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A simple automated method for the speciation of dissolved inorganic nitrogen in seawater

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Abstract

A new flow injection analysis (FIA) method using spectrophotometric detection has been developed for the simultaneous determination of ammonium, nitrite and nitrate in marine waters. The method is based on absorbance measurement of azo dye obtained by the reaction of nitrite with *N*-(1-naphthyl)ethylenediamine dihydrochloride and sulfanilamide. Ammonium was previously oxidized to nitrite by hypochlorite in the presence of large amount of potassium bromide, while nitrate was reduced to nitrite in a copperized cadmium column. The conditions of the former reaction have been studied and its continuous application in seawater has been optimized. With a single sample injection three signals were obtained, corresponding to the concentration of nitrite ($N\text{-}NO_2^-$), nitrite plus ammonium ($N\text{-}NO_2^- + N\text{-}NH_4^+$) and nitrite plus nitrate ($N\text{-}NO_2^- + N\text{-}NO_3^-$). The detection limit for each species was 21 $\mu g\,l^{-1}$ $N\text{-}NO_2^-$, 26 $\mu g\,l^{-1}$ $N\text{-}NH_4^+$ and 32 $\mu g\,l^{-1}$ $N\text{-}NO_3^-$. This method was applied to both synthetic and real samples of seawater, giving average relative errors of 3.2, 4.9 and 4.0% for NO_2^-, $N\text{-}NH_4^+$, and $N\text{-}NO_3^-$, respectively. The present method allows for the simultaneous determination and speciation of nitrogenous with a single injection, in a simple way and a high sampling rate (10 samples h^{-1}). Besides, the method enables the monitoring of nitrogen species in marine ecosystems.

Keywords: Nitrogen; Ammonium; Nitrite; Nitrate; Seawater; FIA; Simultaneous determination; Nutrients monitoring; Flow injection

1. Introduction

Ammonium (1–50 μM), nitrite (0.1–50 μM), and nitrate (1–500 μM) are the three principal forms of dissolved inorganic nitrogen in seawater and they play important roles in marine ecosystems [1]. The introduction of sewage and fertilizers has caused increases in concentrations of these nutrients in coastal waters. Furthermore, in recent years marine aquaculture has been also described as source of high nutrient concentrations in coastal environments [2,3]. Due to these anthropogenic inputs, perturbations on the nitrogen cycle can occur, with the subsequent alteration of marine productivity.

High concentrations of nitrate can originate from nitrification processes or directly from sewage or fertilizers, and may lead to water eutrophication, especially in areas with poor water renovation. The sources of ammonium are mainly fertilizers and marine aquaculture. An increase in its concentration may increase concentrations of ammonia, which is toxic for fishes, and nitrate via nitrification. Nitrite, which is also toxic for fishes, is an intermediate species in the nitrogen cycle.

Nitrite, nitrate and ammonium in seawater are usually quantified by spectrophotometric methods. Nitrites can be analyzed by the Griess method, which

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is based on the measurement of the absorbance of azo dye formed by the reaction with sulfanilamide hydrochloride and *N*-(1-naphthyl) ethylenediamine dihydrochloride [4–8].

Nitrates can be quantified in seawater via its reduction to nitrites by copper-coated cadmium granules. Determination proceeds by the spectrophotometric reaction described for nitrites. This indirect method allows for the simultaneous determination of nitrite and nitrate in a seawater sample [9].

Ammonium is analyzed in seawater, as the sum of NH_4^+ and NH_3, measuring the absorbance of indophenol. The reaction, first described by Berthelot [10], can be catalyzed by, e.g. nitroprusside [11]. The method was first applied to the determination of ammonia in seawater by Sagi [12].

Although the continuous determination of nutrients in seawater has been studied by many authors [13–15], the works dealing with the simultaneous determination of more than one nutrient are very scarce, nitrite and nitrate being the couple of nutrients mostly studied [14]. The simultaneous determination the three forms of inorganic nitrogen in seawater has not been described. This analysis may potentially be performed by the Griess method if nitrate and ammonium are converted to nitrite by reduction and oxidation, respectively. While the reduction of nitrate is well established, the oxidation of ammonium to nitrite in seawater has not been reported.

Richards and Kletsch [16] described the oxidation of ammonium to nitrite by reaction with hypochlorite in the presence of potassium bromide. The reaction was later applied to non-saline waters by using reversed flow injection analysis (r-FIA) [17]. In this work, we have studied its application to the oxidation of ammonium in seawater. Our method enables to determine the concentrations of ammonium, nitrite and nitrate in seawater with a single sample injection.

2. Experimental section

2.1. Apparatus

The FIA manifold (see Fig. 1) consisted of two peristaltic pumps: an eight-path Perimax 12 (Spetec, Germany) and a four-path Minipuls 3 (Gilson, France) equipped with Tygon tubing, which were used to control the flow of reagents and sample solutions. A higher flow rate for reagent R5 was needed, making a second pump necessary (P2). A Model 1106 injection valve (Omnifit, UK) and a Lambda 11 UV-Vis spectrophotometer (Perkin-Elmer, Germany) equipped with a quartz flow cell with a 10 mm pathlength (Hellma, Germany) completed the manifold.

Transport lines and reaction coils were made of 0.8 mm i.d. PTFE tubing (Omnifit, UK), and connections were made of polypropylene (Omnifit, UK).

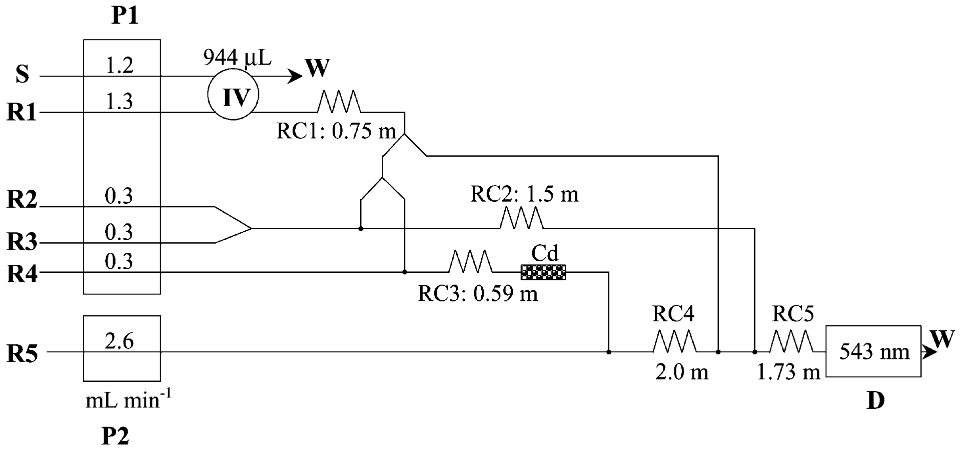

Fig. 1. Flow injection system for simultaneous determination of nitrite, ammonium and nitrate in seawater. S: sample; P1/P2: peristaltic pumps; R1–R5: reagents; IV: injection valve; RC1–RC5: reaction coils; D: detector; W: waste. Optimum conditions as indicated.

Nitrate was reduced to nitrite with 15 g cadmium granules (0.3–1.6 mm diameter), copperized according to Rodier [18], and packed into a glass chromatographic column (100 mm × 25 mm i.d.) with adjustable end-pieces plugged with 25 mm polyethylene frits (Omnifit, UK).

2.2. Reagents and solutions

Stock solutions of 100 mg l^{-1} N-NO_2^-, 100 mg l^{-1} N-NO_3^- and 100 mg l^{-1} N-NH_4^+ were prepared by dissolving $NaNO_2$, KNO_3 and NH_4Cl (Merck, Germany), respectively. Further dilutions were made daily, and a salinity of 35 g l^{-1} was maintained using synthetic seawater according to Grasshoff et al. [9]. Optimization experiments were performed with solutions containing 400 μg l^{-1} of each nitrogen species. The reagents were prepared daily as follows: reagent R1: 2.5% w/v sodium citrate (Scharlau, Spain); reagent R2: 0.008 M NaClO (Scharlau, Spain); reagent R3: 15% KBr (Merck, Germany) in 4 M NaOH (Merck, Germany); reagent R4: 2.4 M KCl (Merck, Germany) and 0.05 M EDTA disodium salt at pH 8.5 (Merck, Germany); reagent R5: 0.06 M sulfanilamide (Merck, Germany), 5% HCl (Merck, Germany), 0.5% polyvinyl alcohol (Sigma) and 0.05% *N*-(1-naphthyl)ethylenediamine dihydrochloride (Merck, Germany). All reagents, except sodium hypochlorite (pure) were of analytical grade.

2.3. Procedure

The FIA manifold was assembled to simultaneously determine the three nitrogenous inorganic compounds (see Fig. 1). Seawater (S) was injected into a sodium citrate carrier solution (R1) to avoid the precipitation of calcium and magnesium present in seawater. After homogenization in a reaction coil (RC1), the sample was divided in two streams. One was directly treated with the color-forming reagent (R5) and passed through the detector, where the increase in absorbance at 543 nm originates a first peak, used to measure the concentration of nitrite in the sample. The second portion of the sample was then divided in two streams. One was mixed with sodium hypochlorite solution (R2) and potassium bromide solution (R3) and mixed in a reaction coil (RC2), where ammonium was oxidized to nitrite, which reacts with colorimetric reagent R5, giving a second peak corresponding to the concentration of ammonium plus nitrite in the sample. The last stream was mixed with potassium chloride and EDTA (R4) and then passed through the copper-coated cadmium packed column, where nitrate was reduced to nitrite. After colorimetric reaction with R5, the sample was subsequently passed through the spectrophotometer giving a third peak proportional to the concentration of nitrite plus nitrate in the sample. The concentrations of ammonium and nitrate were determined from second and third peak heights, respectively, by subtracting the concentration of nitrite, obtained from the height of first peak. All samples were analyzed in triplicate.

3. Results and discussion

3.1. Oxidation of ammonium

Prior to the simultaneous determination of the three nutrients, we examined the conditions allowing the continuous oxidation of ammonium in seawater and its determination as nitrite. FIA manifold shown in Fig. 2 was used to study the single determination of ammonium in seawater after its oxidation to nitrite. The figure also shows the sample volume, flow rates and reaction coils lengths used. For initial chemical conditions, we used those described by Liu et al. [17] for the determination of ammonia in non-saline waters by r-FIA based on its oxidation to nitrite, i.e. we generated the oxidant solution by on-line mixing 0.1 M sodium hypochlorite (R1) with a solution of 15% potassium bromide in 4 M sodium hydroxide (R2). Reagents R3 and R4 contained sulfanilamide and *N*-1-naphtylethylenediamine, respectively. In these conditions, we observed turbidity that caused instability in the response. Liu et al. found this turbidity, but they did not describe any adverse effect, probably because of the small volume of reagent injected in an r-FIA system. In our case, to eliminate this turbidity, we varied the concentration of hypochlorite in the oxidant solution within the range 0–0.15 M. The results obtained are shown in Fig. 3, where an increase of the signal can be observed up to 0.08 M hypochlorite. For higher concentrations, slight turbidity (0.1 M) or even strong precipitation (0.15 M) appeared. Thus, 0.08 M sodium hypochlorite

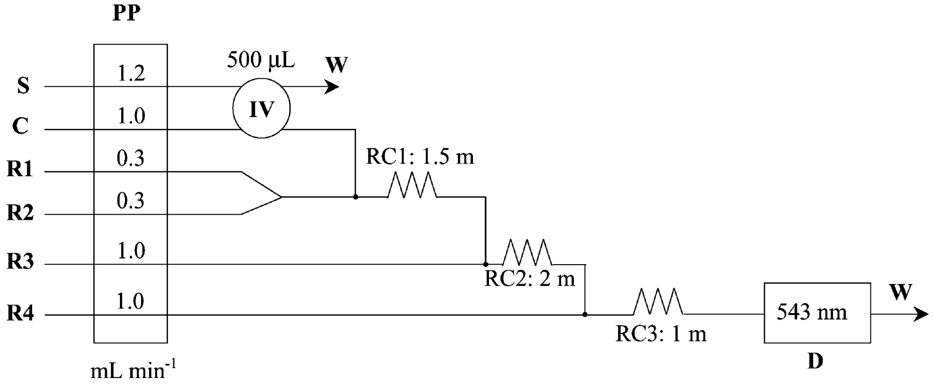

Fig. 2. Flow injection system used for the oxidation and determination of ammonium. PP: peristaltic pump; S: sample; C: aqueous carrier; R1–R4: reagents; IV: injection valve; RC1–RC3: reaction coils; D: detector; W: waste. Operational conditions as indicated.

solution was used henceforth. At these conditions, calibration plot was constructed within 0–800 $\mu g\,l^{-1}$ N-NH_4^+, with a slope of 1.82×10^{-4}, a y-intercept of -1.45×10^{-5} and a regression coefficient of $r^2 = 0.999$. The limit of detection was 19 $\mu g\,l^{-1}$ N-NH_4^+, calculated as $3s_b/m$, where s_b represents the standard deviation of the y-intercept and m represents the slope of the calibration straight line.

3.2. Simultaneous determination of ammonium, nitrite and nitrate

For a proper performance of the determination system, two requirements need to be met: maximization of the signal to increase sensitivity, and correct separation of the three peaks to allow their measurement. For this reason, the optimization of the FIA manifold

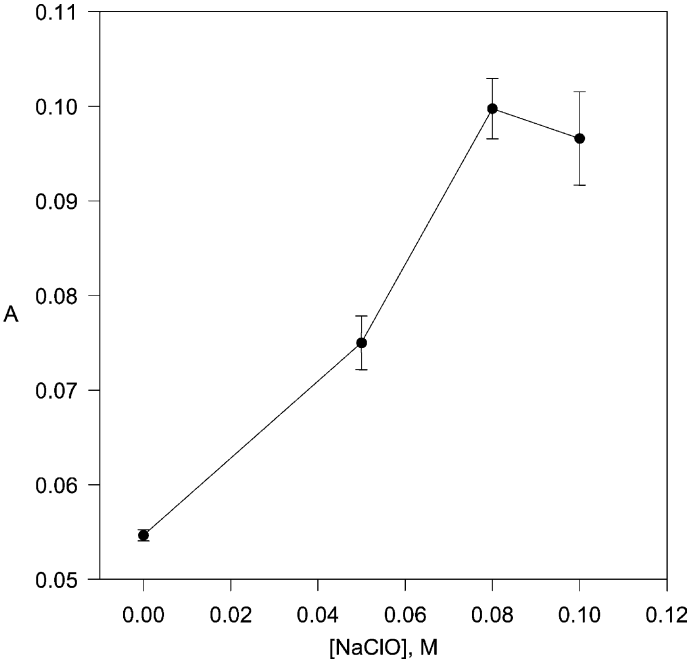

Fig. 3. Influence of hypochlorite concentration on the oxidation of ammonium. Error bars indicate standard deviations.

was done in two steps: first, the reagent flow rates and the lengths of the reaction coils RC2–RC4 were varied to get correct peak separation. Second, the sample injection volume and the lengths of RC1 and RC5 were varied to obtain maximum absorbance. The complexity of the manifold recommended the use of an univariate optimization method.

3.2.1. Optimization of peaks separation

The separation of the three peaks was controlled by the reagent flow rates and the length of the reaction coils RC2–RC4 (see Fig. 1). These variables were changed to obtain the best situation as a compromise between time and peaks separation. The conditions selected for a correct quantification of the signals are shown in Fig. 1. Thus, after a single sample injection, three peaks appeared at 30 s (nitrite), 2 min 30 s (nitrite plus ammonium) and 5 min (nitrite plus nitrate). The delay between sample injections was 6 min, which allowed the system to equilibrate and obtain an original baseline level. At these conditions, up to 10 samples h^{-1} could be analyzed.

3.2.2. Optimization of method sensitivity

The influence of sample injection volume (316–1126 μl) and the length of reaction coils RC1 (0.01–1.75 m) and RC5 (0.73–2.36 m) on the intensity of the three signals were also examined. To reach the best operational conditions, we used an univariate method, where one variable is varied by maintaining constant the rest.

The effect of different sample injection volumes is shown in Fig. 4. It was found that the absorbance increased gradually with increasing injection volume. To select the optimum value, a curve of weighted response function was constructed by giving each signal a weight inversely proportional to its sensitivity. The response function, RF, was calculated as follows:

$$\mathrm{RF} = \frac{A_1 + 3A_2 + 5A_3}{3}$$

where A_1, A_2 and A_3 represent the three successive absorbance signals of the peaks used to quantify nitrite, ammonium and nitrate, respectively. As optimum volume, 944 μl was selected. Finally, the influence of the

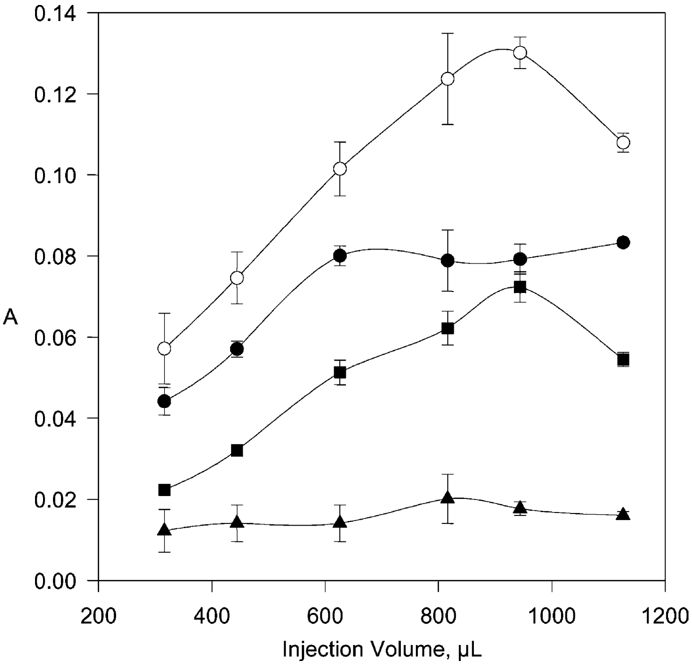

Fig. 4. Dependence of absorbance on injection volume. N-NO_2^- (●); N-NO_2^- + N-NH_4^+ (■); N-NO_2^- + N-NO_3^- (▲); response function (○); RC1: 0.01 m; RC5: 0.73 m. Error bars indicate standard deviations.

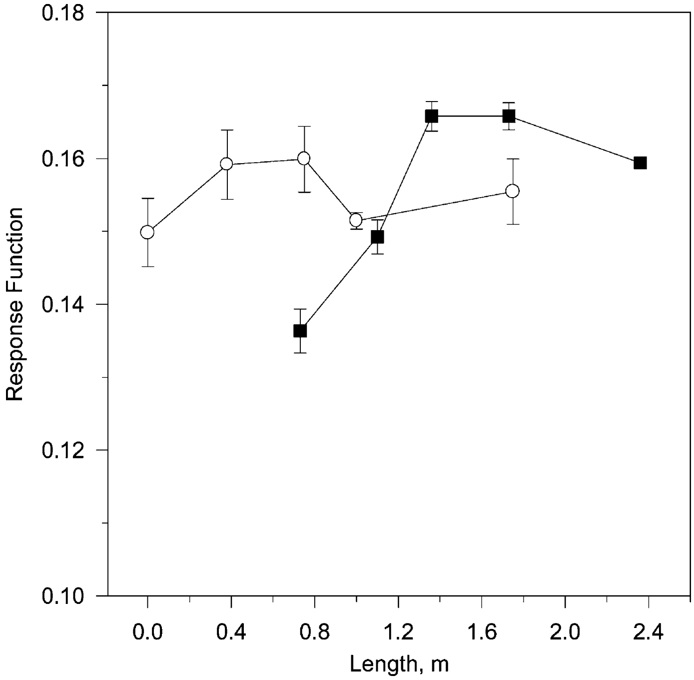

Fig. 5. Dependence of response function on reaction coils lengths. RC1 (○); RC5 (■); injection volume: 944 μl. Error bars indicate standard deviations.

length of the reaction coils RC1 (0.01–1.75 m) and RC5 (0.73–2.36 m) was studied. The results of the RF values are plotted in Fig. 5. The variations in RF were very similar for both coils. After an initial increase in the signals caused by a better mixing of sample and reagents, a maximum was reached and then, a decrease of signal was observed due to the higher dispersion of the sample into the reaction coils. Coils of 0.75 m (RC1) and 1.73 m (RC5) were selected as optima.

The best operational conditions were selected at injection volume, 944 μl, length of the reaction coil RC1 = 0.75 m, and RC5 = 1.73 m. At these conditions, calibration curves were constructed for each analyte to determine the linear range for each signal. Table 1 shows the features of the method. Higher linear ranges for ammonium and nitrate are due to both higher dilution and dispersion affecting to these nutrients. The limits of detection were calculated as three times the standard deviation of the y-intercept divided by the slope of the straight line. As nitrate and ammonium are measured together with nitrite, their detection limits are dependent of the nitrite concentration in the sample. The limits of detection reported are calculated assuming the absence of nitrite in the samples. Increases in nitrite concentrations imply a decrease in detection limits for both ammonium and nitrate. The sensitivity and the determination ranges were acceptable for all samples within the concentration ranges of nitrogen in most marine environments including those affected by nutrient

Table 1
Characteristics of the proposed method

Species	Intercept	Slope	r^2	Linear range (mg l^{-1})	Limit of detection (μg l^{-1})
N-NO_2^-	−0.029	2.09×10^{-4}	0.999	0.05–1.0	21
N-NH_4^+	−0.030	7.68×10^{-5}	0.999	0.06–2.0	26
N-NO_3^-	0.004	3.27×10^{-5}	0.990	0.08–2.0	32

Table 2
Results of nitrite, ammonium and nitrate analysis in seawater samples

Sample	$N\text{-}NO_2^-$ $(\mu g\,l^{-1})$				$N\text{-}NH_4^+$ $(\mu g\,l^{-1})$				$N\text{-}NO_3^-$ $(\mu g\,l^{-1})$			
	Reference	This method	ε_r[a]	t_{exp}[b]	Reference	This method	ε_r[a]	t_{exp}[b]	Reference	This method	ε_r[a]	t_{exp}[b]
1	100	99 ± 2	−1.0	0.69	160	186 ± 13	16.2	3.52	200	212 ± 18	6.0	1.20
2	80	88 ± 3	10.0	3.48	400	397 ± 6	−0.8	0.72	200	213 ± 31	6.5	0.76
3	180	197 ± 7	9.4	4.20	520	514 ± 11	−1.2	0.87	100	99 ± 9	−1.0	0.12
4	0	n.d.[c]	–	–	220	226 ± 11	2.7	0.91	0	n.d.[c]	–	–
5	225	232 ± 3	3.1	3.59	0	n.d.[c]	–	–	0	n.d.[c]	–	–
6	0	n.d.[c]	–	–	0	n.d.[c]	–	–	800	806 ± 31	0.8	0.34
7	185	187 ± 5	1.1	0.73	392	404 ± 9	3.1	2.37	260	257 ± 25	−1.2	0.23
8	204	210 ± 5	2.9	2.51	388	386 ± 10	−0.5	0.37	273	267 ± 15	−2.2	0.71
9	187	199 ± 7	6.4	2.69	384	383 ± 12	−0.3	0.08	188	211 ± 29	12.2	1.36
10	143	151 ± 18	5.6	0.78	310	312 ± 40	0.6	0.11	328	335 ± 29	2.1	0.45
Average			4.9				3.2				4.0	

Samples 1–6: synthetic seawater; samples 7–10: real seawater taken at marine fish farm effluents.

[a] ε_r: relative error.

[b] t_{exp}: t experimental (critical value: 4.30).

[c] n.d.: non-detected.

enrichment by wastewater, aquaculture effluents, etc. Nevertheless, real samples with very low concentration of one or several nitrogen compounds could easily be analyzed by adding a small volume of solution containing a known concentration of $N\text{-}NO_2^-$ (e.g. $0.05\,mg\,l^{-1}$) or using the standard addition method.

3.3. Application

The method was validated by simultaneous determination of nitrite, ammonium and nitrate in 10 samples. Samples 1–6 consisted of synthetic seawater while samples 7–10 were real seawater taken from outputs of a marine fish farm devoted to the intensive culture of seabream. Precision was similar for both types of samples, with average relative standard deviations of 3.9, 4.5 and 9.2% for $N\text{-}NO_2^-$, $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$, respectively. The results, obtained by a triplicate injection of each sample were compared with those obtained by applying the standard batch methodology used for the determination of nitrite, ammonium and nitrate in seawater [9,18]. As can be seen in Table 2, the results obtained by the proposed methods were in good agreement with those used as reference. Averaged relative errors were 3.2, 4.9 and 4.0% for nitrite, ammonium and nitrate, respectively. The accuracy of the results obtained was confirmed using a t-test. Experimental values of t were always less than the critical value $t = 4.30$ ($P = 0.05$, $n = 3$), thus the null hypothesis is retained and there is no significant difference between known and calculated concentrations (see Table 2).

4. Conclusions

The continuous oxidation of ammonium to nitrite in seawater has been studied and optimized. This allowed for the development of an accurate and rapid flow method for the determination and speciation of dissolved inorganic nitrogen in seawater, by using a relatively simple and low-cost methodology, easily applicable to the on-line monitoring of marine ecosystems. The new method has demonstrated its applicability to the analysis of both synthetic and real seawater samples.

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