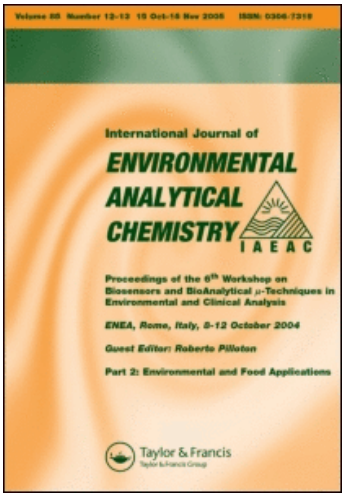

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

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To cite this Article Pinto, Paula C. A. G. , Lima, José L. F. C. and Saraiva, M.Lúcia M. F. S.(2005) 'An enzymatic flow analysis methodology for the determination of nitrates and nitrites in waters', International Journal of Environmental Analytical Chemistry, 85: 1, 29 — 40
To link to this Article: DOI: 10.1080/0306731042000303161
URL: http://dx.doi.org/10.1080/0306731042000303161

Intern. J. Environ. Anal. Chem.
Vol. 85, No. 1, 15 January 2005, pp. 29–40

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An enzymatic flow analysis methodology for the determination of nitrates and nitrites in waters

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(Received 2 April 2004; in final form 6 September 2004)

An enzymatic flow analysis approach, based on the Griess–Ilosvay reaction, was developed for the evaluation of nitrite and nitrate, aiming at an environmentally benign alternative reduction method using nitrate reductase and the development of an in-line dilution procedure for the analysis of water samples, with a wide range of nitrate concentrations. Nitrate concentrations up to 250 mg/L can be analysed directly in the automatic system, by changing the aspirated sample volume or by applying the in-line dilution procedure provided by the automatic system. Under the optimum conditions, the calibration curves were linear up to 50 mg/L of nitrate and 3 mg/L of nitrite, with the detection limits being 0.73 mg/L and 0.03 mg/L, respectively. Nitrate and nitrite were determined in different water types by the flow method with rsd < 5%. Comparison with the reference methods showed no statistically significant differences for a 95% confidence level.

Keywords: Water; Green flow analysis; Nitrate reductase; In-line dilution

1. Introduction

Nitrite and nitrate are widespread contaminants of natural waters, and their levels are normally high, particularly due to increased use of nitrogen-based fertilizers and their subsequent leaching from agricultural land [1]. This has an impact not only on the environment in disturbing both local and global nitrogen cycles [2] but also on health, as it represents a more immediate risk to normal human metabolism [3, 4] due to nitrite toxicity. Consequently, in the last decades, International and European institutions have worked on directives about the levels of nitrites and nitrates in waters from different origins (table 1).

It is therefore not surprising that nitrite and nitrate are currently determined in waters, on a routine basis, and that a large number of methods have been developed over the last decade for their determination. Spectroscopic, electrochemical

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International Journal of Environmental and Analytical Chemistry
ISSN 0306-7319 print: ISSN 1029-0397 online © 2005 Taylor & Francis Group Ltd
DOI: 10.1080/0306731042000303161

Table 1. International directives for the levels of nitrate and nitrite in water.

Regulation organism	Water	Guide level (mg/L of N)	Maximum level (mg/L of N)	Reference
European Union	Water intended for human consumption	$NO_3^- = 5.6$	$NO_3^- = 11.3$	[5]
		NO_2^- –	$NO_2^- < 0.1$	
WHO	Drinking water	$NO_3^- < 11$	$NO_3^- < 22$	[6]
U.S. EPA	Drinking water	$NO_3^- = 10$	$NO_3^- = 10$	[7]
		$NO_2^- = 1$	$NO_2^- = 1$	
		$NO_3^- + NO_2^- = 10$	$NO_3^- + NO_2^- = 10$	
European Union	Wastewater discharges	–	Total nitrogen[a] = 10–15	[8]

[a]Total nitrogen = Kjeldahl nitrogen + nitrate + nitrite.

and chromatographic techniques are those mainly adhered to [9]. The search for automation, in view of its speed, low sample/reagent consumption and less expensive methodologies has already forged the development of several flow-injection methods. However, a further impetus in the development of automatic flow methodologies and in the way the fluids are manipulated emerged with the utilization of a multiposition selection valve normally associated with sequential injection analysis (SIA). This introduced several advantages such as robustness, simplicity, versatility, computer compatibility and the ability to reduce even further the consumption of solutions and, consequently, waste generation [10]. Besides this, the use of the multiposition selection valve now offers easy and feasible alternatives to perform in-line sample pre-treatments, as concentrations or dilutions, since its use makes many operations within the system possible. Inevitably, SIA methods for sequential determination of nitrites and nitrates have also been presented in recent years, almost all using spectrophotometric detection. As in conventional methods, many reducing agents have been used to convert nitrate into nitrite, such as copper-coated cadmium column, hydrazine and UV radiation [11–17].

For the detection of nitrite, the most frequently used procedure involves the formation of an azo dye after diazo-coupling. To yield high concentrations of nitrate, some of these methods involve sample dilutions, but outside the SIA system [11, 13], others use the zone sampling strategy to increase the concentration range [15].

In the development of the methodology presented here for the evaluation of nitrate and nitrite in water samples, there were two underlying objectives: (1) an environmentally benign alternative reduction method with specificity for nitrate; and (2) the realization of a procedure that permitted the analysis of water samples with a wide concentration range. This avoids the need to perform a previous treatment of the samples (outside the system) to adjust the concentrations of the ions. Thus, in the developed flow system, the reduction of nitrate to nitrite was carried out using the enzyme nitrate reductase. Enzymes are known by their specificity, selectivity and efficiency, and are also biodegradable natural products. This therefore obviates handling hazardous materials, such as cadmium or hydrazine and the further contamination of wastes that should be avoided. Furthermore, interferences that are present in some cases, namely the presence of significant quantities of phosphate

anion in the water samples, and the reduction in activity of the cadmium columns with residual waters are minimized [12, 17].

For the evaluation of nitrate in diverse water types, the time-based sampling capacity of the SIA systems was explored. A system was developed that permitted, through the aspiration of different samples volumes or recourse to a process of in-line dilution, selection of only a slice of the zone sample profile to the evaluation of nitrate up to 250 mg/L.

The main goal of this work was therefore to develop a greener method for the determination of nitrate and nitrite. To achieve this, we developed an automatic flow system with in-line dilution and low consumption of sample and reagents, thus leading to a reduction in waste. To reduce the pollutant side effects of the method even further, we avoided toxic reductants and instead focused on using the enzyme nitrate reductase.

2. Experimental procedures

2.1 *Reagents and solutions*

All solutions were prepared with analytical-reagent-grade and high-purity water (milli Q) with a specific conductance of $< 0.1\,\mu S/cm$.

A stock solution of nitrate reductase (10 units/mL) was prepared by dissolving the lyophilized powder (300 U/g solid), from *Aspergillus niger*, in water. At this point, 200 μL portions were then separated and frozen (protected from light). Immediately before use, each aliquot was reconstituted in phosphate buffer (0.5 M; pH 7.5) to obtain a 1.5 U/mL solution. The NADPH solution (1 mM) was also prepared daily in the same phosphate buffer. A solution of L-lactic dehydrogenase (ammonium sulphate solution from rabbit muscle, 1.2 U/mg protein) 6.64 U/μL and Pyruvate 2.6×10^{-4} mg/L was prepared in phosphate buffer (0.5 M; pH 7.5). Throughout use, these solutions were kept in ice and protected from light.

The Griess reagent was prepared by adding equal volumes of 4% (w/v) solution of Sulphanilamide and 0.4% (w/v) solution of *N*-(1-naphthyl)ethylenediamine (NED). The solids were dissolved in 25 mL of water and 2.5 mL of 85% phosphoric acid.

Nitrite and nitrate stock solutions 1 g/L were prepared in water from the corresponding sodium and potassium salts. Working standard solutions were prepared daily by correct dilution of each stock solution with water.

2.2 *Apparatus*

The SIA system (figure 1) was made of a Gilson Minipuls 3 peristaltic pump (Villiers-leBel, France), equipped with a PVC pumping tube of 1.02 mm i.d. and a 10-port multi-position Valco selection valve (Valco, Houston, TX; VICI C25–3180EMH). All tubular paths were made of 0.8 mm i.d PTFE tubing. A home-made perspex mixing chamber with an inner volume of *ca.* 400 μL (including a magnetic stirrer) was placed on an Arex heating magnetic stirrer (Velp Scientifica). This was connected to the flow system by one of the inlets of the selection valve.

An Nresearch 161 T031 (W.Caldwell, NJ) valve and a contact device (on the peristaltic pump) were introduced into the system to guarantee reproducibility in the

volumes of aspirated or propelled solutions [18]. These devices were controlled by a home-made program written in QuickBasic language, implemented in a microcomputer equipped with an Advantech PCL 711B interface card, which also permitted signal acquisition.

A UV-VIS Jenway 6105 spectrophotometer, with a Helma 178712 QS flow cell (Mullheim/Baden, Germany) of 18 μL inner volume, was also used as detection system. Analytical signals were recorded on a Kipp & Zonen BD 111 strip chart recorder or via computer equipped with a convenient interface.

A Perkin Elmer, Lambda 45 UV/Vis spectrophotometer was used for analysis by the comparison methods.

2.3 *Flow procedure*

The analytical cycle (table 2) described here basically consists of four phases: the dilution phase that includes the aspiration of sample to the holding coil and its propulsion to the dilution coil (figure 1, DC) and the cleaning of the holding coil (steps 1–3); reduction of nitrates to nitrites involving the aspiration of NADPH intercalated between two air bubbles, the aspiration of nitrate reductase followed by the sample and propelling the aspirated sequence to the mixing chamber where the reaction mixture remains for 240 s (steps 4–10); determination of the nitrites initially present in the sample made of the sequential aspiration of sample and Griess reagent to the holding coil followed by propulsion of the reaction zone formed to the detector (steps 11–13); and determination of the total nitrites (initial nitrite plus nitrite reduced from nitrate) that includes the aspiration of L-lactic dehydrogenase and pyruvate

Table 2. Analytical cycle for the determination of nitrates and nitrites in water.

Step	Position	Volume (μL)	Time (s)	Flow direction[a]	Flow rate (mL/min)	Event
1	1	70	8.6	A	0.5	Sample (nitrates)
2	9	128	9.6	P	0.8	Dilution
3	10	250	10	P	1.5	Clean (holding coil)
4	8	–	1	A	0.8	Air
5	3	75	5.6	A	0.8	NADPH
6	8	–	1	A	0.8	Air
7	2	60	4.5	A	0.8	Nitrate reductase
8	1 or 9	25	3	A	0.5	Sample or diluted sample
9	4	320	15	P	0.8	Mixing chamber
10	4	–	240	–	0	Stop
11	1	150	11.25	A	0.8	Sample (nitrites)
12	6	50	1.5	A	2	Griess reagent
13	7	–	80	P	2	Detection (nitrites)
14	5	100	7.5	A	0.8	LDH and pyruvate
15	4	147	11	P	0.8	Mixing chamber
16	4	–	120	–	0	Stop
17	6	75	2.25	A	2	Griess reagent
18	4	140	4.2	P	2	Mixing chamber
19	4	–	20	–	0	Stop
20	4	266	20	A	0.8	Mixing chamber
21	7	–	80	P	2	Detection (nitrates)
22	4	–	30	P	3	Clean (mixing chamber)
23	4	–	20	A	3	Empty (mixing chamber)

[a]A: aspiration; P: propulsion.

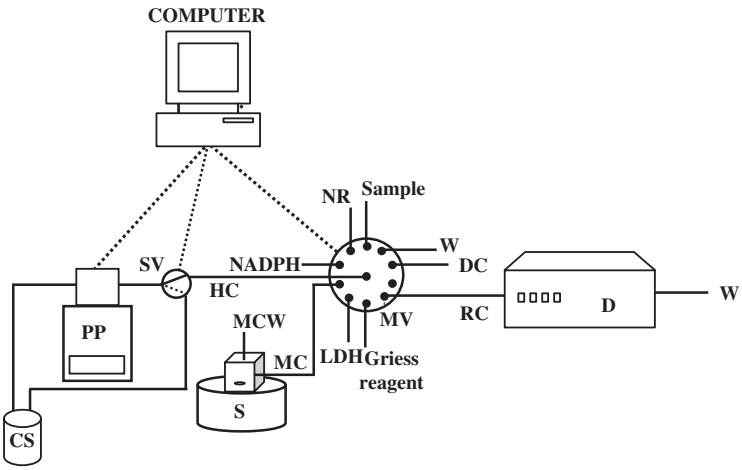

Figure 1. PP—peristaltic pump; CS—carrier solution (deionized water); SV—solenoid valve; MV—multiposition valve; MC—mixing chamber; S—stirrer; HC—holding coil; RC—reaction coil; DC—dilution coil; D—spectrophotometric detector; MCW—mixing chamber waste; W—waste; NR—nitrate reductase; LDH—L–lactic dehydrogenase.

solution and its propulsion to the mixing chamber (steps 14–16), the aspiration of Griess reagent and its propelling to the mixing chamber (steps 17–19), and the aspiration of the final mixture and its propulsion to the detector (steps 20 and 21); at the end of each cycle, the mixing chamber is cleaned and emptied (steps 22 and 23).

The in-line dilution phase is used for samples with a concentration of nitrates greater than 50 mg/L. The diluted sample is subsequently analysed according to the optimized analytical sequence. The individualization of the different ports of the selection valve permitted the reduction of nitrates as well as determination of nitrites initially present in the sample to be carried out simultaneously.

In each analytical cycle, two peaks were obtained; the first corresponding to the quantification of nitrite alone, and the second referring to the evaluation of total nitrite after the reduction of nitrate.

3. Results and discussion

One of the objectives of this work was to develop a flow system using a reducing agent which was environmentally benign and which presented an elevated degree of selectivity. With this in mind, an enzyme-based assay system was developed using the enzyme nitrate reductase (NaR), with the corresponding consequences on instrumentation and methodology.

Nitrate is reduced in the active site after the enzyme has been reduced by nicotinamide adenine dinucleotide phosphate (NADPH). The catalytic rate of NaR is about 200 nitrate to nitrite conversions per second per molecule of NaR. One of the advantages of systems containing a multiposition selector valve is the fact that a different task can be allocated to each entry of the valve. A mixing chamber was therefore set, connected to one of the valve entries, to effectively undertake the

mixing of the solutions involved in the reduction and carry out the determination of initial nitrite at the same time as the reduction of the nitrate. The mixing chamber was placed above a magnetic stirrer, which also served to maintain the chamber temperature at approximately 37°C, the temperature at which the enzymatic activity was more intense. It was then observed that the order of addition and mixture of the solutions involved in the process of enzymatic reduction was important. As in the flow system, the mixture of the solutions depends on the way they are aspirated, since the dispersion with the carrier and other solution zones starts with the aspiration to the holding coil, some prior optimization tests were carried out. It was verified that the best results were obtained when NADPH was aspirated separately and added to the sample and NaR only in the mixing chamber. To achieve this goal and guarantee the individuality of the NADPH zone, the best approach was to isolate the NADPH zone with two air bubbles. This means combining the advantages of the monosegmented flow analysis approach (MSFA) [19] with the versatility of the SIA systems. This procedure allows no zone dispersion in the carrier with complete individualization of samples and/or reagents and therefore high sensitivity. In this way, the solution could flow in the system and be added to the other reagents only at the right time. Apart from this, the presence of the bubbles ensures the stopping of the propulsion flow to the mixing chamber at a precise time, just after the entrance of the last bubble. Normally in the MSFA, a mechanism is required for bubble detection in order to remove the air bubbles that will affect detection. In this case, this problem does not exist as the bubbles escape from one of the inlets of the mixing chamber that is open (figure 1). This procedure was then followed during the optimization. The NADPH was then initially aspirated to the holding coil between two air bubbles followed by the sample zone and NaR zone. On route to the mixing chamber, the NADPH zone was the last to enter, with the flow being stopped just after the entrance of the last bubble. This guaranteed that no carrier was dispersing the mixture, as it did not enter the mixing chamber.

The greater the volume of sample sent to the chamber, the greater the volume of the chamber had to be, which would correspond to an increase in the consumption and dispersion of the solutions. Therefore, volumes between 15 and 40 μL were tested, with the studies proceeding with 25μL, thus guaranteeing a compromise between the factors cited and the sensitivity obtained. For the reduction process, it was necessary to optimize the quantity of enzyme to use and the time needed for it to act in the chamber, through the implementation of a stopped flow period. Using a 1.5 U/mL NaR solution, the number of units employed in each assay was varied by altering the volume of enzyme solution aspirated. Studies were carried out in an interval between 30 and 70 μL of enzyme solution (0.045–0.105 U) and varying the period of enzymatic reduction between 60 and 600 s. It was verified that with 0.09 units of NaR and a period of 240 s, it was possible to promote the reduction of nitrate to nitrite.

Another factor to be optimized is related to the balance between the quantity of NADPH to use and the quantity of nitrate ions to reduce [20]. It was verified that high concentrations of NADPH interfered with the Griess reaction, subsequently carried out to evaluate the concentration of total nitrite (nitrate plus initial nitrite) and that excessively low concentrations limited the enzymatic activity of NaR. An enzyme regenerating system incorporating lactate dehydrogenase [21] was then used to limit the NADPH concentration. Optimizations were then carried out to evaluate

the minimum NADPH concentration that permitted adequate reduction in the full range of nitrate concentrations. This was with a view to accomplishing the conversion into $NADP^+$ of the NADPH not consumed under the action of L-lactic dehydrogenase and pyruvate in the least time interval. With a 1 mM NADPH solution and following addition of 100 μL of a solution of L-lactic dehydrogenase and pyruvate, it was possible to effectively eliminate the NADPH not consumed in a period of 120 s.

After the reduction of the ions nitrate to nitrite, two procedures were tested for the quantification of total nitrite: (1) aspiration of an aliquot from the mixing chamber to the holding coil and subsequent mixture with the Griess reagent; or (2) sending the colorimetric reagent to the mixing chamber and subsequent aspiration of an aliquot of the product formed therein to the holding coil. After carrying out studies with reagent volumes falling between 50 and 150 μL and testing the two procedures, it was verified that sending 75 μL of Griess reagent to the mixing chamber gave rise to analytical signals of higher intensity, given that the reagent was mixed with the total volume of sample present in the mixing chamber.

Regarding the determination of the ions nitrite initially present in the samples, the optimization demonstrated that a sample volume of 150 μL (between 50–300 μL) and a Griess reagent volume of 50 μL guaranteed linearity of the calibration curve in the range of nitrite concentrations normally present in waters. Concentrations of the two components of the Griess reagent were also tested by varying each one separately. A mixture of 40 g/L solution of sulphanilamide and 4 g/L NED (1:1, v:v) was used since, for higher concentrations, there was no substantial gain in sensitivity.

Another of the objectives at the base of the development of this system was the possibility of in-line analysis of water samples of diverse origin with a concentration of nitrate ranging over a significantly enlarged concentration interval. This did not present a problem for the nitrite, since the interval of concentrations in which they normally arise in waters was covered by the optimized procedure. The recourse to dilution or concentration of the samples in a discrete way, before insertion into the flow system, leads to an effective increase in the time necessary to carry out the determinations. A procedure was then developed whereby, with only small alterations in the analytical cycle, it was possible to carry out a quick in-line dilution of the samples and therefore adjust the concentration of nitrate ions present in the samples, inside the automatic system. Two of the procedures that permit this objective to be achieved more easily, applied in distinct cases, take advantage of the dispersion imposed on the sample either by mixing with an appropriate volume of diluent [22] or by analysing a section of the concentration gradient generated in a dedicated tube of the selection valve (dilution coil) [22–27].

In the flow method developed, dilution was carried out using a dilution coil, placed in one of the inlets of the selection valve (figure 1, DC) and adding three steps to the analytical sequence, corresponding to steps 1–3 in table 2. The degree of dilution in this kind of situation depends on (as defined Baron *et al.* [22]): sample volume (amount of sample aspirated via the sample port to the holding coil), transfer volume (includes the sample volume and carrier solution transferred from the holding coil to the dilution coil) and analysis volume (volume of the aliquot taken from the dilution coil to the holding coil). The three parameters were studied and optimized to achieve a dilution degree of about 5. The analysis volume had already been established, and so all studies were then performed with 25 μL. The gradient

concentration profile formed in the dilution coil and consequently the slice corresponding to this 25 μL of sample resulted from the combined effect between the sample volume utilized and the dispersion of this sample zone in the carrier on the way to the dilution coil. A dilution factor of 4.4 with a repeatability of less than 3% was obtained with a sample volume of 70 μL (15–80 μL) and a transfer volume of 128 μL (55–160 μL). All dilution studies were developed by comparing the results obtained with standards made by manual volumetric dilution.

The reaction coil used during the optimization was 200 cm in length and with an eight-shaped figure. Its selection was a compromise between reaction development in the determination of nitrite and the least dispersion in nitrate determination.

Under the selected conditions, linear calibration curves up to 50 mg/L and 3 mg/L were obtained for nitrate and nitrite, respectively. The analytical curves were represented as Abs (AU) = 0.008 (± 0.0003) [mg/L NO_3^-] + 0.0005 (± 0.0082), $R^2 = 0.9991$, and Abs (AU) = 0.1671 (± 0.0099) [mg/L NO_2^-] + 0.0071 (± 0.0129), $R^2 = 0.9992$. The absorbance values used in the equations are the flow signal after subtraction of the blank value. Detection limits were calculated, using 3σ of the blank signal (Abs units) [28], as 0.03 mg/L for nitrite and 0.73 mg/L for nitrate, after processing the blank solution 15 times.

Relative standard deviations (rsd %; $n = 15$) of 3.8 for nitrite (0.31 and 0.54 mg/L), and 2.5 and 3.6 for nitrate (23.3 and 94.3 mg/L) were obtained, showing small differences in a large range of concentrations. The measurements were made in real water samples containing nitrites and nitrates.

The flow system developed was used for the sequential determination of nitrates and nitrites in 15 real water samples intended for human consumption from different origins. According to the sampling requirements [29], the water samples were collected in glass containers and refrigerated. The analysis took place as soon as possible after the collection. Water samples with a nitrate concentration greater than 50 mg/L were subjected to the process of in-line dilution developed. The accuracy of the proposed method was evaluated by comparing the results obtained with those achieved by reference methods (table 3). Nitrite was determined using Griess reagent, and nitrate was quantified by measurements of absorbance values at 220 and 275 nm [30].

A linear relationship between the two methods was established: Flow (mg/L) = C_0 + SRef (mg/L), along with the evaluation of the correlation significance using the *t*-test, carried out as a bilateral coupled test, according to Miller and Miller [28, 31], using $t = (\bar{X}/S)\sqrt{n}$ with $n-1$ degrees of freedom. $\bar{X}$ is the mean difference between each pair, S is the standard deviation and n is the number of measurements. The calculated t value, when compared with the tabulated value at the 95% confidence level, confirms the good agreement between the two methods, since the null hypothesis is accepted (table 4).

In this case, the test involving the means of two samples was not appropriate because the amounts of analyte in each sample are substantially different, which could swamp any difference between the two methods [32].

The results were also compared using the significance *F*-test, which compares the variances of the two methods and can evaluate them with regard to their random errors. The F values for nitrates (1.039) and nitrites (1.069) obtained confirm the null hypothesis (equal variances) by comparison with the critical value of F (2.483 and 3.178, respectively).

Table 3. Results obtained by the proposed flow methodology and the comparison batch procedure for the determination of nitrates and nitrites in water samples.

Samples	Flow (mg/L) ± SD[a]		Reference method (mg/L) ± SD		Relative error (%)	
	Nitrite	Nitrate	Nitrite	Nitrate	Nitrite	Nitrate
1	<LD	15.0 ± 0.3	<LD	14.7 ± 0.2	–	2.0
2	0.201 ± 0.008	36.2 ± 0.6	0.210 ± 0.008	36.8 ± 0.5	4.2	0.6
3	0.253 ± 0.021	20.7 ± 0.4	0.243 ± 0.009	20.2 ± 0.3	4.1	3.5
4	0.275 ± 0.009	35.9 ± 0.1	0.266 ± 0.011	36.8 ± 0.8	3.3	2.4
5	0.261 ± 0.009	24.2 ± 0.2	0.268 ± 0.012	24.6 ± 0.1	2.6	1.6
6	0.225 ± 0	13.1 ± 0.1	0.215 ± 0.006	13.4 ± 0.2	4.6	2.2
7	0.144 ± 0.004	27.2 ± 0.2	0.149 ± 0.008	26.2 ± 0.3	3.3	3.8
8	0.078 ± 0.006	113.0 ± 0.4	0.080 ± 0.009	112.0 ± 0.9	2.4	0.9
9	0.500 ± 0.008	141.2 ± 0.4	0.520 ± 0.021	143.7 ± 1.2	3.8	1.7
10	0.140 ± 0.005	89.7 ± 1	0.130 ± 0.007	85.7 ± 0.8	4.4	4.4
11	<LD	3.3 ± 0.3	<LD	3.4 ± 0.1	–	2.9
12	<LD	2.7 ± 0.2	<LD	2.8 ± 0.3	–	3.6
13	<LD	106.7 ± 0.8	<LD	111.9 ± 0.6	–	4.6
14	0.030 ± 0.002	153.7 ± 1.7	0.031 ± 0.008	158.2 ± 1.2	2.3	2.9
15	<LD	2.6 ± 0.1	<LD	2.5 ± 0.1	–	4.0

[a]SD: standard deviation of three replicates.

Table 4. Parameters of the equation flow (mg/L) = $C_0 + S$Ref for comparing the results obtained by both SIA methodology (flow) and the comparison procedure (Ref) and the results of Student's t-test.

	C_0	S	r^a	$t^b_{0.05}$	$t^c_{0.05}$
Nitrites	0.007 (±0.01)[d]	0.9645 (±0.0548)[d]	0.9952	0.155	2.26
Nitrates	0.5437 (±1.6010)[d]	0.9800 (±0.0215)[d]	0.9987	0.869	2.14

[a] Correlation coefficient. [b] Calculated values for a two tail test. [c] Tabulated values (95% confidence level). [d] Values in parentheses are the limits of the 95% confidence level.

The developed automatic methodology allowed 12 determinations per hour, six for nitrates and six for nitrites. The automatic system developed can therefore be considered an alternative tool to the conventional methods for environmental monitoring of nitrate and nitrite, and is intended as a contribution towards greening analytical chemistry. It is fully automated and can work for long periods of time with minimum intervention from the analyst.

The introduction of an in-line dilution led to a versatile and flexible method, enabling direct sampling of waters with concentration ranges up to 250 mg/L, greater than all others developed to date by sequential injection. As prior dilution treatment was therefore not necessary, a reduction of the time spent and of the generated effluents was achieved. Besides, samples with lower concentrations of nitrate down to 0.4 mg/L can be analysed by simply changing the sample volume. The method developed here can be used in nitrate and nitrite determination in a large variety of waters such as natural, mineral, waste and agriculture waters.

The use of nitrate reductase made the method very selective and sensitive, and offered distinct advantages since interferences from reducing agents like cadmium [33], hydrazine [34] or UV radiation [35] were avoided. Indeed, nitrate reductase activity was ensured by controlling its optimum conditions and was only affected by high concentrations of a small number of ions [36]. These ions are not usually presented, in such concentrations, in the waters analysed. Reduction using nitrate

Table 5. Some flow methods for the determination of nitrites and nitrates in waters.

Water type	Reducing agent	Linear range (mg/L)	Flow technique	Detection	Reference
Natural water	Copperized cadmium	0–3 NO_3^-	Segmented flow	Spectrophotometric	[37]
Seawater	Copperized cadmium	0.2–2 NO_3^-	Segmented flow	Spectrophotometric	[38]
River and seawater	Copperized cadmium	0–12.4 NO_3^-	FIA	Fluorimetric	[39]
Rainwater	Copperized cadmium	0.006–0.12 NO_3^-	FIA	Fluorimetric	[40]
Natural water	Copperized cadmium	0.0009–0.013 NO_3^-	FIA	Fluorimetric	[41]
Natural water	Copperized cadmium	0.06–62 NO_3^-	FIA	Voltametric	[42]
River and lake water	Copperized cadmium	0.1–1.3 NO_3^- 0.08–6.6 NO_2^-	FIA	Spectrophotometric	[43]
Environmental water	Copperized cadmium	0.1–3.5 NO_3^- 0.01–2.2 NO_2^-	FIA	Spectrophotometric	[44]
Natural water	Copperized cadmium	1.0–5.0 NO_3^- 0.1–0.5 NO_2^-	FIA	Spectrophotometric	[45]
Waste and coastal marine water	Copperized cadmium	0.06–4 NO_2^-	FIA	Spectrophotometric	[46]
Tap, rain and river water	Copperized cadmium	0.01–53 NO_3^-	FIA	Spectrophotometric	[47]
River water	Copperized cadmium	0–53 NO_3^-	FIA	Spectrophotometric	[48]
Wastewater	Copperized cadmium	0.25–50 NO_3^- 0.05–5 NO_2^-	FIA	Spectrophotometric	[49]
Surface, ground and domestic water	Copperized cadmium	0–5 NO_3^- 0–0.5 NO_2^-	FIA	Spectrophotometric	[33]
Wastewater	Copperized cadmium	0.092–4.6 NO_3^- 0.023–1.84 NO_2^-	SIA	Spectrophotometric	[12]
Wastewater	Copperized cadmium	0.05–15 NO_3^- 0.05–25 NO_2^-	SIA	Spectrophotometric	[13]
Natural water	Copperized cadmium	0.01–5 NO_3^-	SIA	Spectrophotometric	[14]
Natural and wastewater	Copperized cadmium	0.09–4.4 NO_3^- 0.06–3.3 NO_2^-	SIA	Spectrophotometric	[15]
Environmental water	Copperized cadmium	0.4–17.7 NO_3^- 0.1–13 NO_2^-	SIA	Spectrophotometric	[16]
Surface water	Copperized cadmium	2.2–221 NO_3^- 0.16–3.3 NO_2^-	SIA	Spectrophotometric	[17]
Natural water	Hydrazine	1–10 NO_3^-	FIA	Spectrophotometric	[34]
River and seawater	UV radiation	0.003–0.6 NO_3^-	FIA	Spectrophotometric	[35]
Drinking and natural water	UV radiation	4.96×10^{-7} to 6.2×10^{-4} NO_3^-	FIA	Chemiluminescent	[50]
Industrial effluents	Nitrate reductase	0.017–7 NO_3^-	FIA	Spectrophotometric	[51]
River and lake water	Nitrate reductase	0.31–6.2 NO_3^-	FIA	Spectrophotometric	[52]
River water	Titanium chloride	0.03–12.4 NO_3^-	FIA	Fluorimetric	[53]
Tap and mineral water	Zinc	0.9–6.6 NO_3^-	FIA	Conductivity	[54]
Hydroponic fluids	HCl/H_2SO_4	5.6–300 NO_3^-	FIA	Amperometry	[55]

reductase is environmentally safer when compared with that performed by cadmium or hydrazine, well known for their toxicity. Effectively, the developed sequential injection method for the determination of nitrates and nitrites when compared with the analytical flow methodologies developed in the last decades showed some advantages, mainly in terms of sensitivity and safety (table 5).

Acknowledgements

Paula C.A.G. Pinto thanks Fundação para a Ciência e Tecnologia and FSE (III Quadro Comunitário de Apoio) for the Ph.D. grant (SFRH/BD/2915/2000). This work was developed under the scope of the Programme Agros—Project 273.

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