



Reduction with zinc — Impact on the determination of nitrite and nitrate ions using microfluidic paper-based analytical devices

Mika I. Umeda^{a,b}, Kaewta Danchana^b, Takatoshi Fujii^a, Eiichi Hino^a, Yusuke Date^a, Kaoru Aoki^a, Takashi Kaneta^{b,*}

^a National Institute of Technology, Yonago College, 4448 Hikona-cho, Yonago, Tottori 683-8502, Japan

^b Okayama University, 3-1-1 Tsushimanaka, Kita-ku, Okayama 700-8530, Japan

ARTICLE INFO

Keywords:

Microfluidic paper-based analytical device
Nitrite ion
Nitrate ion
On-site analysis
Environmental analysis

ABSTRACT

We used a microfluidic paper-based analytical device (μ PAD) to investigate the influence that zinc reduction exerts on the determination of nitrite and nitrate ions in natural water samples. The μ PAD consists of layered channels for the reduction of nitrate to nitrite with zinc powder and the subsequent detection of nitrite with Griess reagent. The amount of zinc, number of layers, and reaction time for the reduction were optimized to obtain an intense signal for nitrate. Initially, the sensitivity to nitrate corresponded to 55% that of nitrite, which implied an incomplete reduction. We found, however, that zinc decreased the sensitivity to nitrite in both the μ PAD and spectrophotometry. The sensitivity to nitrite was decreased by 48% in spectrophotometry and 68% in the μ PAD following the reaction with zinc. One of the reasons for the decreased sensitivity is attributed to the production of ammonia, as we elucidated that both nitrite and nitrate produced ammonia via the reaction with zinc. The results suggest that the total concentration of nitrite and nitrate must be corrected by constructing a calibration curve for nitrite with zinc, in addition to developing curves for nitrate with zinc and for nitrite without zinc. Using these calibration curves, the absorbance at different concentration ratios of nitrite and nitrate ions could be reproduced via calculation using the calibration curves with zinc for nitrite and nitrate. Eventually, the developed μ PAD was applied to the determination of nitrite and nitrate ions in natural water samples, and the results were compared with those using a conventional spectrophotometric method. The results of the μ PAD are in good agreement with those of conventional spectrophotometry, which suggests that the μ PAD is reliable for the measurement of nitrite and nitrate ions in natural water samples.

1. Introduction

In water, particularly natural water, nitrogen exists as nitrite, nitrate, and ammonium ions (or ammonia), which is relative to the environmental pollution caused by human activities. Among them, nitrite and nitrate ions are naturally found in low concentrations in the natural environment, and small amounts of nitrate salt are essential nutrients for animals and plants. However, agricultural water, nitrogen-based fertilizers, animal manure, and sewage are sometimes sources of contamination that increases the levels of these ions.

Nitrite ions form toxic and carcinogenic compounds such as nitrosamines and nitrosamides [1] when they reach the acidic condition of the stomach in the presence of amines or amides. Conversely, salivary microorganisms possibly convert nitrate to nitrite, which can be absorbed by blood. Nitrite reacts with the iron in hemoglobin, and irreversibly

converts it to methemoglobin, which blocks oxygen transport [1,2] and results in methemoglobinemia. This reaction is known to occur particularly in infants [3]. For these reasons, the environmental emissions of all forms of these ions must be strictly regulated.

Several analytical methods that include spectrophotometry [4,5], ion chromatography [6], flow injection analysis [7], capillary electrophoresis [8], and electrochemical techniques [9] are used to measure the concentrations of nitrite and nitrate ions. All of these techniques require extensive instrumentation that must be operated in a laboratory. However, nitrite ions require rapid measurements because the concentration is easily changed during transportation and storage due to oxidation. Therefore, nitrate ions should also be measured before they are converted to nitrate ions.

A common spectrophotometric method employs Griess reagent to measure nitrite and nitrate ions by coupling with a reducing reaction. In

* Corresponding author.

E-mail address: kaneta@okayama-u.ac.jp (T. Kaneta).

<https://doi.org/10.1016/j.talo.2024.100347>

Received 2 February 2024; Received in revised form 13 July 2024; Accepted 5 August 2024

Available online 10 August 2024

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the absence of a reductant, nitrite ions directly react with Griess reagent whereas nitrate ions are converted to nitrite ions via reductants such as cadmium [10], cadmium-copper [11-13], zinc [14], and nitrate reductase to react with Griess reagent. However, the spectrophotometric method requires a large and expensive spectrophotometer and large volumes of reagents, samples, and waste.

To achieve onsite environmental analysis, a microfluidic paper-based analytical device (μ PAD) was developed by the Whitesides group in 2007 [15], and it has since attracted much attention. The μ PAD is a device that consists of hydrophilic channels surrounded by hydrophobic barriers on paper, and has the advantage of generating a spontaneous flow of liquid due to capillary action. In addition, paper as a substrate is light, low-cost, and the color change is easy to observe because of the low interference from the original white color. There are many reports on μ PADs for nitrite and nitrate measurements using Griess reagent. The μ PADs allow the direct measurement of nitrites in clinical settings [16-21], in food [18,22-25], and in environmental samples [18,19,23,26-29]. For simultaneous measurements of nitrite and nitrate, the μ PADs require two detection methods, one relies on a reductive reaction and the other does not. These μ PADs have been applied to the analysis of nitrites and nitrates in clinical settings [30], in food [31-33], and in environmental samples such as saliva, tap water, and vegetables [34,35].

Although many researchers have used μ PADs to determine the levels of nitrite and nitrate, the reduction of nitrate continues to present challenging issues; for example, individual devices show different reduction efficiencies and lack information on the effect of zinc in the simultaneous determinations of nitrite and nitrate. Therefore, in the present study, we designed a μ PAD with a layered structure for the determination of nitrite and nitrate ions in natural water samples and clarified the effect of zinc in a colorimetric reaction using Griess reagent. We found that zinc influences the sensitivity to nitrite ions so that calibration curves for nitrite ions must be constructed under conditions both with and without a reduction by zinc. We proposed a correction method to measure the concentrations of both nitrite and nitrate ions. Consequently, nitrite and nitrate ions in natural water samples were measured simultaneously using the developed μ PAD and the results were compared with those of conventional UV-VIS spectrophotometry.

2. Experimental

2.1. Materials

Sulfanilamide, citric acid, N-(1-naphthyl)-ethylenediamine dihydrochloride, zinc dust, nitrate nitrogen standard solution (N 0.1 mg mL⁻¹), nitrite ion standard solution (NO₂ 1000 ppm), and an ammonia assay kit (LabAssay Ammonia) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). A zinc suspension containing 0.2 g mL⁻¹ of zinc was prepared by suspending zinc dust in water. The Griess reagent contained 50 mM sulfanilamide, 330 mM citric acid, and 10 mM N-(1-naphthyl)-ethylenediamine dihydrochloride. Chromatography paper (200 × 200 mm, 1CHR, WhatmanTM) served as the substrate for the μ PADs. Filter paper, (5B or 5C, ADVANTEC, Tokyo, Japan) was used to remove zinc powder from the solutions in spectrophotometric measurements. PARAFILM (Bemis Company, Inc., WI, USA) controlled the time for the reduction of nitrate ions on the μ PADs.

Natural water samples were collected from wells, ponds, and a brackish lake in and nearby the National Institute of Technology, Yonago College and Okayama University. The amounts of nitrite and nitrate ions were measured via both the μ PADs and a spectrophotometer.

2.2. Apparatus

The μ PADs were designed using Microsoft Office Power Point 2016. The μ PADs were printed using a wax printer, Color Qube 8580N (Xerox,

CT, USA), and heated at 120 °C for 1 min in an oven (EO-300V, AS ONE, Tokyo, Japan) to melt the wax so that it would penetrate to the backside of the μ PADs. The images of the detection zones on the μ PADs were scanned using a CanoScan LiDE400 scanner (Canon, Tokyo, Japan). We used a V-730 spectrophotometer (JASCO, Tokyo, Japan) to measure the absorbance of the reaction product with Griess reagent for conventional absorption spectrophotometry. A Synergy Neo2 microplate reader (Agilent BioTek, CA, USA) was employed for the measurement of ammonia.

2.3. Fabrication of the μ PADs

Two types of μ PADs, 2D and 3D, were designed in this study as shown in Fig. 1(a) and (b). Both types of μ PADs were composed of sample introduction zones (one introduction zone for the 2D- μ PAD), reduction zones, and detection zones. In the 2D- μ PAD, the sample introduction zone, the reduction zones, and the detection zones were horizontally and perpendicularly connected to flow the sample solution. Two reduction zones were aligned successively, and each contained 2 mg of zinc (5 μ L of 0.2 g mL⁻¹ zinc suspension was added 2 times). Each of the four detection zones contained 2 μ L of Griess reagent. Forty μ L volumes of sample solutions were added at the center of the μ PADs.

In the 3D- μ PAD, the two reduction zones were layered with untreated zones to prevent their contact (Fig. 1(b)). The 3D- μ PAD measured five samples in one operation to enhance the throughput of the analysis. All detection zones contained 2 μ L of Griess reagent, which was similar to the 2D- μ PAD. The analytical procedure for use of the 3D- μ PAD was as follows: (1) The device had a folding design that would allow the formation of a layered channel; (2) a sample solution was added to layer 2; (3) parafilm was inserted between layers 1 and 2 to prevent contact between the Griess reagent and the sample solution; (4) the device was flipped and the parafilm was removed, and then 10 μ L of water was added to start the colorimetric reaction; and, (5) finally, a pink color appeared on layer 1.

2.4. Data processing

The images of the μ PADs were captured via a scanner and were processed via ImageJ software. The intensity of the detection zone was marked by the green values in an RGB color system and the intensity of the green color was converted to absorbance using Eq. (1).

$$\text{Absorbance} = -\log_{10} \frac{I}{I_0} \quad (1)$$

In Eq. (1), I_0 is the color intensity of the blank and I is the color intensity of the sample. The absorbance values were plotted against the concentrations of nitrogen (mg L⁻¹) in the forms of nitrite and nitrate ions in the calibration curves.

2.5. Spectrophotometric method

In the spectrophotometric method, nitrate ions were reduced using the following procedure: 5-mL aliquots of a 0.2 g mL⁻¹ zinc suspension were added to 10 mL of nitrate ion solutions in the centrifuge tubes. The solutions were shaken for 10 s or 6 times, and were then filtered with filter paper after 10 min [5]. A 1-mL aliquot of the filtrate was transferred to a microtube, and 50 μ L of the Griess reagent was added to react with the resultant nitrite ions. After 30 min, absorbance was measured at a wavelength of 520 nm [36].

3. Results and discussion

3.1. Comparison of the 2D- and 3D- μ PADs

The literature cites reports of several types of μ PADs with 2D [31,32] and 3D [30,34] structures being used for the simultaneous

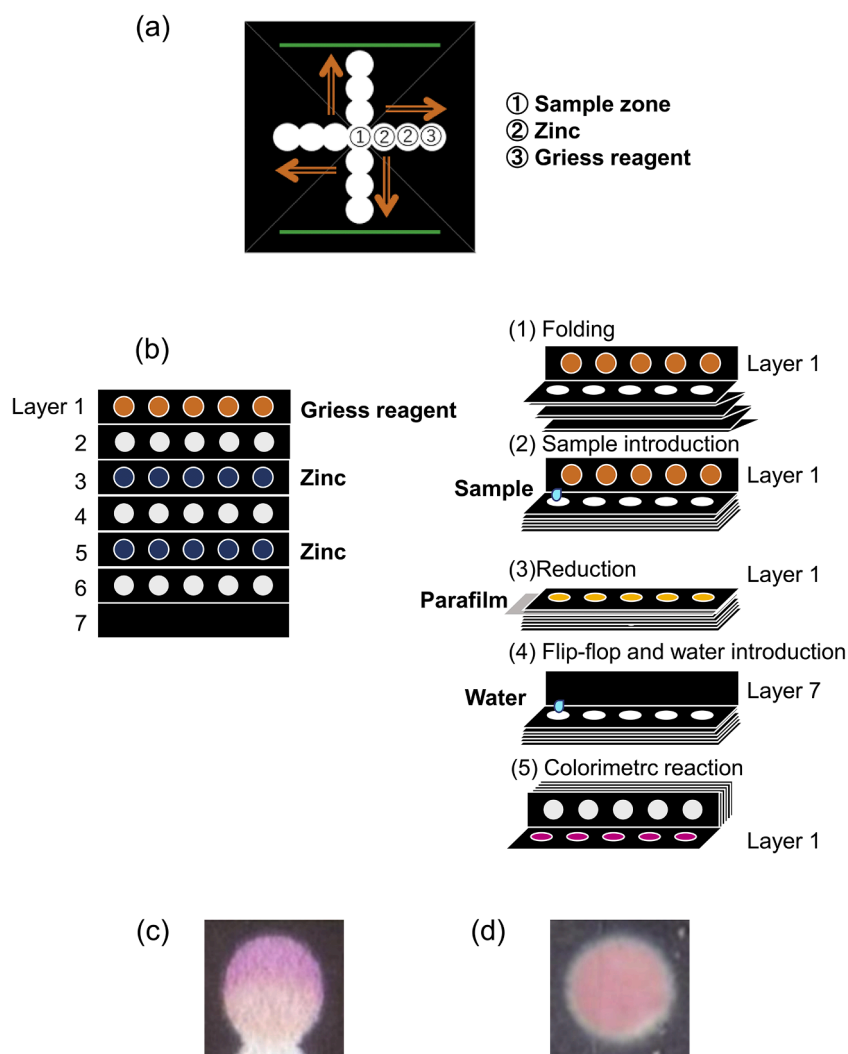


Fig. 1. The design of 2D- and 3D- μ PADs.

(a) 2D- μ PAD; (b) 3D- μ PAD, 1) folding 3D- μ PAD, 2) sample introduction, 3) reduction with zinc, 4) elution of the sample, and 5) colorimetric reaction; (c) and d), images of the color change on 2D- μ PAD and 3D- μ PAD, respectively. The sample concentration for (c) and (d) was $2.8 \text{ mg L}^{-1} \text{ N-NO}_3^-$.

measurements of nitrite and nitrate ions. In the present study, μ PADs with 2D and 3D structures were examined to measure the nitrite ions without zinc powder and to observe the color intensity. When 2.8 mg L^{-1} of a nitrite solution was introduced into the 2D- μ PAD, the colored product was flushed to the edge of the detection zone, which resulted in a heterogeneous color, as shown in Fig. 1 (c). Conversely, the 3D- μ PAD showed a homogeneous color in the detection zone due to the vertical flow of the sample solution (Fig. 1 (d)). A heterogeneous color induces errors in measurement, because the color intensity depends on the selection of the measured area. Therefore, the 3D- μ PAD was employed in this study and was optimized for the measurement of nitrate ions.

The number of reduction layers (number of reduction zones) in the 3D- μ PAD was optimized for the efficient reduction of nitrate ions. The number of the reduction layers varied from 1 to 4 (total number of layers, 10). The resultant color was the most intense when the μ PAD involved 2 layers in the reduction zones. Consequently, the optimized design for nitrate ions consisted of one detection zone and an additional six layers in which zinc powder was added to 2 layers, as shown in Fig. 1 (b).

3.2. Reduction time

To obtain the most intense color for nitrate ions, the reduction time

was varied from 5 to 90 min in 5 min intervals after sample introduction. Two sample volumes, 25 and 40 μL , were also examined to clarify the effect of sample volume. It is noteworthy that the sample solution overflowed when the sample volume exceeded 40 μL . The relationship between the reaction time and the absorbance is shown in Fig. 2. When adding 25 μL of the sample, the absorbance gradually increased with increases in the reaction time. Conversely, a reaction time range of 15 to 30 min resulted in the largest absorbance when introducing 40 μL of the sample. As Fig. 2 shows, the absorbance for 40 μL was always larger than that for 25 μL because of the larger absolute amount of nitrate ions. Based on these results, 40 μL samples were chosen for the reactions with zinc powder for durations of 20 min.

3.3. Analytical performance of the 3D- μ PAD

A calibration curve for nitrate ions was constructed using 40 μL samples with reaction times of 20 min. Nitrate showed a linear relationship that ranged from 1 to 10 mg L^{-1} of nitrogen ($y = 0.0151x + 0.006$, $R^2 = 0.994$) whereas the calibration curves for nitrite deviated from a linear relationship when the concentration range exceeded 10 mg L^{-1} . A linear relationship for nitrite was obtained within a range of from 0.05 to 1.5 mg L^{-1} in the absence of zinc ($y = 0.0421x + 0.0327$, $R^2 = 0.960$). The limit of detection (LOD) and limit of quantification (LOQ)

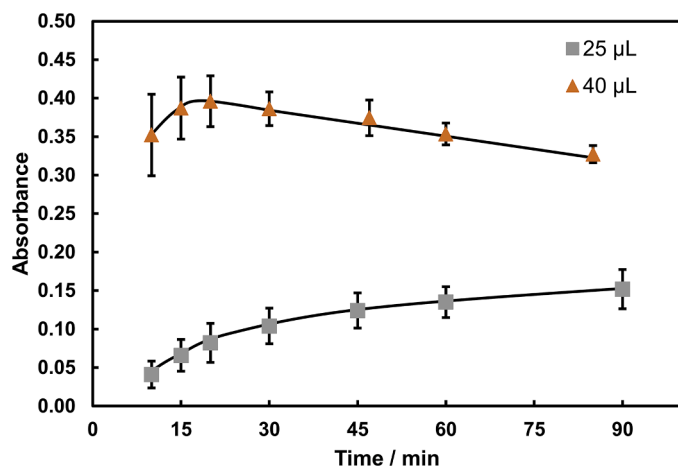


Fig. 2. Effect of reduction time on 3D- μ PADs at different sample volumes. Sample: 10 mg L^{-1} N- NO_2 . Parafilm was removed from the device after introducing the sample solutions. The average absorbance of five measurements on each of the μ PADs was plotted against the reduction time. The error bar indicates the standard deviations for five measurements.

were calculated from concentrations calculated using the IUPAC recommendation [37]. The LODs and LOQs were 0.03 mg L^{-1} and 0.21 mg L^{-1} for N- NO_2 (0.1 and 0.7 mg L^{-1} in the form of NO_2) and 0.9 mg L^{-1} and 3.9 mg L^{-1} for N- NO_3 (4.2 and 17 mg L^{-1} in the form of NO_3).

3.4. Effect of zinc on the measurements of nitrate and nitrite

Several μ PADs demonstrated simultaneous measurements of nitrite and nitrate ions when nitrite ions were measured without a reduction whereas the total concentrations of nitrate and nitrite ions were determined by the calibration curve of nitrate ions following a reduction with zinc. Table 1 summarizes the limit of detection (LOD) for nitrite and nitrate and the reduction efficiency from nitrate to nitrite ions using a reductant reported in the literature. Here, the reduction efficiencies were calculated from the ratio of the slopes in the calibration curves for nitrate to that for nitrite — with the noted exception of that reported by Teepoo et al. [31] because they omitted the slopes of the calibration

Table 1
Comparison of LODs, LOQs, and conversion efficiencies for different μ PADs.

Type of μ PADs	Reductant	LOD of NO_2 ^a	LOD of NO_3 ^b	Reduction efficiency from NO_3 to NO_2 / % ^c	Ref.
3D	Zn	$0.05 \mu\text{M}$ (0.0023 mg L^{-1})	$80 \mu\text{M}$ (5.0 mg L^{-1})	15	30
2D	Zn	1.2 mg L^{-1}	3.6 mg L^{-1}	33*	31
2D	Zn	0.4 mg L^{-1}	0.4 mg L^{-1}	58	32
2D	VCl_3	0.1 mg L^{-1}	0.4 mg L^{-1}	25	33
2D, 3D	Zn	$1.0 \mu\text{M}$ (0.046 mg L^{-1})	$19 \mu\text{M}$ (1.2 mg L^{-1})	20	34
3D	Zn	0.1 mg L^{-1}	4.2 mg L^{-1}	55	Present study

^{a,b} LODs are expressed according to the concentrations of nitrite and nitrate ions in the table although they refer to N- NO_2 and N- NO_3 in the text.

^c The reduction efficiency was calculated from the ratio of the slopes in the calibration curves for nitrite and nitrate ions.

* The reduction efficiency was calculated from the ratio of LODs because no data for the slopes of the calibration curves were given.

curves. The LOD and the reduction efficiency in this study are comparable to those reported in other studies. The reduction efficiencies ranged from 15 to 58%. Obviously, zinc incompletely converts nitrate to nitrite, and the reduction efficiency was influenced by the types of the μ PADs as well as possibly by the type of zinc.

The calibration curves for nitrite and nitrate were constructed in wide ranges of concentrations via the use of reduction with zinc. Fig. 3 (a) shows the calibration curves for nitrate with zinc reduction and for nitrite both with and without zinc reduction using the μ PADs. It should be noted that the sensitivity to nitrite decreased to 68% when zinc was added to the reduction zones. Fig. 3 (b) shows similar results obtained using conventional spectrophotometry where the sensitivity was decreased to 48%, which is more significant than the decrease observed when using the μ PADs. These results indicate that zinc incompletely converts nitrate to nitrite ions and also decreases the amount of nitrite ions.

Although many studies have reported simultaneous determinations of nitrite and nitrate using μ PADs, only Jayawardane et al. reported that zinc showed no effect on the quantification of nitrite ions [34]. Unfortunately, the other reports could have simply assumed that zinc resulted in no change in the sensitivity to nitrite ions [30-33]. In the present study, the sensitivity to nitrite ions decreased to 68% after treatment with zinc. Merino reported a similar decrease in the sensitivity using conventional spectrophotometry, although the decrease in sensitivity was much smaller (9%) than that observed in the present study when the slopes of the calibration curves are compared [14]. Therefore, the sensitivity to nitrite ions was obviously decreased after pretreatment with zinc. The inconsistency with Merino [14] and Jayawardane et al. [34] would have been caused by different conditions, such as the concentration range of nitrite ions, the amounts of zinc, and the reaction time. Zinc would have no, or even less, influence on the determination of

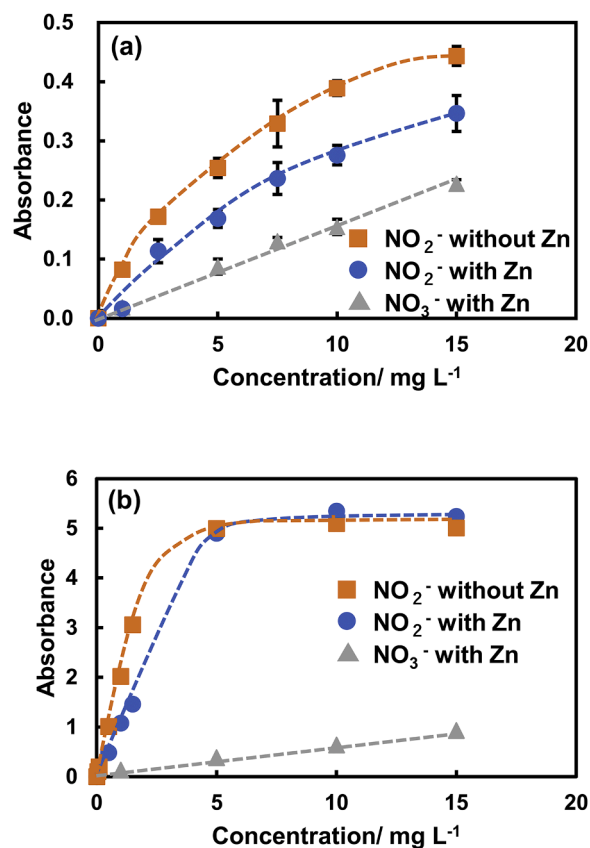


Fig. 3. Calibration curves of nitrite and nitrate obtained by 3D- μ PADs and spectrophotometry.

(a) μ PADs; (b) spectrophotometry.

nitrate ions if the concentrations of nitrite ions were sufficiently low to negate the effect on the measurement of nitrate ions.

Zinc reduces nitrite to ammonia and nitrogen gas, as described in the literature [38,39]. To elucidate the cause for the decrease in sensitivity to nitrite ions in the presence of zinc, ammonia was measured after the reaction with zinc. Table 2 shows the production of ammonia at 100 mg L⁻¹ of nitrite and nitrate ions using different amounts of zinc. When reacting with zinc, both nitrite and nitrate generated ammonia due to their reduction. So, one of the possible reasons for the decrease in sensitivity to nitrite ions is attributed to the reduction of nitrite ions to ammonia. However, the amount of ammonia was too small (1-2% of nitrite and nitrate ions) to explain the decrease in sensitivity. Therefore, further investigation is needed to clarify the complete mechanism of the decreased sensitivity.

To achieve a simultaneous determination of nitrite and nitrate, we considered whether the total concentration could be correctly obtained following pretreatment with zinc, because the apparent conversion efficiency from nitrate to nitrite is 53%, which is the ratio of the sensitivity to nitrate treated with zinc to that for nitrite treated with zinc. This suggests that if the decreased sensitivity to nitrite in the presence of zinc depends on the concentration ratio of nitrate to nitrite in the sample, we could not have measured the total concentrations of nitrite and nitrate.

To elucidate the effect of zinc on the calibrations of nitrite and nitrate, the calibration curves at different ratios of nitrate to nitrite were constructed within a range of from 1 to 15 mg L⁻¹-N via spectrophotometry. The concentrations of nitrate and nitrite were varied using ratios of 0.25:0.75, 0.5:0.5, and 0.25:0.75 to achieve different concentrations. In Fig. 4, the circle markers indicate the experimental data. It should be noted that linear relationships were obtained in the range of $A < 5$, and, therefore, data higher than 5 were removed from the graph. The calibration curves were linear and showed different sensitivities, depending on the ratio of nitrite and nitrate. The sensitivity increased with increases in the ratio of nitrite, as expected. These results indicate that the total amounts of nitrite and nitrate must be corrected for the ratio between nitrite and nitrate. Then, we attempted to reproduce the experimental results using the calibration curves of nitrite and nitrate with zinc treatment. The absorbances, A , shown by the triangle markers in Fig. 4 were calculated using Eq. (2).

$$A = S_{NO_2} C_{total} (1 - X_{NO_3}) + I_{NO_2} + S_{NO_3} C_{total} X_{NO_3} + I_{NO_3} \quad (2)$$

In Eq. (2), S and I indicate the slopes and intercepts of the calibration curves for nitrite and nitrate in the presence of zinc, and X_{NO_3} is the ratio of nitrate ions ($0 \leq X_{NO_3} \leq 1$) to the total concentration of nitrite and nitrate ions. Obviously, the equation reproduces the calibration curves that were experimentally obtained, as seen in Fig. 4, where the calculated results are represented by triangles and dashed lines. Therefore, Eq. (2) must be employed for the determination of the total concentration if the concentration of nitrite ions is too high to be ignored against that of the nitrate concentration. In practical analyses, therefore, Eq. (3) should be employed to determine the concentration of nitrate ions.

$$A = S_{NO_2} C_{NO_2} + I_{NO_2} + S_{NO_3} C_{NO_3} + I_{NO_3} \quad (3)$$

In Eq. (3), A is the absorbance with zinc treatment and C_{NO_2} is the concentration of nitrite ions, which must be measured without zinc treatment. In addition, S_{NO_2} , I_{NO_2} , S_{NO_3} , and I_{NO_3} also must be measured

Table 2

Measurement of ammonia produced by the reduction of nitrite and nitrate ions.

Ions	N-NO _x / mg L ⁻¹	Zinc/ g mL ⁻¹	NH ₃ -N/ mg L ⁻¹
NO ₂	100	0	0.0
	100	0.5	1.1
	100	2.5	1.9
NO ₃	100	0	0.0
	100	0.5	0.9
	100	2.5	1.5

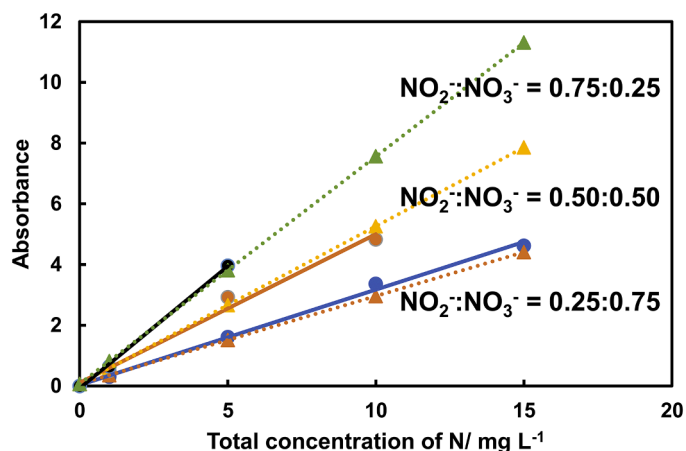


Fig. 4. Calibration curves of nitrite and nitrate at different ratios of nitrite and nitrate.

The circles represent the data experimentally obtained, and the triangles and the dashed lines indicate the values calculated from the calibration curves only for nitrite and nitrate after zinc treatment according to Eq. (2).

by constructing calibration curves for nitrite and nitrate with zinc treatment.

3.5. Analysis of natural water

With respect to the impact of zinc on the determination of nitrite and nitrate, natural water samples were analyzed using the developed μ PADs and spectrophotometry to demonstrate a practical application and validate the proposed correction method. Table 3 shows the concentrations of nitrite and nitrate ions in natural water samples taken from wells, ponds, and a brackish lake. As seen in Table 3, the results for water samples are in good agreement with the results from the μ PADs and spectrophotometry. The level of N-NO₂ in the brackish lake water sample was too low to be detected by the μ PADs (LOD is 0.03 mg L⁻¹). This result is reasonable because spectrophotometry showed 0.01 mg L⁻¹ of N-NO₂. Although the concentration of nitrate in the pond water showed a relatively large difference between the μ PAD and spectrophotometry, the μ PAD would be useful to estimate concentrations of nitrite and nitrate roughly because the results showed the same order of magnitude. Conversely, it is unclear why N-NO₃ was undetectable in the spectrophotometric measurement. As expected from the results of the decreased sensitivity to nitrite ions in the presence of zinc, the level of N-NO₃ would be overestimated if not corrected using the calibration curve for nitrite with zinc treatment (see Table 3). We also attempted recovery tests for pond samples, revealing recovery values of 76 to 107% for nitrite and 64 to 116% for nitrate. These values would be acceptable for rough estimation, although some of the results for nitrate were slightly lower than 70%.

The tolerance limit of nitrogen formed by the combination of nitrite and nitrate in tap water is 10 mg L⁻¹ in Japan. Thus, the obtained values were much lower than the tolerance limit, which means that the water samples were relatively uncontaminated.

4. Conclusions

The 3D- μ PADs were developed for the measurement of nitrite and nitrate ions contained in natural water samples. In general, natural water contains low or undetectable concentration levels of nitrite ions, so nitrate ions could be directly determined using the μ PADs with a zinc reduction. When a sample contains an amount of nitrite comparable to that of nitrate, we must measure the concentration of nitrite without zinc reduction and the total concentrations of nitrite and nitrate after the reduction of nitrate to nitrite. However, we found that zinc reduction

Table 3

Concentrations of nitrogen in the form of nitrite and nitrate ions in natural water samples.

	$\mu\text{PAD}/\text{mg L}^{-1}$			$\text{UV-VIS}/\text{mg L}^{-1}$		
	N-Nitrite	N-Nitrate with correction	N-Nitrate without correction	N-Nitrite	N-Nitrate with correction	N-Nitrate without correction
Well water 1 (Yonago)	0.18 ± 0.29	3.10 \pm 0.90	3.75 \pm 0.86	0.20 ± 0.00	4.79 \pm 0.00	8.80 \pm 0.00
Well water 2 (Yonago)	< LOD	-	2.84 \pm 0.56	< LOD	-	2.05 \pm 0.00
Brackish lake water (Yonago)	< LOD	-	2.37 \pm 0.54	0.01 ± 0.00	< LOD	1.16 \pm 0.00
Pond water 1 (Yonago)	0.04 ± 0.05	4.17 \pm 0.51	4.88 \pm 0.51	0.01 ± 0.00	3.16 \pm 0.00	7.37 \pm 0.00
Pond water 2 (Okayama)	< LOD	-	1.31 \pm 0.47	< LOD	-	0.79 \pm 0.00
Pond water 3 (Okayama)	< LOD	-	1.26 \pm 0.71	< LOD	-	0.80 \pm 0.00

converts nitrite and nitrate to ammonia, which decreases the sensitivity to nitrite. So, the total concentrations of nitrite and nitrate ions must be corrected using an additional calibration for nitrite with zinc treatment. We proposed a correction method to measure the total concentration and validated it by comparing the calibration curves for mixtures containing nitrite and nitrate at different ratios with the calculated values using individual calibration curves for nitrite and nitrate with zinc treatment. Consequently, we successfully measured the concentration of nitrate ions in natural water samples using the developed μPADs . The results were in good agreement with those obtained via conventional spectrophotometry, i.e., the developed μPAD is expected to be useful for the determination of nitrate ions in natural water samples under the conditions of under-equipped facilities, which would be particularly useful in developing countries.

CRedit authorship contribution statement

Mika I. Umeda: Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kaewta Danchana:** Supervision, Investigation, Funding acquisition, Formal analysis. **Takatoshi Fujii:** Supervision. **Eiichi Hino:** Supervision. **Yusuke Date:** Supervision. **Kaoru Aoki:** Supervision. **Takashi Kaneta:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

This work was supported by JSPS KAKENHI Grant Number JP23K04830, The Kurita Water and Environment Foundation (KWEF) Grant Number 23H035, and Okayama Foundation for Science and Technology.

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