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A simple miniaturised photometrical method for rapid determination of nitrate and nitrite in freshwater

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ABSTRACT

A rapid, simple miniaturised photometrical method was developed for the determination of nitrate and/or nitrite in freshwater samples. All procedures, including sample buffering, reduction by copperised cadmium granules, colour development and absorbance determination, were completed in a 96-well microplate. The factors governing the nitrate reduction and its recovery were investigated in detail, and the optimised analysing conditions were established. Nitrate was quantitatively reduced by copperised cadmium granules with a high reduction efficiency (96.59 ± 0.96%). The proposed method gave a linear calibration ranging from 0.01 to 1.50 mg L^{-1} for NO_2^--N and 0.02 to 1.50 mg L^{-1} for NO_3^--N. The detection limits for nitrite and nitrate were 2 and 4 μg L^{-1}, respectively. The proposed method allowed at least 48 samples to be simultaneously analysed in duplicate, with good precision, within 90 min for nitrate and 30 min for nitrite, and was successfully applied to actual freshwater sample analysis with a recovery of 98.02 ± 1.04% for nitrite and 99.72 ± 1.39% for nitrate. This method produced accurate results comparable to standard methods, provided a much higher sample throughput than conventional methods and could be routinely used in actual freshwater sample monitoring.

1. Introduction

Nitrogen and phosphorus species are key factors in the control of eutrophication and play an important role in determining the ecological status and health of freshwater ecosystems. Both nutrients, particularly in dissolved species such as nitrate, nitrite, ammonium and phosphate, are easily assimilated by phytoplankton for growth and act as significant factors in the regulation of primary productivity in bodies of water. Nitrogen concentrations in river and ground water increase as a response to increased agricultural activity and point-source discharges and were one of the most implicated species in water eutrophication [1].

Nitrate and nitrite are naturally occurring ions and are ubiquitous in the environment. Nitrate contamination of surface- and groundwater is a pervasive, worldwide problem, while nitrite is an important indicator of faecal pollution in water. Monitoring variations of nitrate and nitrite is important for understanding their biogeochemical processes in aquatic environments and for developing better methods of managing water quality. The determination of nitrite and nitrate in freshwater has received increased attention in recent years because of increasing eutrophication of natural water worldwide and the growing need to control point and non-point pollution.

In the last couple decades, many strategies have been developed to facilitate the detection, determination and monitoring of nitrate and/or nitrite [2]. Among which, spectroscopic methods are by far the most widely used for nitrite and nitrate determination due to the excellent limits of detection, the simplicity of the protocols and the wide availability of the instrumentation involved. For nitrate analysis, the most current methods are based on reduction of nitrate to nitrite by copperised cadmium (Cu–Cd) columns, with subsequently colorimetric determination of nitrite by a Griess diazo-coupling reaction [3]. This method is very selective for nitrate analysis and has been extensively used in a variety of flow injection techniques which obtain a high throughput with excellent results [4–11]. Nitrite present in the original sample can be determined without the use of the reductor column.

Preparation of Cu–Cd columns, however, is laborious, time consuming and requires considerable manual skill, and unsatisfactory column performance (e.g., low reduction efficiency and short lifetimes) has been experienced during the analysis [12]. Moreover, some drawbacks, such as the large quantity of sample needed, excessive analysis time and progressive decrease in column efficiency, have been documented [13]. Perhaps for these reasons, the cadmium reduction method has been seldom applied in China and is being gradually replaced by other methods, with the con-

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ventional phenoldisulfonic acid method being the most popular method for the determination of nitrate. Nevertheless, the phenoldisulfonic acid method is also laborious, time consuming and requires a large water volume for samples (usually 100 mL).

Routine analysis of nitrate and nitrite in surface water usually constitutes a major workload in agricultural and environmental monitoring laboratories due to the short holding time for nitrate and nitrite (<24 h for refrigerated samples). Thus, there is a definite need to develop a relatively simple, rapid, reliable and sensitive method for quantification of nitrate and nitrite in natural waters, especially for a large set of samples or for extensive environmental monitoring programs.

The objectives of the present study were to develop a simple and rapid analytical method by coupling the nitrate-selective reduction method of Cu–Cd granules to the more robust microtiter plates and microplate reader detection for routine determination of nitrate and nitrite in water samples. All analytical steps were completed in a 96-well microplate. The effects of various factors on the reduction efficiency and colour development were thoroughly investigated. The experimental conditions were optimised and successfully applied to natural water samples with precise and accurate results.

2. Experimental

2.1. Reagents and solutions

All chemicals were of analytical reagent grade and double distilled water was used in all experiments.

Ammonium chloride–ethylenediaminetetraacetic acid (NH_4Cl–EDTA) stock solution was prepared by dissolving 58.50 g NH_4Cl and 7.65 g EDTA in 900 mL water, adjusting to pH 8.5 with ammonia solution, and diluting the solution to 1000 mL.

NH_4Cl–EDTA buffer solution was prepared by dissolving 13.00 g NH_4Cl and 1.70 g EDTA in 900 mL water, adjusting to pH 8.5 with ammonia solution, and diluting the solution to 1000 mL.

Dilute NH_4Cl–EDTA solution was prepared by diluting 300 mL NH_4Cl–EDTA buffer solution to 500 mL with water.

Griess chromogenic reagent (Chr-R) was prepared by mixing 4.00 g sulphanilamide, 0.20 g N-(L-naphthyl) ethylenediamine dihydrochloride (NED), and 10 mL phosphoric acid in 50 mL water, stirring to complete dissolution, and diluting the solution to 100 mL. This reagent is stable for at least one month when stored in a brown bottle at 2–5 °C. Experimental Chr-R solution was prepared by diluting 8 mL in a 50 mL volumetric flask with water before use. The structure of Chr-R and diazo-coupling reaction is showed in Fig. S1 in the Supplementary Data (SD).

Nitrate and nitrite stock standard solutions (1000 mg L^{-1} $NO_3{}^-$-N or 250 mg L^{-1} $NO_2{}^-$-N) were each prepared in 100 mL volumetric flasks by dissolving 0.7218 g potassium nitrate (pre-dried at 105 °C for 24 h) or 0.1232 g sodium nitrite (pre-dried at 105 °C for 1 h) in water and diluting to volume. The stock solutions were preserved with 0.2 mL $CHCl_3$ and stored at 4 °C in a refrigerator. Working standard solutions were prepared by diluting these stock solutions with water.

2.2. Preparation of Cu–Cd granules

Cadmium granules (purity $\geq$ 99.99%) were purchased from Tianjin Kermel Reagents Development Centre, China. To prepare Cu–Cd granules, 10.0 g of cadmium granules (20–40 mesh size, each with 16 ± 6 mg) were initially washed with acetone to remove any organic contamination on the surface, followed by washing thoroughly with water to eliminate acetone. These granules were then washed twice with 10% HCl for a few minutes. The acid was decanted and the granules were immediately rinsed with 50 mL water at least three times. The water was then decanted and 10 mL of 2% (w/v) $CuSO_4$ solution added. The granules were gently swirled until the partial fading of the blue colour of the $CuSO_4$ solution. The surface of the granules immediately turned gray upon coming into contact with $CuSO_4$ solution, which is indicative of coating with copper. The faded solution was then decanted, repeated with fresh $CuSO_4$ solution and swirled again until the blue colour of the solution did not fade and a brownish colloidal precipitate of copper began to develop. During this process, special care should be taken not to excessively disturb the newly formed copper layers coated on the surface of the granules, because exposure of new cadmium surfaces to $CuSO_4$ solution will lead to the continuous dissolving of the cadmium and the generation of a useless lump of copper “mud”.

The copperised granules were then gently flushed with water several times to remove the precipitated copper, followed by washing with dilute NH_4Cl–EDTA solution until the supernatant was clear and free of fine particles, also done with care not to damage the copper layer coating on the cadmium granules. The prepared Cu–Cd granules were preserved in dilute NH_4Cl–EDTA solution for at least two days. Subsequently, the Cu–Cd granules were vigorously swirled several times to remove loosely coated copper until no copper particles were found. Further conditioning was carried out by adding 10 mL of 40 μM nitrite and NH_4Cl–EDTA buffer mixture solution (1:3, v/v) to the Cu–Cd granules, gently swirling for 5 min and then discarding the washings, repeating this treatment three times. Finally, Cu–Cd granules were rinsed with dilute NH_4Cl–EDTA solution to thoroughly eliminate nitrite, and then preserved until further use.

2.3. Analytical procedures

The reduction of nitrate to nitrite and the determination of nitrite were performed in a microplate (Polystyrene, Costar, Corning Inc., NY, USA), which was comprised of 96 wells (8 by 12 configuration) with a cell volume of 375 μL (i.d. 7 mm, 11 mm depth). To analyse nitrate, 250 μL of nitrate working standard solutions or water samples (prior filtered through a 0.45 μm membrane filter) and 50 μL of NH_4Cl–EDTA stock solution were pipetted into each well by an eight-channel multipipette (10–100 μL and 100–300 μL; Eppendorf, Germany) and mixed, followed by the addition of two Cu–Cd granules (dried on filter paper) into each well. The microplate was then shaken manually or automatically on a rotary shaker ($\leq$100 r/min) for 60 min. Subsequently, 250 μL of the mixture was transferred into another microplate and 50 μL of the Chr-R was added with mixing. Special care was taken to ensure complete mixing and to avoid formation of air bubbles in wells during all these manipulations. The microplate was then covered and the colour was allowed to develop for 20 min at room temperature. The reddish purple coloured azo dye was measured at 540 nm against a reagent blank by an ELx800 absorbance microplate reader (BioTek Instruments, Inc.) equipped with a 540 nm filter. The nitrite present in the original samples was analysed separately by mixing 250 μL of the filtered sample and 50 μL of the Chr-R. Each sample was analysed in triplicate, and the used Cu–Cd granules could easily be removed, washed with water and stored in dilute NH_4Cl–EDTA solution for future use. The schematic illustration of the principle of the proposed and conventional method is shown in Fig. S2 in the SD.

The reduction efficiency of Cu–Cd granules was calculated from the ratio of the absorbance of the nitrate solution to a standard nitrite solution of the same concentration. To optimise the experimental variables, various factors affecting the reduction efficiency of the copperised granules, including the number of Cu–Cd granules added, reaction time, pH value, NH_4Cl–EDTA buffer solution and the reactivity of freshly activated granules, were investigated. The influence of fine copper particles on the colour development of the

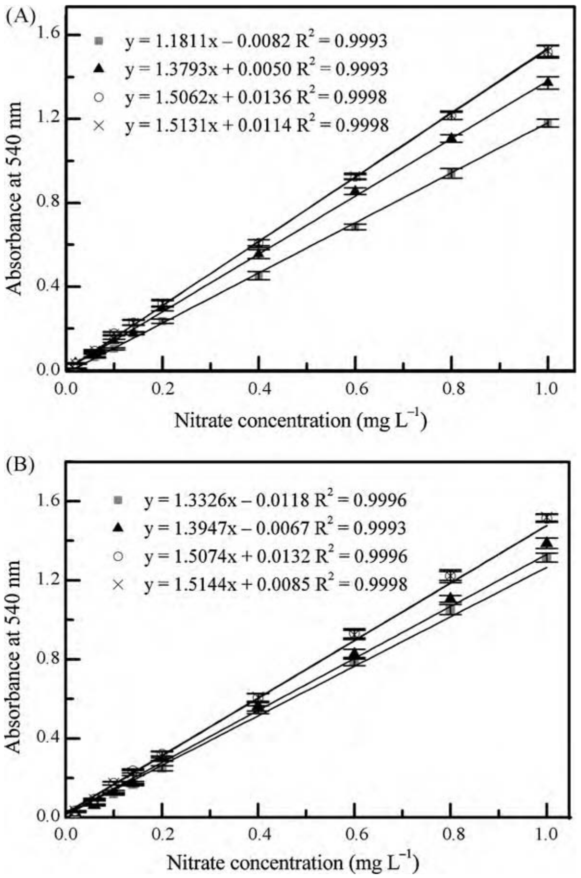

Fig. 1. Nitrate reduction by (A) two and (B) four Cu–Cd granules during different reaction times (■ 20 min; ▲ 40 min; ○ 60 min; × 80 min). Values are presented as mean ± SD ($n = 6$).

azo dye and the potential interference effects caused by commonly occurring ions were also tested. The linearity, accuracy, precision and detection limits of the method were established using nitrate and nitrite standard solutions. Applicability to real samples was evaluated and verified by analysis of natural water samples.

3. Results and discussion

3.1. Optimisation of experimental conditions

3.1.1. Effects of the number of Cu–Cd granules added and reaction time on nitrate reduction

A set of nitrate working standard solutions ranging from 0.02 to 1.00 mg L^{-1} were used to assess the number of Cu–Cd granules needed and the reaction time required to complete the reduction. The absorbance increased with increasing reaction time from 20 to 60 min, reaching a maximum after 60 min, because there was no significant difference between 60 and 80 min ($P > 0.05$). This result suggested a reaction time of 60–80 min gave complete reduction of nitrate (Fig. 1). These observations agreed with those of other reports that showed that shaking for at least 60 min was required for complete reduction of nitrate by spongy cadmium [13], cadmium powder [14] and by a Cu–Cd alloy [15]. The correlation between the absorbance and the concentrations of nitrate showed a quite good linear response, as indicated by a high correlation coefficient (R^2) of above 0.999. A comparison of the slopes of the regression equations between two granules and four granules further indicated that there was no significant difference under either set of conditions except for the case of 20 min of reaction time (Fig. 1a and b). Taking these results into consideration, we chose two Cu–Cd granules reacting with nitrate solution for 60 min as an optimum reduction condition for further experiments.

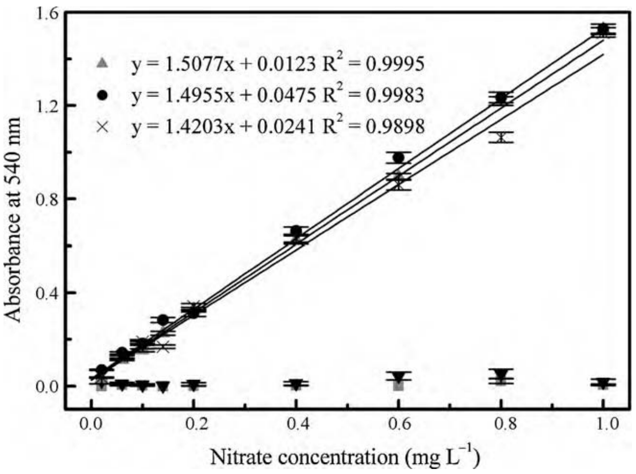

Fig. 2. Effects of different pH values on the reduction and measurement of nitrate standards. Values are presented as mean ± SD ($n = 6$). ■ pH = 1.60; ▼ pH = 1.98; ▲ pH = 8.31; ● pH = 9.38; × pH = 9.69.

3.1.2. Effects of pH values on nitrate reduction

To evaluate the effects of pH on reduction efficiency of Cu–Cd granules, experiments of nitrate reduction were conducted under different pH values adjusted by adding 10–20 μL of 1.2 M hydrochloric acid or 3.5 M ammonia to the reaction solution. Nitrate could be quantitatively reduced to nitrite under basic conditions, with little difference across a pH range of 8.3–9.7 (Fig. 2). However, the correlation coefficients of the linear equations decreased slightly from 0.9995 to 0.9898 when the pH increased from 8.31 to 9.69, suggesting that too high of a pH value led to a slight decrease in stability of the reduction efficiency. This result agreed with that reported by Nydahl [16], who observed a tendency for deviation of the reduction efficiency of a cadmium column was larger and more apparent at a higher pH (>9.5). Under acidic conditions, however, only trace nitrite was detected (Fig. 2). To test whether the nitrite produced was further reduced to lower nitrogen compounds under acidic conditions, another experiment was performed where 6 mL of 1 mg L^{-1} nitrate working standard solution was pipetted into a 10 mL beaker and its pH value was adjusted to 2.5 with 1.2 M HCl. The absorbance at 220 and 275 nm, before and after forty Cu–Cd granules were added to the solution, were measured at intervals in a UV–vis spectrophotometer (Shanghai Spectrum Instruments Co., Ltd., Shanghai, China) using a 1.00 cm optical path length quartz cuvette. NH_4Cl–EDTA stock solution was not added to the nitrate solution because EDTA has a strong absorbance at 220 nm and could cause serious interference with the measurement. Both the absorbance at 220 nm and the corrected absorbance dropped dramatically from 0.323 to 0.115 and from 0.269 to 0.051 within 60 min, respectively (Fig. 3). Given that nitrate and nitrite have identical absorbance at 220 nm at the same concentration, the results obtained indicated that nitrate was reduced to nitrite and further reduced to lower nitrogen compounds in acidic conditions. This can be explained by the observation that, in acidic solution, both nitrate and nitrite are strong oxidising reagents, with a standard potential of 0.94 V for the reduction of nitrate ion to nitrite ion and 1.297 or 0.983 V for the reduction of nitrite ion to N_2O or NO, respectively. Because the reduction potential of Cu^{2+} to Cu and Cd^{2+} to Cd is 0.337 and −0.403 V respectively, nitrate and nitrite can be thermodynamically reduced to their lower valence nitrogen compounds under these conditions. Nydahl [16] reported that a specific hydrogen ion concentration was required for quantita-

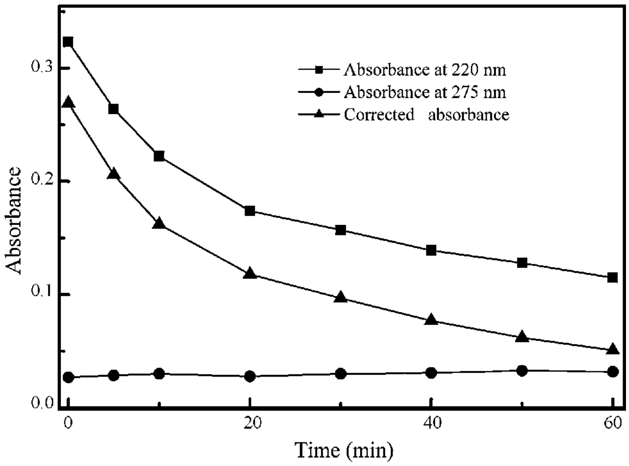

Fig. 3. Changes in absorbance at 220 nm and 275 nm for 1 mg L^{-1} nitrate solution reduction by copperised cadmium granules under acidic conditions (pH = 2.5). The absorbance was measured in a UV–vis spectrophotometer using a 1.00 cm optical path length quartz cuvette. Corrected absorbance was calculated by subtracting two times the absorbance reading at 275 nm from the reading at 220 nm.

tive reduction of nitrate to nitrite on a Cu–Cd reductor column. A pH range of 8.0–9.5 has been reported to be most suitable, while too high a hydrogen ion concentration (e.g., pH 1–3) can cause further reduction of nitrite to such species as NH_3OH^+ or NH_4^+. At pH ranging from 3 to 5, the nitrate reduction tends to stop at the formation of nitrite, while a pH below 8.0 leads to a decrease in the quantitative reduction of nitrate. Lambert and DuBois [17] also demonstrated that, under acidic conditions, the Cu–Cd prepared by precipitation of copper on cadmium powder reduced the nitrate to an oxidation state lower than nitrite too rapidly to quantitatively detect the nitrite that was momentarily present. Our results were consistent with those reported by these authors. Although the pH of the NH_4Cl buffer can be varied between 5 and 10 without affecting nitrate reduction [18], the colour development step was found to be pH dependent [13]. Therefore, considering the buffer capacity of NH_4Cl–EDTA solution (Table S1 in the SD), most natural water samples can be analysed directly with no adjustment of pH value of the solution; otherwise prior adjustment of the pH to between 2.5 and 8.5 was needed.

3.1.3. Effects of NH_4Cl–EDTA buffer on nitrate reduction

In the standard method for the determination of nitrate by cadmium reduction, NH_4Cl–EDTA buffer was usually added to water samples (e.g., mixing 25 mL of sample with 75 mL NH_4Cl–EDTA buffer solution) before passing through a Cu–Cd column [3]. To test whether NH_4Cl–EDTA buffer was necessary for achieving a quantitative reduction of nitrate to nitrite, three experiments were conducted with the same protocol as proposed above, except for those specified as follows: first, using distilled water instead of NH_4Cl–EDTA stock solution; second, using distilled water (presetting its pH to 8.5 with sodium hydroxide) instead of NH_4Cl–EDTA stock solution; and finally, using the same concentration of the NH_4Cl solution (also presetting its pH to 8.5 with sodium hydroxide) instead of the NH_4Cl–EDTA stock solution. Quantitative reduction of nitrate to nitrite only occurred under NH_4Cl–EDTA buffer conditions, whereas much lower absorbencies were recorded in distilled water (Table 1). When nitrate was reduced at different pH values, a narrower pH range (7.96–8.38) was obtained for the NH_4Cl–EDTA stock solution than that (pH 2.55–8.43) for distilled water, despite preconditioning of its pH to 8.5 with sodium hydroxide, and much higher reduction efficiencies and smaller relative standard deviations (RSD) were achieved for the NH_4Cl–EDTA buffer (Table 2). Similarly, lower reduction efficiency, but higher RSD was produced when using NH_4Cl in place of the NH_4Cl–EDTA stock solution (Table S2 in the SD). These results indicated that the NH_4Cl–EDTA stock solution was essential for a quantitative conversion of nitrate to nitrite.

NH_4Cl is commonly used as a buffer that presumably complexes oxidised cadmium during the reduction of nitrate to nitrite [19], but NH_4Cl alone was insufficient to yield high reduction efficiencies, as indicated in the present study. EDTA was found to have an important role in the nitrate reduction, as concentrations of iron, copper, or other metals above several milligrams per liter have been documented to decrease reduction efficiency [3]. Precipitation of these metal salts onto the Cu–Cd surface can cause serious interference, but can be prevented by the addition of EDTA to the buffer solution. During nitrate reduction by cadmium, added EDTA appeared to be chelated with Cd^{2+}, forming a soluble complex with this ion according to the equation proposed by Wood et al. [20]: $NO_3^- + H_2O + EDTA^{4-} + Cd \rightarrow NO_2^- + 2OH^- + Cd(EDTA)^{2-}$. Without EDTA, $Cd(OH)_2$ or other hydroxides would precipitate on the surface of the Cu–Cd granules and lower their reduction efficiency. Although a complete reduction of nitrate to nitrite has also been obtained using borate buffer (pH 8.9) or carbonate buffer (pH 9.0) instead of the NH_4Cl–EDTA buffer (pH 8.5) [15,21], so far only NH_4Cl–EDTA has been the most widely used buffer to sustain a high reduction activity on the Cu–Cd granules or a reductor column.

3.1.4. Effects of the processes of conditioning and aging of Cu–Cd granules on nitrate reduction

A conditioning process has been commonly carried out before routine analysis of nitrate reduction to nitrite by a Cu–Cd column [3]. Over-reactivity of newly prepared Cu–Cd columns has been reported by several authors. Zhang et al. [12] found that freshly prepared Cu–Cd columns without conditioning reduced all nitrate to nitrite and further reduced 40.5% of the nitrite produced to even lower oxidation state nitrogen compounds. Wood et al. [20] also observed that a slight decrease occurred on a new Cu–Cd column, probably due to the reduction of some NO_3^- to states lower than NO_2^-. To check whether this case happened in the present study, experiments were carried out to (1) compare the reduction efficiency of Cu–Cd granules prepared with and without conditioning and (2) investigate whether nitrite produced could be further reduced by Cu–Cd granules prepared with and without conditioning. The fact that (1) the slope of the reduction of nitrate to nitrite by Cu–Cd granules prepared with conditioning (1.5077) was significantly higher than that of granules used without conditioning (1.2544) (Fig. 4), (2) some nitrite was further reduced to lower oxidation state nitrogen compounds by Cu–Cd granules prepared without conditioning (absorbance significantly decreased from 0.330 to 0.265), and (3) no significant changes ($P > 0.05$) occurred in Cu–Cd granules prepared with conditioning compared to the blank (Fig. 5), indicated that some extent of over-reactivity existed in newly prepared Cu–Cd granules. These results demonstrated that a conditioning process was necessary to obtain satisfying nitrate reduction efficiencies. The detailed mechanism of the conditioning process is not clear but probably involves re-arranging of the reactive surface site to create a uniform reactive surface for selective reduction of nitrate to nitrite [12].

The influence of the aging process on nitrate reduction was also tested and the results are shown in Fig. 4. The slope (1.5077) of the reduction of nitrate to nitrite by Cu–Cd granules prepared with aging was significantly higher than that (1.2686) without aging, suggesting that this process had an important role in improvement and stability of reduction efficiency and, hence, could not be omitted. Without aging, newly coated copper on the surface of the cadmium was easily flushed off, and these loosely attached copper particles then entered the solution, resulting in interference with absorbance measurements. More importantly, even after the aging

Table 1
Reduction of nitrate to nitrite with distilled water or NH_4Cl–EDTA stock solution.

Nitrate concentration ($mg\,L^{-1}$)	Distilled water added Absorbance[a]	RSD (%)	NH_4Cl–EDTA buffer added Absorbance[a]	RSD (%)
0.02	0.002 ± 0.002	100.0	0.030 ± 0.002	6.57
0.10	0.013 ± 0.008	66.67	0.153 ± 0.006	4.11
0.20	0.033 ± 0.012	35.48	0.328 ± 0.011	3.21
0.40	0.049 ± 0.020	40.43	0.625 ± 0.018	2.86
0.60	0.044 ± 0.018	40.48	0.895 ± 0.015	1.64
0.80	0.082 ± 0.016	19.23	1.219 ± 0.019	1.55
1.00	0.054 ± 0.014	25.49	1.527 ± 0.022	1.44

[a] Values are presented as mean ± SD ($n = 6$).

Table 2
Changes in pH and absorbance of 1 $mg\,L^{-1}$ nitrate when mixed with water or NH_4Cl–EDTA buffer and reduced by copperised cadmium granules.

Initial pH in nitrate solutions	Mix with distilled water (pH preconditioned to 8.5) pH obtained	Absorbance[a]	RSD (%)	Mix with NH_4Cl–EDTA stock buffer pH obtained	Absorbance[a]	RSD (%)
2.50	2.55	0.074 ± 0.038	51.1	7.96	1.605 ± 0.037	2.31
4.02	4.15	0.328 ± 0.193	58.8	8.34	1.606 ± 0.030	1.89
5.75	6.19	0.166 ± 0.057	34.0	8.37	1.597 ± 0.060	3.77
8.40	8.43	0.339 ± 0.149	44.1	8.38	1.610 ± 0.043	2.69

[a] Values are presented as mean ± SD ($n = 6$).

treatment, some fine copper particles could be generated during the swirling process and subsequently affect the colour development of the diazo-coupling reaction, as will be discussed in the next section. In order to overcome this disadvantage, a vigorous swirling step was recommended to remove loosely coated copper after aging.

3.1.5. *Effect of copper particles on the colour development of the diazo-coupling reaction*

Our preliminary experiments revealed that some fine copper particles generated during the swirling process might have affected the colour development of the diazo-coupling reaction. To verify this point, 2.50 mL of 0.2 $mg\,L^{-1}$ or 0.6 $mg\,L^{-1}$ nitrite and 0.50 mL of the Chr-R were added into a 1.00 cm cuvette, mixed, allowed to sit for 20 min for the colour to develop, and then the absorbance of the mixture was measured on a spectrophotometer at 540 nm against a reagent blank. Subsequently, 0.1 mL of suspension solution containing fine copper particles, generated during the processes of either washing with water or swirling vigorously in dilute NH_4Cl–EDTA solution, was pipetted into the azo dye solution and mixed. The absorbance of the mixture measured at the intervals was shown in Fig. 6. The absorbance decreased dramatically during the colour development, with higher decreasing rates associated with larger amounts of copper particles generated during the process of washing with water than that of swirling vigorously in dilute NH_4Cl–EDTA solution, indicating that the copper particles had significant effects on the colour development of the diazo-coupling reaction. Given that at least 20 min was required for full colour development of the azo dye [3], much more attention must be paid to eliminate these copper particles, even though their amount was much lower in actual measurements when compared with those in the suspension solution. Furthermore, it has been reported that the copper precipitate washed off from the Cu–Cd granules affected the rate of reduction of nitrate to nitrite [16]. Therefore, the control of shaking speed (e.g., swirling manually or automatically on a rotary shaker ≤ 100 r/min) was

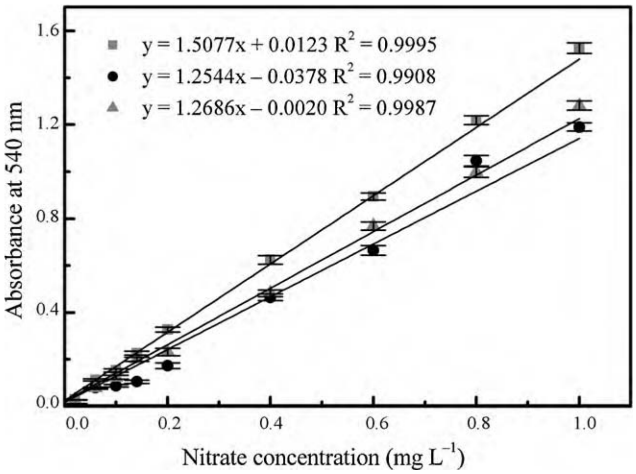

Fig. 4. Effects of the processes of conditioning and aging of the Cu–Cd granules on nitrate reduction. Values are presented as mean ± SD ($n = 6$). ■ Cu–Cd granules prepared by the recommended method; ● Cu–Cd granules prepared by aging 48 h without conditioning; ▲ Cu–Cd particles prepared with conditioning but without aging.

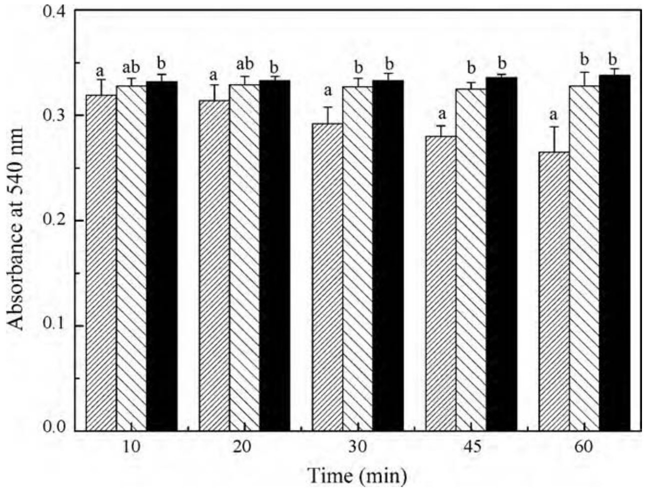

Fig. 5. Effects of Cu–Cd granules prepared with or without conditioning on nitrite reduction. 250 μL of 0.2 $mg\,L^{-1}$ NO_2^--N was mixed with 50 μL of NH_4Cl–EDTA stock solution, swirled gently and reduced by two Cu–Cd granules, prepared by the recommended method (▧); aging 48 h but without conditioning (▨); no Cu–Cd granules added (■). 250 μL of the mixture was pipetted at 10 min, 20 min, 30 min, 45 min and 60 min, mixed with 50 μL of Chl-R, and the absorbance was measured at 540 nm by a microplate reader. Each treatment was analysed in six parallels. Bars with different small letters in each figure represent significant differences ($P < 0.05$) among treatments.

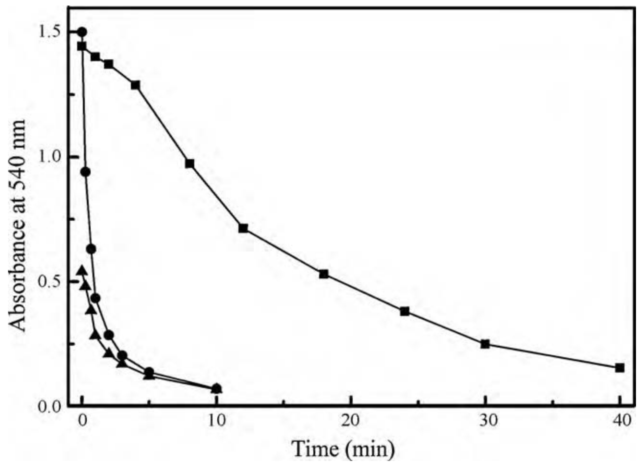

Fig. 6. Effects of fine copper particles on the colour development of the diazo-coupling reaction. ■ Copper particles generated during vigorously swirling and added to a 0.6 mg L^{-1} nitrite solution; ● Copper particles generated during water washing and added to a 0.6 mg L^{-1} nitrite solution; ▲ Copper particles generated during water washing and added to a 0.2 mg L^{-1} nitrite solution. The absorbance was spectrophotometrically measured at 540 nm using a 1.00 cm optical path length cuvette.

Table 3
Effect of interfering ions on the determination of 0.5 mg L^{-1} nitrite or nitrate.

Foreign ions	Added as	Tolerance limits (mg L^{-1})	
		Nitrite	Nitrate
Cu^{2+}	$CuSO_4·5H_2O$	150	150
K^+	KCl	3000	3000
Na^+	NaCl	3000	3000
Mg^{2+}	$MgCl_2·6H_2O$	2000	50[a], 200[b]
Ca^{2+}	$CaCl_2·2H_2O$	2000	50[a], 400[b]
Cl^-	NaCl	3000	3000
$CO_3{}^{2-}$	Na_2CO_3	1000	1000
$HPO_4{}^{2-}$	Na_2HPO_4	200	200
$SO_4{}^{2-}$	Na_2SO_4	5000	5000

[a] Tolerance concentrations in the original solution.
[b] Tolerance concentrations after treatment with sodium carbonate (1000 mg L^{-1}).

necessary for achieving complete and consistent reduction efficiency.

3.2. Study of interferences

The effect of potentially interfering ions was investigated by adding them to a standard nitrate- or nitrite-containing solution. A given ion was considered to interfere with the determination when the relative error reached a value of above 5%. The errors were calculated by comparing the absorbance of nitrite and nitrate solutions containing foreign ions with those obtained from solutions containing no foreign ions. The results of this study are summarised in Table 3. The majority of ions were found to interfere only when in very high concentrations. The presence of Ca^{2+} and Mg^{2+} (>50 mg L^{-1}) did have a significant effect on the reduction of nitrate to nitrite, mainly due to their precipitates as hydroxide in the alkaline medium. Their tolerance levels, however, were as high as 400 and 200 mg L^{-1} for Ca^{2+} and Mg^{2+}, respectively, when treated with sodium carbonate (1000 mg L^{-1}).

Olson [22] demonstrated that 2.5–25 μM $PO_4{}^{3-}$ (0.15–1.5 mg L^{-1}) may decrease reduction of nitrate by 10–40% in Cu–Cd columns. Cerdà et al. [23] also indicated that phosphate was the main interference, even when present at low levels (maximum tolerated concentration was 1 mg L^{-1}). However, we did not find any interference by phosphate until its concentration reached 200 mg L^{-1}. In general, the potential interferences of these foreign ions were much less likely in the proposed method than in a column reduction method because a continuous shaking led to continual scouring of the surface of the Cu–Cd granules. This phenomenon ensured that the granule surface was cleaned during the reduction process, resulting in complete and consistent reduction of nitrate to nitrite. Therefore, the proposed method was selective enough to be used to directly determine nitrite and nitrate levels in natural water samples without significant interference.

3.3. Performance evaluation

The present miniaturised photometric method can determine both nitrate and nitrite. The optimised experimental conditions were found to be 250 μL nitrate-containing samples, buffered with 50 μL NH_4Cl–EDTA solution and shaken for 60 min with two Cu–Cd granules. 250 μL of each mixture was then reacted with 50 μL of Chr-R and the formed azo dye was measured at 540 nm by a microplate reader. Nitrite in the original samples was determined separately by mixing 250 μL of the sample and 50 μL of the Chr-R. Nitrate concentration was then calculated by subtracting this nitrite value from the total nitrite.

The reduction efficiency of Cu–Cd granules was calculated from the ratio of the absorbance of the nitrate solution to that of a nitrite solution of the same concentration. The absorbance of 0.06, 0.20, 0.40 and 1.50 mg L^{-1} of nitrate and nitrite were determined using the method proposed, and the results are listed in Table 4. Considering the dilution of nitrate solution occurred by adding NH_4Cl–EDTA stock solution (250 μL nitrate solution plus 50 μL buffer), a correction coefficient of 5/6 was used in the calculation. The reduction efficiencies for 0.06, 0.20, 0.40 and 1.50 mg L^{-1} of nitrate were 97.32%, 96.85%, 95.18% and 97.02%, respectively, with an average of 96.59 ± 0.96%, indicating that the proposed method had a high and consistent reduction efficiency, as comparable to that determined by a Cu–Cd column.

Using a set of nitrate and nitrite standard solutions, the linearity of the method was established over a range of 0.02–1.50 mg L^{-1} for nitrate and 0.01–1.50 mg L^{-1} for nitrite. A linear calibration of $A = 0.0046 + 1.5416C$ (mg L^{-1}) with $R^2 = 0.9997$ and $A = 0.0162 + 1.9247C$ (mg L^{-1}) with $R^2 = 0.9998$ were obtained for nitrate and nitrite, respectively, concomitant with high sensitivity and good linear responses. This was a significant improvement as compared with conventional Cu–Cd reduction method for nitrate analysis and the Griess diazo-coupling spectrophotometer method for nitrite analysis, both of which are linear over only very narrow concentration ranges. Even compared with the widely used flow injection techniques, the proposed method also had a satisfactory

Table 4
Reduction efficiency of copperised cadmium granules calculated from the ratio of absorbance of the nitrate solution to that of a nitrite solution of the same concentration.

Concentration of nitrate or nitrite (mg L^{-1})	Absorbance of nitrite at 540 nm[a]	Absorbance of nitrate at 540 nm[a]	Reduction efficiency (%)
0.06	0.127 ± 0.003	0.103 ± 0.008	97.32
0.20	0.394 ± 0.005	0.318 ± 0.013	96.85
0.40	0.788 ± 0.006	0.625 ± 0.015	95.18
1.50	2.893 ± 0.029	2.339 ± 0.057	97.02

[a] Values are presented as mean ± SD (n = 6).

Table 5
Determination of nitrite in natural water samples by the proposed method and by the standard method.

Sample	Proposed method (mg L^{-1}, $n = 12$)[a]			Standard method (mg L^{-1}, $n = 3$)[a]		
	Initial	Observed[b]	Recovery (%)	Initial	Observed[b]	Recovery (%)
S1	0.161 ± 0.003	0.337 ± 0.003	98.13	0.161 ± 0.003	0.334 ± 0.003	98.93
S2	0.071 ± 0.002	0.289 ± 0.001	98.88	0.070 ± 0.001	0.286 ± 0.002	99.56
S3	0.075 ± 0.002	0.296 ± 0.003	97.00	0.076 ± 0.002	0.293 ± 0.002	98.14
S4	0.009 ± 0.001	0.263 ± 0.002	97.01	0.008 ± 0.001	0.264 ± 0.002	96.33
S5	0.087 ± 0.003	0.302 ± 0.005	96.99	0.085 ± 0.002	0.302 ± 0.004	96.90
S6	0.010 ± 0.001	0.258 ± 0.003	98.64	0.010 ± 0.001	0.260 ± 0.003	98.15
S7	0.014 ± 0.001	0.258 ± 0.004	99.51	0.014 ± 0.001	0.259 ± 0.003	99.36

[a] Values are presented as mean ± SD.
[b] Addition of 0.5 mg L^{-1} nitrite standard solution to equal volume of original samples.

linear range for nitrate and nitrite analysis (Table S4 in the SD). Furthermore, a reduction efficiency of 96.12% was obtained from the slopes of the two calibration graphs when corrected with a dilution coefficient of 5/6, which was consistent with the average observed reduction efficiency of 96.59% (Table 4).

The detection limit was defined as three times the standard deviation, where standard deviation was measured for an amount near the detection limit itself. Using results obtained for analysis of 0.01 mg L^{-1} NO_3^--N and 0.005 mg L^{-1} NO_2^--N standard solutions ($n = 11$), the limits of detection calculated were 0.004 mg L^{-1} for NO_3^--N and 0.002 mg L^{-1} for NO_2^--N.

Reproducibility was calculated from the relative standard deviations (RSD) for twelve repeated analyses of a standard solution containing 0.3 mg L^{-1} nitrite and 0.6 mg L^{-1} nitrate. These values were found to be 1.10% and 1.51%, respectively.

The microplate reader could read the absorbencies of the 96 wells within 30 s, thus providing a useful advantage for high throughput analysis of larger numbers of samples. Microscale analysis using the microplate reader has also been applied to the determination of nutrients in environmental samples [14,24,25]. In the present study, at least 48 nitrate-containing samples could be simultaneously measured in duplicate within 90 min. For the determination of nitrite alone, only 30 min was required for complete analysis. The sample throughput for nitrate and nitrite analysis by the proposed method was comparable to or better than that of flow injection techniques (Table S4 in the SD). Furthermore, the Cu–Cd granules could be used repeatedly at least six times without any obvious decrease in reduction efficiency, preventing problems with progressive deterioration in reduction capacity, as encountered in column methods. Another important and appealing feature was the very low volume of waste produced by this method compared with the large volumes of waste generated by flow injection techniques and conventional methods. The reduction efficiency of the Cu–Cd granules could be easily checked periodically by analysing a nitrate standard solution followed by a nitrite solution of the same concentration.

These results indicate that the proposed method is an efficient method for the determination of nitrate and nitrite with high sensitivity, precision, accuracy, low limits of detection, low waste generation and over an extensive linear range. The method also has potential to provide high quality data with high temporal and spatial resolution. Such features are attractive for nitrate and nitrite determination in water samples, especially as a suitable tool for analysing a large series of samples.

3.4. *Application to real samples*

The proposed method was applied to the determination of nitrite and nitrate in natural water samples. The samples were collected from different bays of Lake Dianchi (Kunming, China) and its surrounding ponds and rivers, followed by filtration through a 0.45 μm membrane filter to eliminate interference caused by the turbidity of the particles in the samples. Samples in which the concentrations of nitrate or nitrite were beyond the linear range of detection were diluted appropriately.

The recoveries and reproducibility of the proposed method were compared with those obtained for the standard analysis method of phenoldisulfonic acid [26] for nitrate and the Griess diazo-coupling spectrophotometer method [3] for nitrite determination, both of which are commonly used in routine analysis of water quality in China. These results are shown in Tables 5 and 6. The paired-samples *t*-test indicated that there was no difference between the proposed method and the standard methods. The average recoveries of nitrite and nitrate obtained by the proposed method were 96.99–99.51% (mean 98.02 ± 1.04%) and 97.87–102.15% (mean 99.72 ± 1.39%) respectively, matching those obtained by the standard method for nitrite (96.33–99.56%, mean 98.19 ± 1.22%) and nitrate (98.94–101.66%, mean 100.20 ± 1.02%). Reproducibility was evaluated from the relative standard deviations (RSD) of repeated determinations of nitrate or nitrite in real samples containing different contents of NO_2^--N and NO_3^--N. The RSDs obtained for nitrite were < 3.5% when nitrite concentrations were $\geq$ 0.07 mg L^{-1}, or 7–10% when nitrite concentrations were near to 0.010–0.014 mg L^{-1}. Comparable RSD values for nitrate were < 5% when nitrate concentrations were $\geq$0.10 mg L^{-1}, or 6–10% when nitrate concentrations ranged from 0.02 to 0.06 mg L^{-1}. These results indicate that the method has good accuracy associated with small errors during the analysis.

Table 6
Determination of nitrate in natural water samples by the proposed method and by the standard method.

Sample	Proposed method (mg L^{-1}, $n = 6$)[a]			Standard method (mg L^{-1}, $n = 6$)[a]		
	Initial	Observed[b]	Recovery (%)	Initial	Observed[b]	Recovery (%)
M1	0.228 ± 0.009	0.536 ± 0.018	100.16	0.229 ± 0.007	0.538 ± 0.013	99.80
M2	0.607 ± 0.023	0.748 ± 0.020	102.15	0.617 ± 0.017	0.760 ± 0.021	101.40
M3	0.062 ± 0.004	0.447 ± 0.010	97.87	0.064 ± 0.003	0.438 ± 0.008	100.06
M4	2.111 ± 0.096	1.672 ± 0.069	99.66	2.092 ± 0.067	1.651 ± 0.062	100.28
M5	0.035 ± 0.003	0.423 ± 0.011	99.60	0.038 ± 0.003	0.428 ± 0.010	98.94
M6	2.240 ± 0.076	1.773 ± 0.041	98.38	2.277 ± 0.069	1.737 ± 0.045	101.66
M7	1.342 ± 0.036	1.203 ± 0.022	100.22	1.357 ± 0.028	1.223 ± 0.022	99.29

[a] Values are presented as mean ± SD.
[b] Addition of 1.0 mg L^{-1} nitrate standard solution to the original samples in a ratio of 4:6 (v/v).

In addition, when comparing the results obtained by the proposed method with those by the standard method, a linear calibration of $y = 0.9975x + 0.0001$ ($n = 14$) with $R^2 = 0.9999$ for nitrite and $y = 0.9975x + 0.004$ ($n = 14$) with $R^2 = 0.9994$ for nitrate were obtained, where y and x represent the nitrite or nitrate concentrations obtained from the proposed method and the standard method respectively. The slope of 0.9975 suggested a 1:1 relationship between the two methods employed, and the correlation coefficient (R^2) of 0.9998 for nitrite and 0.9994 for nitrate showed a good linear response. These results demonstrated the proposed method had a good reproducibility and accuracy in comparison to the standard methods.

Further investigation, using different amount of real samples to test if the actual samples interfere in the reduction reaction and the diazo-coupling reaction, also verified that there was no interference of real sample upon the proposed method (details are provided in page S1 in the SD, also see Table S3 and Figs. S3 and S4) and that the proposed method was suitable for actual sample analysis.

4. Conclusion

This paper reports a simple, miniaturised photometric method for fast determination of nitrite and nitrate in water samples. The proposed method could simultaneously analyse at least 48 samples in duplicate, with only a 250 μL volume required for each sample, and within 90 min for nitrate and 30 min for nitrite. Nitrate was quantitatively reduced by Cu–Cd granules with high reduction efficiency. A good precision and a good recovery were obtained when the proposed method was applied to actual freshwater sample analysis, and there was no difference when compared with standard methods. All analysing steps, including sample buffering, reduction by Cu–Cd granules, and colour development, were carried out in a 96-well microplate. The main advantages of the proposed method are a high sample throughput, low detection limits (2 and 4 $\mu g\,L^{-1}$ for nitrite and nitrate, respectively), a satisfactory precision and accuracy, only small amounts of reagent needed and the lack of required sample pre-treatment. This method offers a simple, cost-effective and rapid alternative to existing methods for nitrite and nitrate determination.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.06.002.

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