controlled bath. Once the last reagent had been added, the sample solution was transferred via a peristaltic pump to a flow cuvette in a Shimadzu UV-1800 spectrophotometer, and then the sample was held in the cuvette for absorbance monitoring. The cuvette holder was also thermo-controlled by a thermostat jacket. The curve of PAD formation was obtained, and the time for the curve to reach 90% of the maximum absorbance was noted as  $t_{90\%}$ , which provided a comparative indicator of the reaction kinetics.

**Reagents and Standards.** The Griess reagent recipe suggested by a previous study<sup>21</sup> was adopted early in this study. Adjustments of reagent strengths in latter experiments are stated elsewhere in the text. The SUL reagent [2% (w/v)] was prepared by dissolving sulfanilamide (molecular weight of 172.2) in 15% (v/v) HCl. The NED reagent [0.3% (w/v)] was prepared by dissolving *N*-1-naphthylethylenediamine dihydrochloride (molecular weight of 259.2) in doubly distilled water. The V(III) reagent, 1% (w/v)  $VCl_3$ , was prepared in 15% (v/v) HCl. Standard stock solutions, 5000  $\mu M$   $NO_2^-$  and  $NO_3^-$ , were prepared from  $NaNO_2$  and  $KNO_3$ . Ten micromolar nitrite and nitrate working standards were obtained by diluting the stock solutions 500-fold.

## RESULTS AND DISCUSSION

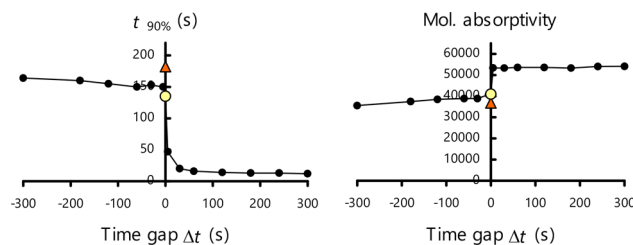
**Acidity for PAD Formation.** Acidity is a key parameter in the Griess reaction. Because it is nitrous acid rather than nitrite ion to react with SUL, an acidic condition would transform most of the species to nitrous acid as the  $pK_a$  of nitrous acid is 3.34. Practically, it is convenient to calculate the amount of acid added through the SUL reagent and divide by the final volume to obtain a final added acidity. Following the previous protocol<sup>21</sup> for nitrite determination, a 25 mL aliquot of the sample with 1 mL of 2% (w/v) SUL in 15% (v/v) HCl and 1 mL of 0.3% (w/v) NED would result in a final added acidity of 67 mM or a final pH of  $\sim 1.4$ . Under these conditions, PAD would have a molar absorptivity of  $\sim 53000 M^{-1} cm^{-1}$ . Increasing the final added acidity to 300 mM does not affect the absorptivity. However, if the final added acidity is  $< 50$  mM, then the absorptivity would decrease significantly.

**Incomplete PAD Formation by the Premixed Reagent.** Traditionally, the Griess reagents, SUL and NED, are added sequentially for nitrite analysis simply because the Griess reaction involves several different steps.<sup>22,23</sup> Sequential addition allows diazonium to form first before the addition of the NED reagent to elicit the pink color; the  $t_{90\%}$  value for the PAD reaction is normally  $< 20$  s, and the maximum absorbance is obtained within 4 min at room temperature (Figure 2, right). However, while using premixed reagents, PAD formation becomes much slower, with a  $t_{90\%}$  of  $> 170$  s, and the complete reaction may take as long as 20 min. It is also observed that the final molar absorptivity is only  $\sim 70\%$  of the value obtained by sequential addition. It appears that a substantial nitrite fraction does not form PAD in premixed reagents. To the best of our knowledge, the cause of this phenomenon has not been investigated.

**Effects of Reagent Addition Order and Timing.** PAD formation kinetics was thus studied by varying the reagent addition order and timing to evaluate the effects on the reaction rates and final absorbance. Experiments were carried out by varying time gap  $\Delta t$ , which is defined as the time between adding SUL and NED. A negative  $\Delta t$  stands for a

reverse addition order, NED first and SUL second. The operation was performed in four ways. (a) Reagents are added in sequential order (SUL first), with  $\Delta t$  from 5 to 300 s. (b) Reagents are added in reverse order (NED first), with  $\Delta t$  from  $-5$  to  $-300$  s. (c) Reagents are added separately and simultaneously ( $\Delta t = 0$ ). (d) Reagents are premixed and added as one shot ( $\Delta t = 0$ ).

The  $t_{90\%}$  and molar absorptivity for PAD formation of the four different treatments are shown in Figure 3. For normal



**Figure 3.** Effects of reagent addition order and timing on  $t_{90\%}$  and molar absorptivity. The experiments were carried out by using a 10  $\mu M$  nitrite standard solution at 25 °C. The final reagent concentrations of SUL and NED were 4.3 and 0.43 mM, respectively. Positive and negative  $\Delta t$  values represent normal and reverse addition procedures, respectively. Empty circles denote the results for reagents added individually and simultaneously. Triangles denote the value obtained by premixed reagents.

sequential addition at room temperature, when  $\Delta t$  was  $+5$  s, the  $t_{90\%}$  value reached 50 s, but when  $\Delta t$  was  $> 30$  s,  $t_{90\%}$  would be  $< 20$  s. In terms of the final molar absorptivity, as long as the SUL reagent was added prior to NED, the final absorptivity would all reach the same value,  $53000 M^{-1} cm^{-1}$ . In terms of the reverse treatment, when NED was added prior to SUL, the reaction rates became comparatively slow and the absorbance continued to increase even after 10 min. For quantitative expression, the absorbance obtained at 20 min was considered to be the maximum value. The  $t_{90\%}$  values for the reverse operation ranged from 150 to 160 s. The final absorbance reached ranged from 67% to 76% of the value obtained by normal sequential addition (also see Figure 3). When the two reagents were added individually but simultaneously, the final absorbance was only 75% of the maximum value obtained by sequential addition, and the  $t_{90\%}$  value reached 145 s. When using a premixed reagent, the final absorbance was  $\sim 69\%$  of the maximum value, and the reaction was even slower, with a  $t_{90\%}$  of  $\sim 180$  s. These results clearly indicate that complete PAD formation occurs only when the sequential addition procedure is used; otherwise, NED may compete with SUL, thus leading to the loss of the pink azo dye.

**Effect of SUL and NED Concentrations.** We have then varied SUL and NED concentrations to observe the effects on PAD formation. Five different SUL concentrations, ranging from 0.43 to 17.2 mM, and eight different NED concentrations, ranging from 0.009 to 0.86 mM, were used for both sequential and premixed procedures (Tables 2 and 3). For sequential reagent addition, the final absorbances were  $\sim 0.494$  for 25 mL of 10  $\mu M$  nitrite standard (to which was added 1 mL each of SUL and NED), very close to the nominal value, except for the lowest-NED concentration treatment (i.e., 0.009 mM). Without sufficient NED concentrations, the absorbances were low, the  $t_{90\%}$  values were  $> 15$  min, and the reaction took 2 h to reach equilibrium. With increasing NED concentrations, the  $t_{90\%}$  values were much shorter,  $< 20$  s when NED concen-

**Table 2. Effect of Reagent Strength on the Final Absorbance<sup>a</sup> of a 10 μM NO<sub>2</sub><sup>-</sup> Standard Using Sequential and Premixed Reagent Additions**

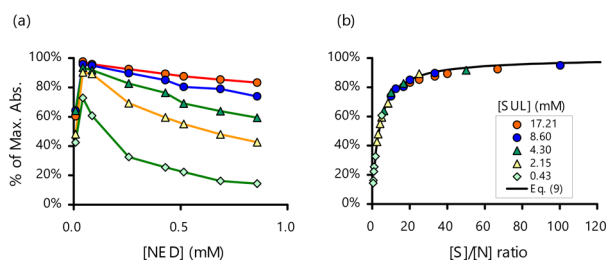
[SUL] (mM)	0.009 mM NED	0.043 mM NED	0.09 mM NED	0.26 mM NED	0.43 mM NED	0.51 mM NED	0.69 mM NED	0.86 mM NED
By Sequential Procedure, SUL Added Prior to NED								
17.21	0.334	0.493	0.493	0.489	0.493	0.495	0.492	0.496
8.60	0.334	0.493	0.492	0.493	0.496	0.494	0.496	0.496
4.30	0.334	0.494	0.495	0.494	0.497	0.494	0.495	0.496
2.15	0.329	0.494	0.494	0.496	0.495	0.494	0.493	0.497
0.43	0.329	0.493	0.494	0.492	0.496	0.494	0.492	0.492
Reagents Added as a Premix								
17.21	0.298	0.482	0.474	0.457	0.441	0.432	0.422	0.411
8.60	0.317	0.473	0.469	0.443	0.420	0.397	0.390	0.365
4.30	0.317	0.462	0.453	0.408	0.377	0.341	0.315	0.293
2.15	0.238	0.446	0.441	0.342	0.294	0.272	0.237	0.211
0.43	0.210	0.360	0.300	0.161	0.126	0.110	0.080	0.071

<sup>a</sup>The absorbance recorded at 20 min was taken as the maximum value.

**Table 3. Effect of Reagent Strength on  $t_{90\%}$  (in seconds) at Room Temperature Using Sequential and Premixed Reagent Additions**

[SUL] (mM)	0.009 mM NED	0.043 mM NED	0.09 mM NED	0.26 mM NED	0.43 mM NED	0.51 mM NED	0.69 mM NED	0.86 mM NED
By Sequential Procedure, SUL Added Prior to NED								
17.21	>900	142	73	19	<15	<15	<15	<15
8.60	>900	144	69	19	<15	<15	<15	<15
4.30	>900	148	79	19	<15	<15	<15	<15
2.15	>900	142	67	21	<15	<15	<15	<15
0.43	>900	142	73	19	<15	<15	<15	<15
Reagents Added as a Premix								
17.21	>900	190	116	112	126	138	161	170
8.60	>900	190	135	140	154	166	175	183
4.30	>900	204	158	170	181	205	203	204
2.15	>900	221	200	190	209	217	224	237
0.43	>900	294	232	226	244	252	257	260

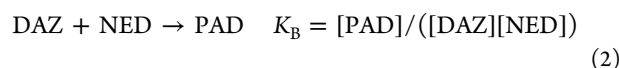
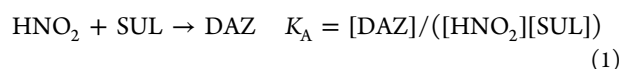
trations were >0.26 mM. For premixed reagents, we found that sufficiently high SUL concentrations were favorable for PAD formation. However, the effect of the increasing NED concentration was somewhat contradictory. A NED concentration of >0.043 mM would lead to a lower final absorbance and slower reaction rates (Tables 2 and 3 and Figure 4a), showing that the missing PAD fraction generally increases with increasing NED concentrations but decreases with increasing SUL concentrations. These results strongly suggest that nitrite may react with NED to form an unknown compound (X) and thus reduce the level of PAD formation (Figure 1)



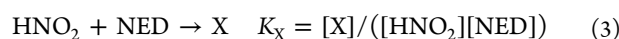
**Figure 4.** (a) Effects of SUL and NED concentrations on PAD formation by using premixed reagents. Original data are listed in Table 2. (b) Comparison of measured values and the curve obtained from eq 9 plotted on the  $[S]/[N]$  axis.

### Validating the Reaction between Nitrite and NED.

The reactions of nitrous acid with SUL and NED can be described with formation constants  $K_A$  and  $K_B$ :



If SUL is added first, all nitrous acid will be transformed eventually into PAD. However, if nitrous acid meets NED first, the formation of PAD would be incomplete. We thus hypothesize that the missing fraction is attributed to a nitrous acid–NED reaction that forms an unidentified compound X (with a formation constant  $K_X$ ):



If both reagents were present at the same time, competition would occur. Assume that the initial concentrations of nitrite, SUL, and NED are  $a$ ,  $b$ , and  $c$ , respectively, and the final equilibrium concentrations of DAZ, PAD, and X are  $d$ ,  $e$ , and  $x$ , respectively. Formation constants  $K_A$  and  $K_X$  can be described as follows:

$$K_A = \frac{d}{(a - d - x)(b - d)} \quad (4)$$

$$K_X = \frac{x}{(a - d - x)(c - x - d)} \quad (5)$$

Because  $a = x + d$ , and all  $d$  will eventually convert to  $e$ , therefore  $a = x + e$ :

$$\frac{K_A}{K_X} = \frac{e(c - a)}{x(b - d)} \quad (6)$$

Reagent concentrations ( $b$  and  $c$ ) are usually much higher than nitrite concentrations ( $a$ ) ( $b \gg d$  and  $c \gg a$ ); eq 6 may be further simplified as follows:

$$\frac{a - x}{x} = \frac{K_A b}{K_X c} \quad (7)$$

The missing fraction of the unknown compound,  $x/a$ , can be presented as

$$\frac{[X]}{[\text{HNO}_2]} (\%) = \frac{1}{1 + \frac{K_A[\text{SUL}]}{K_X[\text{NED}]}} \quad (8)$$

The apparent PAD fraction,  $e/a$ , can be shown as

$$\frac{[\text{PAD}]}{[\text{HNO}_2]} (\%) = 1 - \frac{1}{1 + \frac{K_A[\text{SUL}]}{K_X[\text{NED}]}} \quad (9)$$

The  $K_A/K_X$  ratio can be estimated from experimental results listed in Table 2. By excluding the boundary data sets,  $[\text{NED}] = 0.009$  and  $0.043$  and  $[\text{SUL}] = 17.21$  mM, we found that  $K_A/K_X$  exhibits highly constrained values, with an average of  $0.29 \pm 0.03$  ( $n = 24$ ).

A theoretical curve between the  $[\text{SUL}]/[\text{NED}]$  ratio, hereafter  $[\text{S}]/[\text{N}]$ , and the relative maximum absorbance of PAD formation can be obtained from eq 9 by assuming  $K_A/K_X$  equals 0.29 (Figure 4b). We found that experimental values matched perfectly with the theoretical curve, validating our hypothesis. In brief, an increasing NED concentration would increase the rate of the reaction to form X and lead to PAD loss so that increasing  $[\text{S}]/[\text{N}]$  ratios would result in higher PAD absorbance. For example, the final PAD fractions of 74.4%, 81.3%, 85.3%, 89.7%, and 94.6% corresponded to  $[\text{S}]/[\text{N}]$  ratios of 10, 15, 20, 30, and 60, respectively. A higher  $[\text{S}]/[\text{N}]$  ratio may be obtained by increasing the SUL concentrations and decreasing the NED concentrations. Considering the limitation of SUL solubility [ $\sim 8\%$  (w/v)], we have tried to mix an equal volume of 6% (w/v) SUL and 0.15% (w/v) NED to produce a molar ratio of 60. With this  $[\text{S}]/[\text{N}]$  ratio, the final molar absorptivity reached  $50200 \text{ M}^{-1} \text{ cm}^{-1}$ ,  $\sim 94.7\%$  of the maximum value obtained via sequential addition ( $53000 \text{ M}^{-1} \text{ cm}^{-1}$ ).

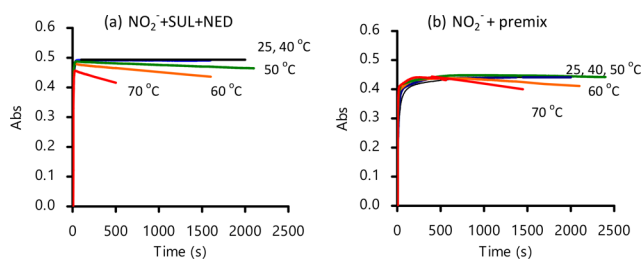
**PAD Formation at Various Temperatures.** Despite the increased molar absorptivity obtained by using a high SUL concentration and an optimally high  $[\text{S}]/[\text{N}]$  ratio, the rate of PAD formation by premixed reagents is still relatively slow at room temperature (Table 3), with a  $t_{90\%}$  of  $>100$  s and a complete reaction time ( $>99\%$ ) of generally  $>15$  min. Heating would be the simplest step to decrease the reaction time. We have further carried out experiments by increasing the reaction temperature from 40 to 70 °C to study the effect on the formation of PAD from nitrite (Figure 5a). For sequential reagent addition, a maximum absorbance can be achieved within 4 min at 25 °C, and the value dropped only 0.5% after 1 h. At 40 °C, PAD formation was quicker, and the fading rate was  $\sim 1.0$ – $1.5\% \text{ h}^{-1}$ . At temperatures of 50, 60, and 70 °C, the maximum absorbance appeared to be low, with very quick

color fading rates of 8, 20, and 45–50%  $\text{h}^{-1}$ , respectively. These results indicate that for sequential reagent addition PAD formation needs to be carried out preferably at room temperature.

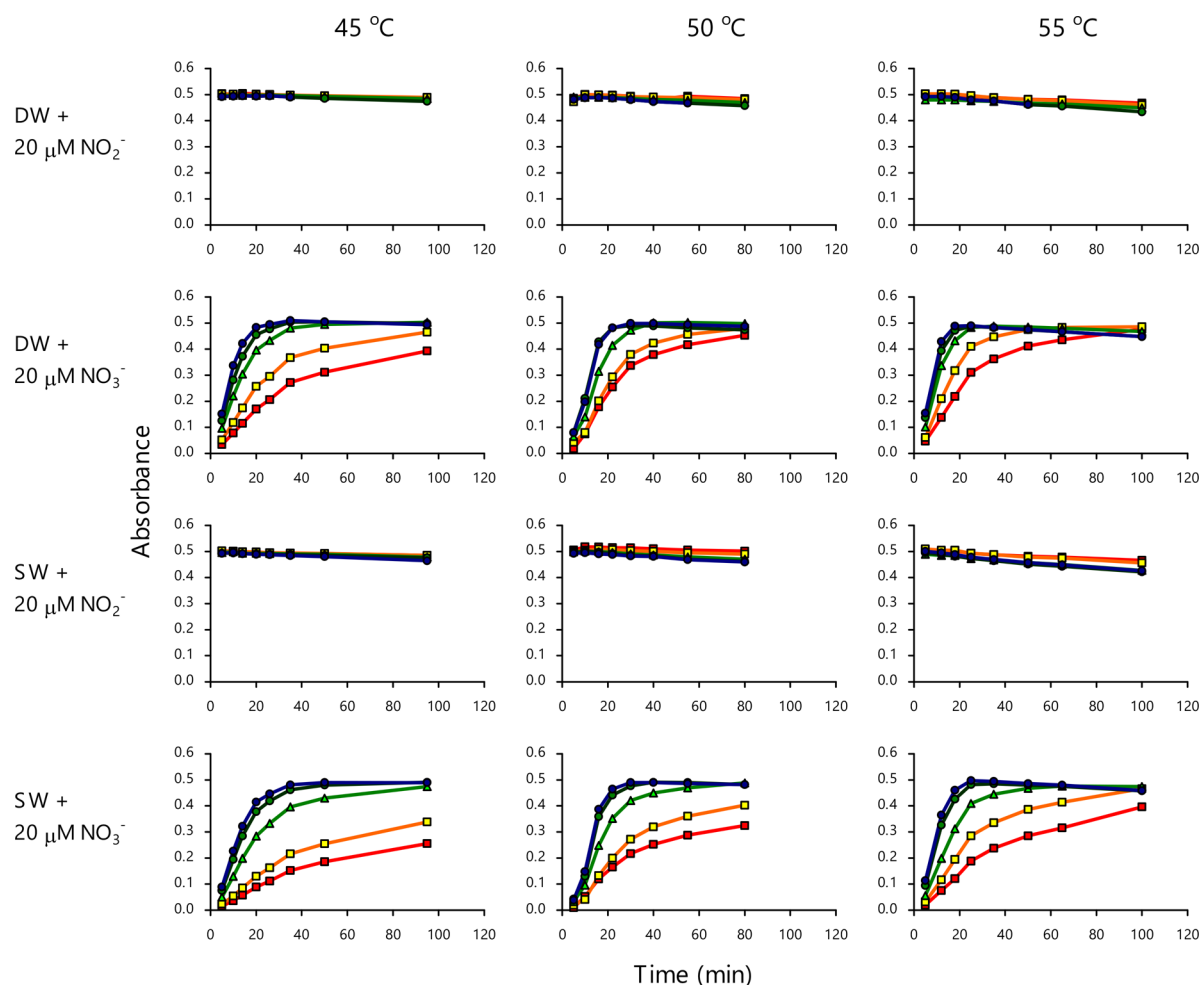
For premixed reagent addition, the formation reaction was quite different (Figure 5b). At 25 °C, the maximum absorbance was stabilized after 20 min. With an increase in temperature, the  $t_{90\%}$  values were shorter but the maximum absorbances were quite similar. At 25, 40, and 50 °C, the absorbances merged gradually to a fixed value and no color fading was observed within 30 min. However, when the temperature was increased to 60 and 70 °C, the absorbances decreased rapidly. These results suggest that for the premixed procedure the temperature should be kept at  $\leq 60$  °C to avoid color fading.

**Optimizing Nitrate Reduction.** In terms of formation of PAD from nitrate reduction by V(III), previous studies have shown that increasing temperatures and V(III) concentrations would enhance nitrate to nitrite reduction rates.<sup>13,15</sup> In this study, experiments were carried out to optimize V concentrations. The Griess reagent and V solution were prepared as follows: 3% (w/v) SUL, 0.3% (w/v) NED, and 4% (w/v)  $\text{VCl}_3$ . The premixed reagent was prepared by mixing 20 mL of SUL, 5 mL of NED, and various amounts of the V(III) solution (1, 2, 4, 6, and 8 mL), and the volume was increased to 100 mL with distilled water. The premixed reagents were added to the sample in a 1:1 volume ratio, which would give final V(III) concentrations of 1.27, 2.54, 5.08, 7.62, and 10.17 mM, respectively. The tested samples were 20  $\mu\text{M}$  nitrite and 20  $\mu\text{M}$  nitrate spiked in either distilled water or an off-shore surface seawater (salinity of 33.8). The bath temperature was set at constant values of 45, 50, and 55 °C. Duplicate samples were prepared in glass sample tubes, and an equal volume of the premixed reagents was added. The tubes were then placed in a water bath at the designated temperature. For every 5–10 min interval, the samples were sipped out for the absorbance measurement.

At higher temperatures, the highest final absorbance of the sample with 20  $\mu\text{M}$  nitrite reached  $\sim 0.500$  within 5 min, equivalent to a molar absorptivity of  $50000 \text{ M}^{-1} \text{ cm}^{-1}$ , for both freshwater and seawater (Figure 6). No significant salt effect was observed at this concentration level. We found that PAD fading rates are related to not only temperature and V(III) concentration but also salinity (Table 4). The fading trends were as low as  $-2\% \text{ h}^{-1}$  at 45 °C for the lowest V concentration (1.27 mM) in freshwater and reached  $-15\% \text{ h}^{-1}$  at 55 °C for the highest V concentration (10.17 mM) in seawater. With respect to nitrate samples, the shapes of the PAD formation curve were significantly different from that of nitrite (Table 5). We found that increasing V concentrations increased reduction rates and the rates were slightly higher in freshwater than in seawater. At 45 °C, the maximum absorbance of 0.500 was attained at 40–50 min using a V(III) concentration of 7.62 mM in both freshwater and seawater samples. At 50 °C and V concentrations of  $\geq 7.62$  mM, both samples reached their maximum absorbance within 30 min. At 55 °C, the reaction was even faster but the fading was also accelerated. In brief, increasing the V(III) concentration increases the reaction rates but also leads to high reagent blank and high PAD fading rates. On the basis of these results, we propose that the optimal condition would include a heating temperature of  $\leq 50$  °C and a V(III) concentration ranging from 7.62 to 10.17 mM.



**Figure 5.** Comparison of PAD formation and stability of an initial 10  $\mu\text{M}$  nitrite sample by (a) sequential and (b) premixed reagent additions at various constant temperatures. The sample and cuvette holder were both preheated to the designated temperature of the water bath.



**Figure 6.** PAD formation curves of 20  $\mu\text{M}$  nitrite/nitrate in distilled water and seawater with various V(III) concentrations (red, 1.27 mM; orange, 2.54 mM; green, 5.08 mM; dark green, 7.62 mM; blue, 10.17 mM) at 45, 50, and 55  $^{\circ}\text{C}$ . All data were subtracted from reagent blanks. The reagent blanks were proportional to V(III) concentrations,  $\sim 0.005$  per millimolar V(III).

**Table 4.** Color Fading Rates (percent per hour) for Nitrite Samples at Various  $\text{V}^{3+}$  Concentrations and Bath Temperatures

$[\text{V}^{3+}]$ (mM)	DW with 20 $\mu\text{M}$ $\text{NO}_2^-$			SW with 20 $\mu\text{M}$ $\text{NO}_2^-$		
	45 $^{\circ}\text{C}$	50 $^{\circ}\text{C}$	55 $^{\circ}\text{C}$	45 $^{\circ}\text{C}$	50 $^{\circ}\text{C}$	55 $^{\circ}\text{C}$
1.27	-2	-3	-7	-3	-3	-7
2.54	-2	-3	-8	-3	-4	-10
5.08	-2	-4	-8	-4	-6	-12
7.62	-3	-5	-10	-4	-7	-14
10.17	-4	-6	-12	-6	-8	-15

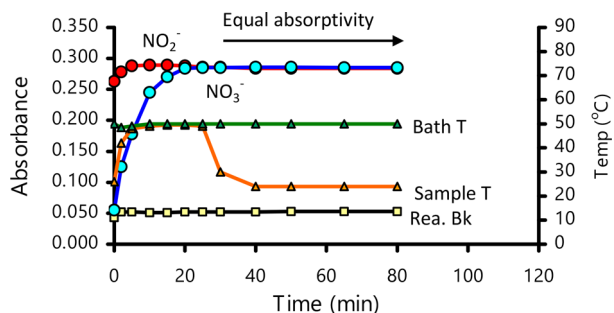
**Table 5.** Times (minutes) for Nitrate Samples to Reach the Highest Absorbance at Various  $\text{V}^{3+}$  Concentrations and Bath Temperatures

$[\text{V}^{3+}]$ (mM)	DW with 20 $\mu\text{M}$ $\text{NO}_3^-$			SW with 20 $\mu\text{M}$ $\text{NO}_3^-$		
	45 $^{\circ}\text{C}$	50 $^{\circ}\text{C}$	55 $^{\circ}\text{C}$	45 $^{\circ}\text{C}$	50 $^{\circ}\text{C}$	55 $^{\circ}\text{C}$
1.27	>100	>100	>100	>100	>100	>100
2.54	>100	>100	50	>100	>100	>100
5.08	>100	30	30	>100	50	40
7.62	30	25	20	50	35	30
10.17	30	20	18	40	30	25

**Slowing PAD Fading.** As mentioned above, a high temperature is a major cause of PAD fading after a heating procedure. To minimize PAD fading rates after heating, we have carried out various tests to decrease the sample temperature after the heating procedure for PAD formation. The procedures include three steps. First, samples are heated gradually from room temperature to  $\sim 50$   $^{\circ}\text{C}$  in a 50  $^{\circ}\text{C}$  water bath. Second, samples remain at this temperature for an additional 10 min for maximum nitrate reduction and an unnoticeable loss of PAD formation produced by nitrite. Third, samples are removed from the water bath and placed in a cold water bath to cool the sample promptly. We found that a sudden icy slurry cooling for 1 min immediately after heating would effectively stop the color fading.

After the heating and cooling procedures had been adjusted, PAD formation curves from both from nitrite and nitrate may turn into a flat plateau after heating (Figure 7). After 25 min, nitrite produced a molar absorptivity of  $50000 \text{ M}^{-1} \text{ cm}^{-1}$  and nitrate would yield a value almost identical to that of nitrite (see also Figure 7). The quantification of nitrite and nitrate concentrations can thus be much simplified.

**Can Nitrite Be Trapped by Only SUL?** Because NED acts as a self-interference factor in PAD formation, there was a primitive idea that if nitrite could be trapped by SUL alone to form DAZ (in the absence of NED), then the subsequent



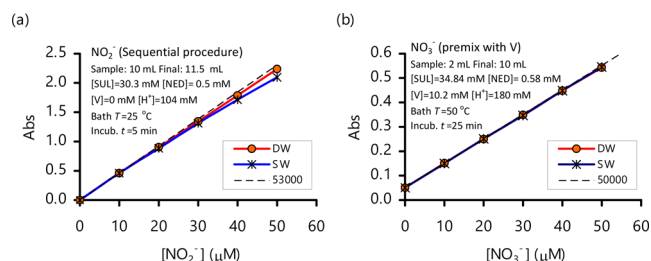
**Figure 7.** Heating and sudden cooling of 10  $\mu\text{M}$  nitrite and nitrate solutions reacted with premixed reagents (1:1 volume ratio) and heated in a 50  $^{\circ}\text{C}$  water bath. The sample reached 50  $^{\circ}\text{C}$  after 15 min, stood for an additional 10–15 min at the same temperature, and then was transferred to an icy water bath for 1 min.

addition of NED would eliminate the premix effect. To check this possibility, the following experiment was performed.

A 25 mL aliquot of a 10  $\mu\text{M}$  nitrite/nitrate standard was placed in 50 mL test tubes and immersed in a hot water bath, to which were added 1 mL of 6% (w/v) SUL and 1 mL of 1% (w/v)  $\text{VCl}_3$ , removed from the test tube after certain incubation times, allowed to cool, and then supplemented with 1 mL of 0.15% (w/v) NED. The absorbance was measured immediately. The results show that, for a nitrite solution, the absorbance decreased immediately during the incubation and the liquid became almost colorless after 1 h. This indicates that nitrite or even DAZ was being continuously reduced to nitric oxide. For a nitrate solution with SUL and V(III) alone, the final absorbance increased during the first 20 min incubation and decreased following the same trend as that for nitrite, clearly showing that nitrite cannot be trapped by SUL alone as DAZ. The only way to sustain the nitrite yield from vanadium reduction is to add the three reagents as a combination in the very beginning.

**Dilution to Improve Linearity and Reduce the Salinity Effect.** Although many previous studies<sup>8,10,12,14,16–18</sup> have emphasized that the method offers a wide linear range, some others have revealed that curvatures do occur at high concentrations or high salinities.<sup>9,13,16,18,20</sup>

In this study, we have also observed that the PAD calibration curve for nitrite is not a perfect straight line with an absorbance of  $>0.5$  (Figure 8a). In addition, salinity causes significant interference at high concentrations, with the negative effect to



**Figure 8.** Linearity deviation and salt effect for nitrite and nitrate analyses. Linearity is generally maintained when absorbances are  $<0.5$ . (a) Sequential procedure for nitrite, with a molar absorptivity of 53000  $\text{M}^{-1}\text{cm}^{-1}$  in a low concentration range. (b) Premixed procedure for nitrate with 5-fold dilution. The molar absorptivity is 50000  $\text{M}^{-1}\text{cm}^{-1}$ , and the linear range can reach 50  $\mu\text{M}$  for both freshwater and seawater media.

be as large as a few percent. For nitrate, we suggest using high reagent strengths and diluting high-concentration samples to reduce the deviations. After 5-fold dilution with reagents (with 2 mL of sample, 2 mL of reagent, and 6 mL of DW), the linear range can be increased to 50  $\mu\text{M}$  for both freshwater and seawater samples with absorbances of  $\leq 0.50$  (Figure 8b). The salinity effect would also become insignificant after 5-fold dilution.

**Recommended Procedures for Routine Analysis.** On the basis of the findings of this study, we have the following suggestions for nitrate analysis by using the V(III) and Griess premixed reagents. (i) The final added acidity should be  $>100$  mM. (ii) The heating temperature for nitrate reduction should be kept at  $\leq 50$   $^{\circ}\text{C}$ , and the total incubation time should be  $\leq 30$  min. (iii) High SUL and NED reagent strengths should be used, e.g.,  $\sim 30$  and  $\sim 0.5$  mM, respectively, which result in a high  $[\text{S}]/[\text{N}]$  molar ratio of 60. (iv) The optimal final V(III) concentration should be  $\sim 10$  mM. (v) A dilution step should be considered to maintain an absorbance of  $<0.5$  for samples with high nitrite and/or nitrate concentrations.

In terms of reagent preparation, the three reagents [SUL, NED, and V(III)] are prepared individually and premixed just before use. The following solutions were used in our protocols for a batch of 40 samples: SUL [6% (w/v)] solution consisting of 6 g of sulfanilamide dissolved in 100 mL of 1.2 N HCl, NED [0.3% (w/v)] solution consisting of 0.15 g of *N*-1-naphthylethylenediamine dihydrochloride dissolved in 50 mL of distilled water, and  $\text{VCl}_3$  [3.2% (w/v)] solution consisting of 0.8 g of vanadium chloride (Merck, 1.12393) dissolved in 25 mL of 1.2 N HCl.

One hundred milliliters of the premixed reagent is prepared by mixing aliquots of 50 mL of a SUL solution, 25 mL of a NED solution, and 25 mL of a  $\text{VCl}_3$  solution. In terms of the stability of an individual reagent, SUL is stable for months at room temperature whereas NED needs to be prepared weekly and stored in a refrigerator.<sup>2</sup> The  $\text{VCl}_3$  solution and the premixed reagents should be prepared daily. Because hazardous fumes may be released upon opening of the bottle containing  $\text{VCl}_3$ , this should be done in a fume hood. Practically, it can be repackaged (e.g., 0.8 g per ampule) and stored in a freezer.

**Nitrite Analysis with a Concentration of  $<10$   $\mu\text{M}$ .** To each 10 mL sample is added 1 mL of SUL, and the sample was mixed and then allowed to stand for at least 30 s before the addition of 0.5 mL of the NED solution. The absorbance at 543 nm is measured after 3 min. The final concentrations of SUL, NED, and  $[\text{H}^+]_{\text{add}}$  are 30.3, 0.5, and 104 mM, respectively, with an  $[\text{S}]/[\text{N}]$  ratio of 60. The nitrite concentration is calculated by

$$[\text{NO}_2^-] (\mu\text{M}) = \frac{(\text{Abs} - \text{RB}_1) \left( \frac{V_f}{V_s} \right)}{b\epsilon_1} \times 10^6$$

or

$$[\text{NO}_2^-] (\mu\text{M}) = \text{Abs}_{(\text{corr})} \times 21.7$$

where  $\text{RB}_1$  is the reagent blank (without V), usually  $<0.002$ ,  $V_f$  the final volume or 11.5 mL,  $V_s$  the sample volume or 10 mL,  $\epsilon_1$  the molar absorptivity (53000  $\text{M}^{-1}\text{cm}^{-1}$ ),  $b$  the cell length (1 cm), and  $\text{Abs}_{(\text{corr})}$  the corrected absorbance. For analysis of nitrite only without using V(III), the precision is better than

1% (relative standard deviation) at 1–10  $\mu\text{M}$ , with a detection limit of  $\sim 0.02 \mu\text{M}$ .

**Nitrate Analysis with Concentrations between 0 and 50  $\mu\text{M}$ .** To a dry 15 mL test tube are added 2 mL of sample, 2 mL of premix reagent, and 6 mL of distilled water. The liquid in the test tube is homogenized by a vibrating mixer, giving final concentrations of SUL, NED, V(III), and  $[\text{H}^+]_{\text{added}}$  of 34.8, 0.58, 10.17, and 180 mM, respectively. The  $[\text{S}]/[\text{N}]$  ratio is 60. All sample tubes are placed in a rack and immersed a thermostated water bath at 50  $^{\circ}\text{C}$  for 25–30 min. The rack is then transferred to a cooling bath for a few minutes (or an icy slurry for 1 min) to decrease the temperature to  $<30 \text{ }^{\circ}\text{C}$ . The samples are measured with a spectrophotometer at 543 nm within 1 h, preferably using a 1 cm flow cuvette equipped with a sipper. A slight color fading ( $\sim 1\%$ ) might take place if detection is performed 1 h later. The final absorbance is produced by both nitrite and nitrate:

$$\text{Abs} = Fb[\text{NO}_2^-]\epsilon_2 \times 10^{-6} + Fb[\text{NO}_3^-]\epsilon_3 \times 10^{-6} + \text{RB}_V$$

whereas  $F$  is the volume factor (0.2),  $b$  is the cell length (1 cm),  $\epsilon_2$  and  $\epsilon_3$  are the apparent absorptivities for nitrite and nitrate, respectively, which can be obtained by measuring nitrite and nitrate standards separately, and  $\text{RB}_V$  is the reagent blank containing  $V$  ( $\sim 0.050$  in this study). When the absorptivities of nitrite and nitrate are adjusted to be equal ( $50000 \text{ M}^{-1} \text{ cm}^{-1}$ ), nitrate concentrations can be directly calculated by subtracting the measured nitrite concentration.

$$[\text{NO}_3^-] (\mu\text{M}) = \frac{\text{Abs} - \text{RB}_V}{Fb\epsilon_3} \times 10^6 - [\text{NO}_2^-]$$

or

$$[\text{NO}_3^-] (\mu\text{M}) = \text{Abs}_{(\text{corr})} \times 100 - [\text{NO}_2^-]$$

For nitrate analysis, a standard addition experiment showed that the absorbance of its reagent blank (1 cm cell) was  $\sim 0.049 \pm 0.0005$  ( $n = 7$ ) and the raw absorbance for a 20  $\mu\text{M}$  nitrate solution with a 5-fold dilution was  $0.248 \pm 0.002$  ( $n = 7$ ) with a precision of  $\sim 1\%$  and a detection limit of 0.2  $\mu\text{M}$ .

In practice, however, it is always advisable to use a working curve comprised of known standards to run under the same conditions to ascertain the accuracy.

## CONCLUSION

We have identified and validated the major pathway causing the loss of PAD in the premixed reagents (Figure 1) and have minimized the loss by increasing the SUL and NED concentrations and their molar ratio. We have also provided a simple heating and cooling procedure to decrease the reaction time and to stabilize PAD formed after the incubation process. The molar absorptivities obtained for both nitrite and nitrate are consistently to be  $\sim 50000 \text{ M}^{-1} \text{ cm}^{-1}$ , the highest ever reported using V(III) and the Griess premixed reagents (Table 1). A batch of 40 samples can be analyzed within 2 h. This new protocol is easy to perform, precise, and accurate for nitrate analysis. We believe that it provides an alternative to the traditional Cd–Cu reduction method for routine nitrate analysis in natural waters and any other aqueous samples.

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### Notes

The authors declare no competing financial interest.

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