A fast and direct spectrophotometric method for the sequential determination of nitrate and nitrite at low concentrations in small volumes

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Abstract
The use of vanadium (III) has been proposed recently as a suitable alternative to cadmium for the reduction of NO$_3^-$ to NO$_2^-$ during spectrophotometric analysis. However, the methods proposed suffer from decreased sensitivity and additional steps for the measurements of nitrite and nitrate. We have developed an improved fast and sequential protocol that permits the determination of low concentrations of nitrite and nitrate in marine and freshwater samples using small volumes. NO$_2^-$ concentration is firstly determined using the common Griess reaction. The subsequent addition of a 2% VCl$_3$ solution in 6N HCl in the same sample and the reaction at 60ºC for 25 minutes results in an efficient reduction of the NO$_3^-$ to NO$_2^-$ (> 95%), which is also detected by the already added Griess reagents. The method has a detection limit <0.05 µM, a high precision (ranging from 0.2 to 11%) and accuracy (0.07 µM) for the determination of NO$_3^-$ + NO$_2^-$ concentrations lower than 30 µM. Comparison of the proposed method with the established Cd column method using samples from a variety of environments (fresh water reservoir, sediment freeze lysable pore water, estuarine water samples and samples from an acid mine drainage impacted reservoir) showed good agreement between the two methods, with a difference between methods of 0.073 ± 0.099 µM. The analysis can be performed in large batches (~60 samples) using small sample volumes (≤1 mL) for the determination of both NO$_3^-$ and NO$_2^-$ in less than one hour.

Keywords (3-6 alphabetical): Nitrite; Nitrate; Griess reaction; Vanadium (III) chloride; spectrophotometric method
**Introduction**

Nitrate is a key compound in the N cycle of natural ecosystems and artificial environments, being substrate or product of several microbial, plant, and animal metabolic processes. In aquatic environments, nitrate is produced by nitrification in a two-step reaction: ammonium oxidation to nitrite and nitrite oxidation to nitrate. Nitrate can then be assimilated by photosynthetic organisms, thus being an important nutrient for primary production. Nitrate is also consumed in a variety of bacterial processes such as the anaerobic denitrification or dissimilatory nitrate reduction to ammonium (DNRA). Denitrification reduces nitrate to nitrous oxide, a potent greenhouse gas (Lashof and Ahuja, 1990), or molecular nitrogen gas, reducing the nitrogen load of the system, whereas DNRA reduces nitrate to biologically available ammonium that remains in the system (Megenigal et al., 2003). Therefore, the measurement of nitrate and nitrite concentrations in aquatic systems is an important aspect of most studies related to the N cycle in order to determine its production and consumption rates.

Numerous methods for the determination of nitrate are available in the literature. Highly sensitive methods are based on the reduction of nitrate to nitric oxide, which is quantified by chemiluminiscence (Aoki et al., 1997; Braman and Hendrix, 1989), or to nitrous oxide, later quantified by gas chromatography (Christensen and Tiedje, 1988). However, both these techniques require expensive and specialized equipment. Other methods involve the use of strong acids often at elevated temperatures (Mir, 2008; Zhang and Fischer, 2006), which complicates handling and analyses of the samples. In contrast, the simplest and most regularly applied method involves the reduction of nitrate to nitrite and its subsequent measurement by colorimetry using the Griess reaction (Grasshoff et al., 1983; Marzinzig et al., 1997). This method has low detection limit, high accuracy and high specificity without using expensive instruments or complex procedures.

The critical step for the accurate determination of nitrate is its efficient reduction to nitrite. Nitrate reduction to nitrite can be accomplished by specific nitrate reductases (Guevara et al., 1998; Marzinzig et al., 1997) or by the use of different reducing metals, with cadmium being the most commonly used one (Grasshoff et al., 1983; Wood et al., 1967). Although various adaptation of the cadmium reduction method have been proposed in order to increase sample throughput and decrease sample volume required (Harris and Mortimer, 2002; Jones, 1984), the method suffers from various shortcomings; it is time consuming, efficiency of the column varies, a continuous activation of the Cd-column is required, and cadmium is highly toxic (European Chemical Agency: www.echa.europa.eu and Occupational Safety and Health Administration, United States Department of Labor: www.osha.gov) making handling of samples and waste hazardous.

Miranda et al. (2001) described a spectrophotometric method using a vanadium solution (VCl3) for the reduction of nitrate. V(III), which is less toxic than cadmium (European Chemical Agency and Occupational Safety and Health Administration, United States Department of Labor), has been commonly used for the reduction of both nitrate and nitrite at high temperatures (80-90°C) to nitric oxide, then measured by chemiluminescence (Braman and Hendrix, 1989). Miranda et al. (2001) showed that at room temperatures nitrate is reduced to nitrite, which can react with Griess reagents and be measured in a spectrophotometer. However, the proposed protocol resulted in a low molar absorptivity for nitrate, indicating low reaction efficiency in the reduction of NO3− to NO2−. As a result, nitrite highly interferes with the determination of nitrate. Beda and Nedospasov (2005) included an initial step in the method for the elimination of nitrite by the reaction with sulfamic acid, reducing thus the high NO2− interference in the NO3− determination. However, as the subsequent steps involving the reduction of NO3− to NO2− were not modified, the overall efficiency of the reaction was not improved. As a result the precision of the method was lower than that using the classic Cd-columns for nitrate reduction.

We describe here an optimised protocol for the sequential measurement of nitrite and nitrate in small volumes (< 1mL) of the same sample by adjusting factors such as vanadium chloride and HCl concentrations, temperature and time of reaction that improved the efficiency of the nitrate reduction to nitrite and the signal measured. As a result, efficiencies higher than 95% were obtained, resulting in a simple, fast and accurate method for the determination of nitrate and nitrite. The proposed methodology was also compared with the most widely used method for NO3− analysis, the Cd column method) by analysing in parallel samples from different environments (fresh water reservoir, sediment freeze lysable pore water, estuarine water samples and samples from an acid mine drainage impacted reservoir).

**Materials and methods**

**Instrumentation**

Spectrophotometric measurements were performed on Unicam UV/Vis UV2-200 spectrophotometer (Unicam, U.K.) using 1-cm path length quartz cuvettes. The spectrophotometer was equipped with a thermostated cuvette holder regulated by an external water bath. The UV-probe software (Unicam, U.K.) was used to control measurements and record absorbance spectra. Spectrophotometric measurements in 96-well mi-
croplates were performed on a microplate reader Powerwave 340 (Bio-Tek Instruments Inc., USA), using the control program KC JuniorTM Data Analysis software. Incubations of samples were performed in a temperature controlled water bath Unitronic OR (JP Selecta, Spain).

Reagents and standards

Reagents were of analytical purity grade. All solutions and dilutions were prepared with pure water (Milli-Q). Griess reagents for the analysis of NO\textsubscript{2}\textsuperscript{-} were prepared as described in Grasshoff et al. (1983). Sulphanilamide reagent was prepared by dissolving 5.0 g of sulphanilamide in 50 mL of concentrated (12N) hydrochloric acid (HCl) diluted in about 300 mL of pure water and after cooling made up to 500 mL with pure water. N-(1-naphthyl)-ethylenediamine dihydrochloride (NED) reagent was prepared by dissolving 0.5 g NED in 500 mL of pure water (MilliQ). Both reagents were mixed in equal proportions just prior to performing the analysis (hereafter referred as Griess-reagent). Reagents were stored in glass dark bottles and maintained at 4ºC. The reagents are stable for at least one month. NED reagent can be used until a brown discoloration occurs.

Vanadium (III) chloride (VCl\textsubscript{3}) reagent 2% w/v was prepared in a 6N HCl solution (VCl\textsubscript{3}-reagent). The time needed for the complete dissolution varies depending on the concentration used but was generally about 1 hour. The complete dissolution was evidenced by the shift from a turbid to a transparent solution. The solution was finally filtered through a 0.7 µm nominal pore size glass fibre filter in order to eliminate any impurities of the reagent. VCl\textsubscript{3}-reagent was prepared weekly and stored in a dark glass bottle at 4ºC. The VCl\textsubscript{3}-reagent is stable for several months.

Artificial seawater was prepared following the complete salt composition described by Grasshoff et al. (1983).

Stock nitrate standard solutions (10 mM) were prepared by dissolving 1.011 g oven dried (100ºC, 1 h) KNO\textsubscript{3} in 1 L pure water. Stock nitrite solution (10 mM) was prepared by dissolving 0.690 g NaN\textsubscript{3}O\textsubscript{2} to 1 L of pure water. Working solutions were prepared from stock solutions as required by dilution with pure water for all the initial optimization experiments. Standard solutions were prepared in artificial seawater of the appropriate salinity for the salinity effect experiment.

Proposed procedure for the sequential determination of NO\textsubscript{2}\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-}

A 2-step protocol was tested and validated, in order to allow the sequential determination of both NO\textsubscript{2}\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-} in the same sample as follows:

\textbf{Step 1: Nitrite determination}

One mL of sample was transferred into 1.5 mL eppendorf vials followed by the addition of Griess-reagent (50 µL) and gently mixed. Vials were incubated at ambient temperature (~25ºC) for 20 min. Then, 350 µL of this solution (sample + reagents) were transferred into 96-well flat bottom polystyrene microplates and absorbance was measured at 540 nm. NO\textsubscript{2}\textsuperscript{-} concentrations was determined by parallel analysis of a set of NO\textsubscript{2}\textsuperscript{-} standards.

\textbf{Step 2: Nitrite plus Nitrate determination}

A volume (70 µL) of VCl\textsubscript{3}-reagent was added to the remaining sample volume (700 µL) in the eppendorf vials. The vials were closed to prevent evaporation, gently mixed and incubated in a temperature-controlled bath at 60ºC for 25 min. Then, the vials were cooled down to room temperature in a water bath and 350 µL of each sample was transferred into 96-well microplates and the absorbance was measured at 540 nm. Parallel analysis of a set of NO\textsubscript{2}\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-} standards were performed simultaneously for the determination of NO\textsubscript{3}\textsuperscript{-} concentration as described below.

\textbf{Calibration curves}

\textbf{- Nitrite determination (Step 1)}

During step 1 a set of NO\textsubscript{2}\textsuperscript{-} standards is used to determine the concentrations of NO\textsubscript{2}\textsuperscript{-} in the standard/sample using the equation:

\[
\text{ABS}^\text{\textsubscript{NOX}} = \text{S}^\text{\textsubscript{NO2}} \times [\text{NO}_2^\text{\textsubscript{-}}] + \text{ABS}^\text{\textsubscript{reagents}} (1)
\]

Where \text{ABS}^\text{\textsubscript{NO2}} is the absorbance of the NO\textsubscript{2}\textsuperscript{-} standards; \text{S}^\text{\textsubscript{NO2}} is the slope of the calibration curve (ABS \text{µM}^{-1}) for NO\textsubscript{2}\textsuperscript{-}; [NO\textsubscript{2}\textsuperscript{-}] is the NO\textsubscript{2}\textsuperscript{-} concentration, and \text{ABS}^\text{\textsubscript{reagents}} is the absorbance of the reagents, i.e. the intercept of the calibration curve.

\textbf{- Nitrate determination (Step 2)}

In the samples, where both NO\textsubscript{2}\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-} are present, it is necessary to discriminate between the contributions from the two compounds. After the reaction with VCl\textsubscript{3}-reagent, the measured absorbance (ABS\textsubscript{NOX}) is a combination of the individual contribution of each compound (NO\textsubscript{2}\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-}) plus the absorbance of the reagents, i.e.:

\[
\text{ABS}^\text{\textsubscript{NOX}} = \text{ABS}^\text{\textsubscript{NO2}} + \text{ABS}^\text{\textsubscript{NO3}} + \text{ABS}^\text{\textsubscript{reagents}} (2)
\]

The use of NO\textsubscript{2}\textsuperscript{-} standards and NO\textsubscript{3}\textsuperscript{-} standards allow performing a calibration of the complete reaction in the presence of VCl\textsubscript{3}-reagent, being:

\[
\text{ABS}^\text{\textsubscript{NO2}} = \frac{\text{ABS}^\text{\textsubscript{NOX}} - \text{ABS}^\text{\textsubscript{reagents}}}{\text{S}^\text{\textsubscript{NO2}} (3)}
\]
\[
\text{ABS}^V_{\text{NO}_3} = S^V_{\text{NO}_3} \times [\text{NO}_3] + \text{ABS}^V_{\text{reagents}} \quad (4)
\]

where, \(\text{ABS}^V_{\text{NO}_2}\) and \(\text{ABS}^V_{\text{NO}_3}\) are the absorbance of the \(\text{NO}_2^-\) and \(\text{NO}_3^-\) standard, respectively; \(S^V_{\text{NO}_2}\) and \(S^V_{\text{NO}_3}\) are the slope of the calibration curves (\(\text{ABS} \mu \text{M}^{-1}\)) for \(\text{NO}_2^-\) and \(\text{NO}_3^-\), respectively; \([\text{NO}_2^-]\) and \([\text{NO}_3^-]\) are the \(\text{NO}_2^-\) or \(\text{NO}_3^-\) concentration of the standards, respectively and \(\text{ABS}^V_{\text{reagents}}\) is the absorbance of the reagents without \(\text{NO}_2^-\) or \(\text{NO}_3^-\), i.e. the intercept of the calibration curve.

The measured absorbance is a combination of the individual contribution of each compound plus the absorbance of the reagents, i.e. the combination of Equations 3 and 4:

\[
\text{ABS}^V_{\text{NOX}} = S^V_{\text{NO}_3} \times [\text{NO}_3^-] + S^V_{\text{NO}_2} \times [\text{NO}_2^-] + \text{ABS}^V_{\text{reagents}} \quad (5)
\]

The actual \(\text{NO}_3^-\) concentration of the sample is calculated as:

\[
[\text{NO}_3^-] = (\text{ABS}^V_{\text{NOX}} - \text{ABS}^V_{\text{reagents}} - S^V_{\text{NO}_2} \times [\text{NO}_2^-]) / S^V_{\text{NO}_3} \quad (6)
\]

Where: \([\text{NO}_3^-]\) is the \(\text{NO}_3^-\) concentration determined in the step 1 and \([\text{NO}_2^-]\) is the \(\text{NO}_2^-\) concentration of the sample.

Environmental samples

A set of environmental samples were collected from different environments and analysed both with the traditional cadmium reduction method on a TRAACS 800 Technicon autoanalyser using standard protocols (Grasshoff et al 1983) and with the protocol described here. Estuarine water column surface samples were collected along a salinity gradient (salinity range 6-26) in the inner Gulf of Nicoya, Costa Rica in July 2011 (Seguro et al. in prep). Freshwater water column samples (0-16 m depth, salinity 0) were collected from the Bornos water reservoir, SW Spain, in August 2010 (Romero-Martínez et al., 2013). Water column samples from an experiment using sediment cores from an acid mine drainage affected water reservoir, Sancho reservoir, SW Spain, were collected in December 2011 (Torres et al. 2013) to test for the effect of high metal concentrations. Water samples were filtered by 0.7 µm glass fibre filters and stored frozen at -20°C until analysis. Pore water nutrient samples extracted from frozen sediment cores (Freeze Lysable Inorganic Nutrients, FLIN) (0-3.5 cm depth, salinity 32-40) were collected from the Rio San Pedro tidal creek, SW Spain, in March 2013, using the procedure described in García-Robledo et al (2010). FLIN samples were stored frozen until analysis. Samples were aliquoted in triplicate and analysed with each method.

Results & Discussion

Effect of temperature, \(VCl_3\) and \(HCl\) concentration

Maximum absorbance and the corresponding minimum time needed to reach it as a function of varying \(VCl_3\) and \(HCl\) concentrations are represented in Figure 1. For \(VCl_3\), maximum absorbance was obtained when concentrations were equal or higher than 1%, being maximum at 2% (Fig. 1A). In order to follow the effect of temperature on colour development, the cuvette holder of the spectrophotometer was maintained at a constant temperature while the absorbance was measured every 5 minutes.

Temperature had a significant effect, with absorbance being highest at 40°C and lowest at 80°C (Fig. 1A). However, the combined effects of temperature and \(VCl_3\) concentration strongly influenced the reaction time (Fig. 1C). Although the maximum colour development was measured at 40°C, reaction times exceeded 2 hours, reducing its application for daily routine analysis. We also tested lower temperatures such as the ambient temperature (25 ºC) which was used in the protocols by Beda and Nedospasov (2005) and Miranda et al. (2001); however, reaction times exceeded 5 hours (data not shown), and therefore temperatures lower than 40°C were not considered during subsequent tests. In contrast, temperatures of 50-70°C combined with 2% \(VCl_3\) solution had high absorbances with reaction times <1 hour. The increase in temperature from 60 to 70°C did not affect the performance of the reaction substantially, so further comparisons were done between 50 and 60°C.

We also tested for the effect of \(HCl\) concentration on the reaction. Maximum absorbance was measured when the concentration was higher than 4 N (maximum at 6-8 N) (Fig. 1B). Reaction time was also influenced by \(HCl\) concentration, decreasing exponentially to reaction time less than 30 min at concentrations ≈6N \(HCl\) (Fig. 1D). Therefore, a concentration of 6N \(HCl\) was determined as the minimum concentration producing the maximum signal with the minimum reaction time. As a consequence, the conditions selected based on the current setup that resulted in the most efficient reaction overall was a solution of 2% \(VCl_3\) in 6N \(HCl\) and a reaction temperature of 60°C.

Optimization of the temperature and reaction time for laboratory use

The dependence of the maxima signal and reaction time on temperature was further tested using a temperature controlled water bath. This heating method is the most common procedure in laboratories and the heat transference efficiency is higher compared to that of the spectrophotometer’s cuvette holder. At the highest tem-
temperatures (70 and 80°C), V(III) reduces nitrate in 5 minutes or less (Braman and Hendrix, 1989) and the produced nitrite reacts quickly with Griess reagent resulting in maximum signal in the first measurement (5 min incubation) (Fig. 2A). However, the addition of reducing compounds has been proved to interfere in complex manners with the Griess reaction (Lebaron et al., 2002), resulting in a progressive reduction of molar absorption with time. In contrast, at 40°C the reaction was slow, resulting in reaction times exceeding 2 hours. At 50 and 60°C, maximum responses were obtained after 40 and 15 min of incubation, respectively, and maintained constant for further 15 min of incubation (Fig. 2A). Considering that, the maximum molar absorptivity was obtained after 25 min of incubation at 60°C, 5% higher than the value obtained at 50°C, the optimal conditions chosen for the method in the previous tests were confirmed.

Optimization of the reagent volume

Reagent volumes were adjusted to produce the minimal dilution of the sample and obtain the maximum molar absorbance. The maximum efficiency was obtained using 100 µL 2% VCl₃-solution per mL of sample (Fig. 2B). With lower volumes, VCl₃ concentration was probably too little to reduce the entire nitrate in the sample within 25 min. In contrast, an excess of VCl₃ favoured the further reduction of nitrite to nitric oxide, resulting in a decrease of the measured signal. On the other hand, Griess-reagent volumes over 25 µL resulted in similar signal, being maxima with the addition of 50 µL per mL of sample (Fig. 2B).

Limit of detection, measuring range and stability

The optimized conditions selected were tested in order to define the measuring range, detection limit and accuracy of the method (Fig. 3). When just NO₃⁻ was present, reaction was linear between 0-40 µM NO₃⁻ (Fig. 3A), similar to the one obtained by cadmium re-
action columns (Wood et al., 1967), and within the range of nitrate concentrations commonly found in unpolluted aquatic environments.

The procedure showed a high precision. The standard deviation of the 6 standard replicates used for the calibration shown in Figure 3B ranged from 0.01 to 0.11 µM, which mean a coefficient of variation between 0.2 to 11%. The limit of detection (LOD) was calculated as 3 times the standard error of the intercept divided by the slope of the calibration as defined by Konieczka and Namiesnik (2009), resulting in a value as low as 0.04 µM NO₃⁻.

As shown above, the reduction of NO₃⁻ and NO₂⁻ with V(III) progresses rapidly at high temperatures, resulting in gradual decrease of the signal after 30-35 min at 60°C (Fib 2A). However, at slightly lower temperatures (e.g. 40°C), the reaction slows down significantly, requiring several hours to reach maximum absorbances (Fig. 2A). Therefore, it is suggested that the reaction time should be controlled carefully to be 20-30 min at 60°C and then the samples to be cooled down to ambient temperature (25°C). In this way, the signal remains stable for at least 1 hour, slowly decreasing with time thereafter (Fig. 3C). This procedure facilitates the analysis of a large number of samples simultaneously without the need to measure the absorbances of the samples immediately.

**Salt effect**

A slight decrease in the molar absorbance was observed with increasing salinities in the determination of NO₃⁻ using the common procedure of the Griess reaction (ABS = 94.163 + 5.822 * exp (-0.120 + salinity), r = 0.958) (Fig. 4), in contrast to previous suggestions (Grasshoff et al., 1983). Norwitz & Kelker (1985) also reported a salt effect but at much higher concentrations than the ones used here and for individual salts. The effect of salinity on the determination of NO₃⁻ was the same when VCl₃ was present in the reaction (ABS = 93.033 + 7.104 * exp (-0.088 + salinity), r = 0.984). The salt effect remained stable in salinities between 20 and 50, producing a molar absorbance about 94% of the value obtained in distilled water. A stronger effect was observed during NO₃⁻ determination in the presence of VCl₃, with the molar absorbptivity decreasing to 85% of the value obtained in distilled water at salinities between 30 and 50 (ABS = 82.519 + 17.469 * exp (-0.055 + salinity), r = 0.998). Therefore, it is suggested that the calibration curves should be performed using standards at salinities similar to those found in the samples to be analyzed.

**Nitrite interference**

Environmental samples of marine and freshwater systems primarily contain NO₃⁻, with NO₂⁻ being either absent or present at low concentrations. However, there are aquatic environments such as marine coastal areas and Oxygen Minimum Zones or fresh water hypolimnion where both NO₃⁻ and NO₂⁻ concentrations can increase and therefore both compounds should be measured accurately. The protocol described by Miranda. et al. (2001) described a fast and simple spectrophotometric protocol using VCl₃ for NO₃⁻ reduction. However, they reported a high interference from NO₂⁻, with the error in the NO₃⁻ determination being proportional to the NO₂⁻ concentration. As a result, the error in a sample with higher concentrations of NO₂⁻ than NO₃⁻ could be superior than the actual NO₃⁻ concentration (Beda and Nedospasov, 2005).

After the complete reaction with VCl₃, both the initial NO₂⁻ and NO₃⁻ from the reduction of NO₃⁻ will con-
Figure 3. (A) Linear range, (B) low concentration calibration with the calculation of the limit of detection (LOD) and (C) decrease of the molar absorptivity over time at ambient temperature (25°C) expressed as percentage of the initial. Reactions were carried out for 25 minutes at 60°C using 1mL NO$_3^-$ standards (n = 3 - 6) + 50 µL Griess reagent + 100 µL VCl$_3$-reagent and absorbances measured using 1 cm cuvettes. Values are means ± SE.

The contribution of each compound to the total absorbance depends on a number of factors. The addition of the VCl$_3$-reagent itself produces a slight dilution (1:1.1) of the usual Griess reagent/sample volume ratio, resulting in a decrease of the expected NO$_3^-$ molar absorptivity. In addition, the azo dye signal decreases with time likely caused by interference from the VCl$_3$ reagent (Lebaron et al., 2002), which also reduces the NO$_2^-$ molar absorptivity with time (Fig. 2). Thus, NO$_2^-$ molar absorptivity, as measured using solely NO$_2^-$ standards, decreases from a value of 0.042 to 0.036 ABS µM$^{-1}$ after the addition of VCl$_3$-reagent (Fig. 5A). This value was slightly lower than the theoretical decrease produced by dilution due to reagents addition (0.038 ABS µM$^{-1}$), emphasising the need to determine the effect of VCl$_3$-reagent addition to the azo dye signal obtained in step 1 using a set of NO$_2^-$ standards in parallel to those of NO$_3^-$.

The efficiency of the NO$_3^-$ reduction to NO$_2^-$ will also influence the measured signal; part of the NO$_3^-$ may not react, the NO$_2^-$ produced could further react with VCl$_3$ or the VCl$_3$ reagent could interfere with the azo dye production or signal, all resulting in efficiencies lower than 100%. Indeed, the molar absorbance obtained for NO$_3^-$ was slightly lower than that for NO$_2^-$, but the efficiency of the reaction was always higher than 90% (94% in the example shown in Fig. 5A) during the tests of the method.

The method developed by Miranda. et al. (2001) achieved much lower molar absorbivities for NO$_3^-$ that the ones observed in the present method due to 1) the larger volume of the reagents used by these authors, resulting in a 1:2 dilution of the sample and 2) a much lower conversion efficiency. The molar absorbities obtained were 0.0089 ABS µM$^{-1}$ which, after consider-
Figure 5. (A) Calibration curves obtained in the sequential measurement of NO$_2^-$ and NO$_3^-$ for NO$_2^-$ after step 1 (standard + Griess-reagent) and separately for NO$_2^-$ and NO$_3^-$ after step 2 (sample + Griess-reagent + VCl$_3$-reagent, 25 minutes reaction at 60ºC). Standard error was <0.0003 for the slopes and <0.003 for the intercepts. (B) NO$_3^-$ calibrations curves (0 to 20 µM) measured in the presence of varied NO$_2^-$ concentrations (0 to 20 µM). Measured absorbance is represented as dots. Solid lines represent the calculated values using the calibration curves shown in (A) and equation 4 of the text.

In contrast, the procedure proposed here achieves a similar absorbance signal for both compounds, resulting in minimal NO$_2^-$ interference and high accuracy. In addition, the signal for each of the NO$_3^-$ and NO$_2^-$ is obtained separately during the calibration, and is constant during each analysis regardless of the NO$_2^-$:NO$_3^-$ ratio present in the sample. This was confirmed by the low error (= 0.5 µM) measured in the determination of NO$_3^-$ in standards with a range of NO$_2^-$ concentrations (Fig. 5B). Nitrate was, however, underestimated when the sum of NO$_2^-$ and NO$_3^-$ (NO$_X^-$) concentrations exceeded 30 µM, which is higher than the upper limit for the measurement of NO$_2^-$ (Grasshoff et al., 1983). Therefore, for measurements of NO$_X^-$ above 25 µM, especially with high NO$_2^-$ concentrations (>30% NO$_X^-$), samples should be diluted in order to obtain an accurate NO$_3^-$ measurement.

We have performed the complete procedure for large series of samples (>60 samples) for the determination of both NO$_2^-$ and NO$_3^-$ in the same water sample (=1 mL) in less than one hour. The method can also be adapted to be performed in its entirety on the same microplate by first taking the absorbance reading for NO$_2^-$ after step 1, heating on a microplate and for NO$_3^-$ after step 2. Thus, sample volumes <300 µL (250 µL sample + 12.5 µL Griess reagent + 25 µL VCl$_3$-reagent) are utilised, making it especially suitable for analysis of sediment porewater samples.

**Comparison of cadmium column and vanadium methods**

Contribution of NO$_2^-$ to NO$_X^-$ in the measured samples ranged from 0.2-32.1% (median 5.1%). Samples were diluted appropriately (dilution factor range 1-12) to obtain NO$_X^-$ concentrations lower than 30 µM. Our diluted samples with a high NO$_2^-$ contribution showed NO$_X^-$ concentrations lower 20 µM. Comparison of the classical cadmium column reduction method and the protocol proposed here over a range of salinities (0-40) and type of samples (i.e. freshwater reservoir, acid mine drainage affected reservoir, estuarine, sediment freeze lysable) gave an excellent agreement between the two methods (Fig. 6). Paired t-test showed that the results from the two methods are homogenous (t = 0.736, p = 0.472, n = 17). Using the Cd column method as the reference methodology, the accuracy of the proposed method with VCl$_3$ could be calculated as the difference between the measured samples (Konieczka and Namiesnik, 2009), resulting in an accuracy of 0.073 ± 0.099 µM. These independent determinations show that the vanadium reduction method using spectrophotometry does not bias the results. Furthermore, 60 samples were analyzed for both NO$_2^-$ and NO$_3^-$ in less than 1 h by the vanadium method without the need of a specially trained technician and an expensive autoanalyser system by the automated cadmium column reduction method.
Spectrophotometric method for nitrate and nitrite determination

Conclusion

We present a protocol for the sequential measurement of low NO$_2^-$ and NO$_3^-$ concentrations in both freshwater and marine samples based on an initial detection of NO$_2^-$ with Griess reagents followed by the reduction of the NO$_3^-$ to NO$_2^-$ with VCl$_3$ and subsequent detection with the excess Griess reagent present. The method has a detection limit < 0.05 µM and a high accuracy and precision in the determination of both NO$_2^-$ and NO$_3^-$ for combined concentrations lower than 30 µM. The procedure does not require specialized equipment, expensive reagents or tedious procedures. The method allows the fast analysis of large series of samples using low volumes, such as those of sediment porewater profiles.

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