

Use of a Digital Image in Flow Analysis: Determination of Nitrite and Nitrate in Natural Waters

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A webcam is proposed as the detector in a flow system with multicommutation, and the feasibility of the approach is demonstrated in the determination of nitrate and nitrite in natural waters. The typical transient signal inherent to the flow system was obtained using a digital video and quantified by ImageJ software. The linear dynamics range for nitrite and nitrate determinations were 0.2 to 2.0 mg L⁻¹ NO₂⁻ and 1.0 to 10.0 mg L⁻¹ NO₃⁻, with relative standard deviation < 2% for both analytes. The limits of detection were 0.01 and 0.04 mg L⁻¹ for nitrite for nitrate, respectively, and the sampling rate were 80 and 103 h⁻¹ for nitrite and nitrate, respectively. The use of webcams has a high potential for analysis in the visible region of the electromagnetic spectrum, and the proposed strategy constitutes a promising alternative to traditional absorbance measurements that depend on conventional equipment. The webcam detection system is attractive, especially in relation to field analysis.

Keywords: webcam, flow analysis, video digital, transient signal, nitrate, groundwater

Introduction

The interest in the nitrite and nitrate determination in waters is due to the toxicological effects of these ions. Excessive ingestion by infants may result in the oxidation of hemoglobin to methemoglobin, reducing oxygen transport to the tissues.¹ Nitrites are also precursors of *N*-nitrosamines, compounds known for their potential carcinogenic and teratogenic actions.²

These species should be monitored, and several analytical methods have been developed highlighting the spectrophotometric methods with the Griess reaction. These methods are mostly chosen due to their simplicity, ruggedness and detection limits.³ To minimize sample and reagent consumption, increase the sampling rate and improve analytical precision, analytical flow systems have been proposed.⁴⁻⁷

Flow analysis systems exploiting multicommutation provide a powerful alternative to enhance the versatility of flow-based procedures, with the advantage of minimizing

both reagent consumption and waste generation,⁸⁻¹⁰ a portable setup may even be developed.^{11,12}

For spectrophotometric flow-based determinations of nitrite and nitrate in conventional spectrophotometers, portable spectrophotometers¹³ and LED-based photometers have been used.¹⁴

Recently, colorimetric methods using digital images have been reported. In most of these methods, the images are captured by digital devices such as digital cameras, webcams or scanners, and the digital images are treated by a custom-built software.¹⁵⁻¹⁷

In this regard, the following method was proposed: determining the ascorbic acid concentration using a scanner for image acquisition and color parameters (RGB) to calculate the analytical response,¹⁵ the relation (equation 1) was used, where I_0 and I are the intensities of the blue component (B) of the digital images obtained from the blank solution and the standard solution, respectively. The standard or sample solutions were placed into transparent bottles positioned on the scanner.

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$$A = \log(I_0/I) \quad (1)$$

Andrade *et al.*¹⁷ propose a digital image-based flow-batch analyzer for determination of Al^{III} and Cr^{VI} in natural waters, using a webcam as a detector and RGB parameters to calculate the analytical response. The analytical figures of merit were similar to those of conventional procedures, demonstrating the webcam's potential as a detector. In both applications, the images were acquired with the static solutions inside a flask or chamber.

In flow systems, the data acquisition of the analytic signal usually has a transient peak shape. Evaluating the shape of the recorded transient signal is important for system optimization and monitoring analysis, and evaluating the transient peak shape of the flow systems is important for system optimization, as it provides relevant information, including the dispersion coefficient, mixing conditions, mean residence time, carry over and the presence of spurious signals (e.g., bubbles or the Schlieren effect).¹⁸

The aim of this work was to develop a multicommuted flow system that used a webcam as a photometric detector, allowing the acquisition of the transient analytical signal. This system was applied to nitrite and nitrate determination in groundwater.

Experimental

Reagents and solutions

All solutions were prepared with double-distilled and deionized water (18.0 M Ω cm) and chemicals with analytical grade quality.

The 1000 mg L⁻¹ tartrazine ($\lambda = 422$ nm), Porceau 4R ($\lambda = 507$ nm) and bright blue ($\lambda = 603$ nm) were prepared by direct dissolution of the clean dried substances (BASF, Germany) in water.

Reference solutions (0.025-1.0 mg L⁻¹ NO₂⁻ and 0.10-5.0 mg L⁻¹ NO₃⁻) were prepared by dilution of 1000 mg L⁻¹ stock solutions prepared from NaNO₂ and NaNO₃ (Merck, USA). The nitrite stock solution was treated with a few drops of chloroform and standardized against potassium permanganate.⁴ The reagent (R) was 2.0% (m/v) sulphanilamide plus 0.1% (m/v) *N*-1-naphthylethylenediamine dihydrochloride (NED) solution, also 0.5 mol L⁻¹ in phosphoric acid. The carrier stream was 0.5% (m/v) Na₂B₄O₇ plus 0.3% (m/v) Na₂EDTA buffer solution with pH adjusted to 7.25 with HCl. Cadmium fillings were copperized¹⁹ and packed into a glass tube (6 cm long, 3 mm internal diameter, i.d.) retained by glass wool.

Sample preparation and reference method

Groundwater samples were collected from wells in

Barreiras-BA, Brazil. The samples were filtered through 0.45 μ m cellulose membrane filters before analysis.²⁰

The reference method was the one proposed by the American Public Health Association (APHA),²¹ which is based on the Griess reaction; it was performed in batches, using the 800 XI model Femto spectrophotometer (São Paulo, Brazil).

The multicommuted flow analyzer

The multicommuted flow system comprised a model IPC-4 peristaltic pump (Ismatec, Switzerland) equipped with TygonTM pumping tubes, three-way 161T031 solenoid valves (NResearch, USA), polyethylene tubing (0.8 mm i.d.) and acrylic confluence connectors. System control and data acquisition were performed with a Pentium 2.1 GHz microcomputer equipped with a commercial electronic interface (National Instruments, USA) and a lab-made electronic circuit similar to that previously described.²² The software was developed in Labview 7.0 and the Windows XP operating system.

The multicommuted flow system (Figure 1) was operated according to the valve-switching course in Table 1. The manifold comprised 04 three-way solenoid valves: one valve for each managed solution (V₁, V₂, and V₄) and one for nitrite or nitrate determination (V₃).

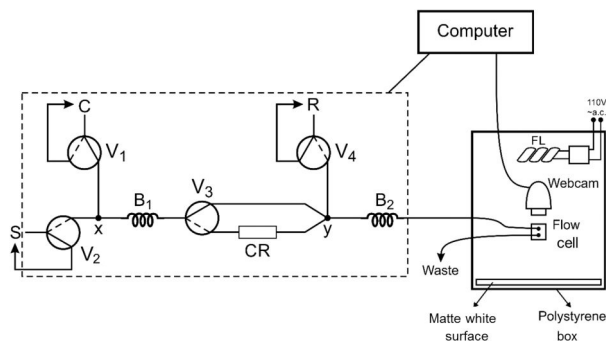


Figure 1. Flow diagram of the system for nitrite and nitrate determination. V₁-V₄: solenoid valves; C: buffer solution (pH = 7.2) (4.3 mL min⁻¹); S: sample (4.3 mL min⁻¹); R: Griess reagent (2.1 mL min⁻¹); CR: Cd reduction mini-column (3 mm i.d. × 6 cm); B₁ (0.8 mm i.d. × 60 cm) and B₂ (0.8 mm i.d. × 90 cm): coiled reactors; x and y: confluence sites; FL: fluorescent lamp; webcam: detector.

The system operation started with all valves switched off, and the carrier solution was pumped through V₁, V₂ and V₃ towards the flow cell, while S was recycled by V₂. For nitrite determination, the V₁ and V₂ valves were simultaneously switched on for 5 s, and the B₁ reactor was filled with the sample solution (Step 1). In the next step, the V₄ valve was switched on, and the sample zone received the Griess reagent at the y confluence point;

Table 1. Valve switching course

Step	V ₁ ^a	V ₂ ^a	V ₃ ^a	V ₄ ^a	time / s	Description
Nitrite						
1	1	1	0	0	5	fill B ₁ ^b with sample
2 ^c	1	0	0	1	1	sampling R ^d
3 ^c	0	0	0	0	2	sampling S ^e
4	0	0	0	0	15	measure signal
Nitrate + Nitrite						
5	0	0	1	0	10	fill B ₁ ^b and C _R ^f with buffer solution
6	1	1	1	0	10	fill B ₁ ^b and C _R ^f with sample
7 ^c	0	0	1	1	1	sampling R ^d
8 ^c	0	0	1	0	2	sampling S ^e
9	0	0	0	0	15	measure signal

^aV₁-V₄: solenoid valves, 0 and 1, solenoid valves switched off or on, respectively; ^bB₁ (0.8 mm i.d. × 60 cm); ^crepeated steps (5 sampling cycles); ^dR: Griess reagent (2.1 mL min⁻¹); ^eS: sample (4.3 mL min⁻¹); ^fC_R: Cd reduction mini-column (3 mm i.d. × 6 cm).

sample aliquots were intercalated with the reagent, as described in Steps 2 and 3 (Table 1). This sequence was repeated until the B₂ reactor was filled (0.45 mL) with the binary string (5 sampling cycles). In the final step, all valves were switched off, and the sample zone was transported to the flow cell, allowing the nitrite determination (Step 4).

For nitrite and nitrate determination, the V₃ valve was switched on for 10 s, directing the flowing sample through the cadmium column (Step 5). In the meantime, the V₁, V₂ and V₃ valves were switched on for 10 s, and the B₁ reactor and cadmium column (CR) were filled with the sample solution (Step 6). During this step, the nitrate ions were converted to nitrite. Steps 7 and 8 were analogous to the determination of nitrite (in Steps 2-4), and the obtained signal (Step 9) was proportional to the concentration of nitrate plus nitrite. All measurements were based on digital image analysis and carried out in triplicate.

Parameters such as sample and reagent volume, carrier and sample flow rate, mixing coil length and number of sampling cycles were evaluated using a single-variable optimization procedure. The NED and sulphanilamide concentrations were evaluated by a central composite design (Table 2).

Digital images sensor

A Leadership® (Brazil) brand webcam (5.0 Mega model) with a (charge-coupled device) CCD sensor was used to

capture the digital images. The webcam was configured to capture 24-bit digital images (16.7 million colors) at a rate of 30 frames s⁻¹ and 640 × 480 pixels of spatial resolution.²³ The images were captured and stored as AVI files using the software provided by the webcam manufacturer.

For digital image acquisition, the flow cell (70 μL inner volume) was positioned in front of the webcam. To avoid the influence of stray environmental light, the webcam and flow cell were placed inside a polystyrene box (21 × 30 × 18 cm) and illuminated with a white 5 W fluorescent lamp (Figure 1), as suggested elsewhere.²⁴

For each determination, a video file with 90 s of recording was created, and, using JPG Converter® software, one image was extracted for every second of the video file, meaning 90 image files were automatically saved in JPEG format. These files were subsequently regrouped with ImageJ software,²⁵ using the stack image tool. With the images in a stack, the oval tool was used to select an area of 9500 pixel² in the observation window of the flow cell (Figure 2). Automatically, the RGB values in the selected area were acquired for all images in the stack. In this process, it was necessary to use a plugin developed in Java.

A mathematical approach aiming at a linear relationship between the proposed RGB-based value and the analyte concentration was already described.²⁶ This model used the concept of an RGB-based value associated with a vector (v) of the color value. The RGB-based value was calculated by equation 2, where R²_{s-b}, G²_{s-b} and B²_{s-b} are the differences

Table 2. Central composite design. NED and sulphanilamide concentration

Factor	Level				
NED ^a / (% , m/v)	0.03	0.05	0.10	0.15	0.17
Sulphanilamide / (% , m/v)	1.3	1.5	2.0	2.5	2.7
Coded values	-1.41	-1	0	1	1.41

^aN-1-Naphthylethylenediamine dihydrochloride (NED).

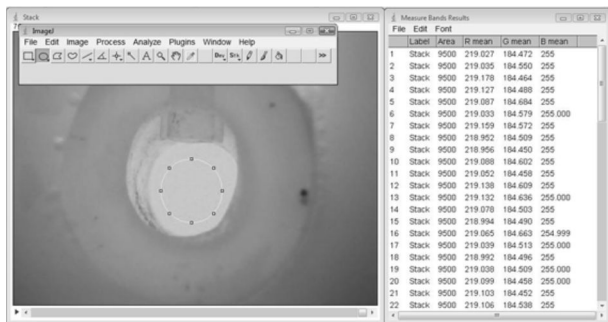


Figure 2. Image acquisition of flow cell and area selected in ImageJ.

in the mean values of the RGB components between the sample or standard solution and the blank solution.

$$\text{RGB based value} = \sqrt{(R_s^2 + G_s^2 + B_s^2)} \quad (2)$$

The detection and quantification limits were estimated as $LD = 3S_b/\beta$ and $LQ = 10S_b/\beta$, respectively, where S_b is the standard deviation of the blank solution's data and β is the angular coefficient of the analytical curve.

Analytical figures of merit, such as detection and quantification limits, linearity and the correlation coefficient of the analytical curve were considered for the dyes in solutions containing red, green and blue components. This was done in order to compare detector performances for the webcam and the conventional detector, a model 800 XI spectrophotometer (Femto, São Paulo, Brazil).

Results and Discussion

Webcam detector optimization

The dye solutions were deliberately used to evaluate the performance of the webcam detector, as the maximum wavelengths were distributed in the visible spectrum (507 nm for red dye, 422 nm for green and 630 nm for blue).

Figure 3 shows the transient signals recorded for increasing concentrations of red, green and blue dyes, using the spectrophotometer and the webcam. For both techniques, a linear regression between the analytical responses and the concentrations was observed, this is shown in Table 3.

Analysis of Table 3 reveals that the webcam detection method yielded analytical curves with high slopes and good linear regression coefficients for all different colors of solution. Coefficients of variation ($n = 10$) of 1.4%, 1.8% and 1.6% were obtained for 400 mg L⁻¹ solutions of red, green and blue, respectively.

Multicommutated flow system

To optimize the flow system, parameters such as sample

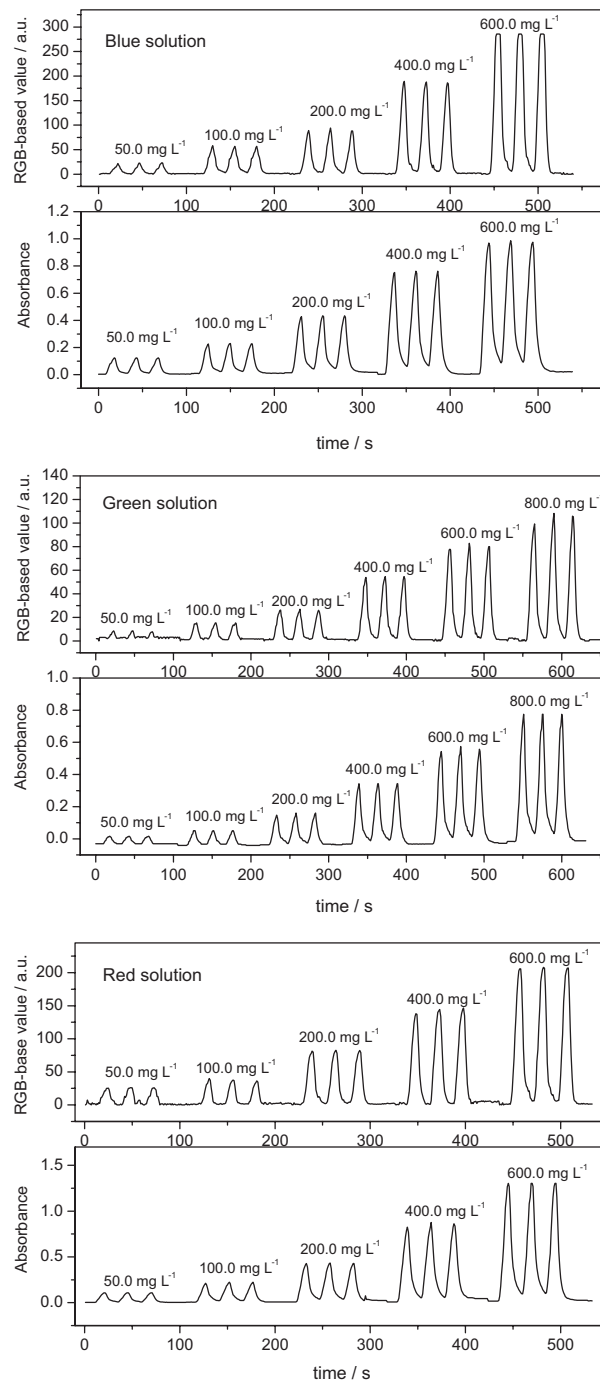


Figure 3. Transient signals to increasing concentrations of the dyes red, green and blue, obtained by the flow analysis system and detection by webcam and spectrophotometer.

and reagent volume, number of sampling cycles, reactor lengths and flow rates were evaluated, and the tested range and selected values are shown in Table 4.

The best concentrations determined by the experimental design of sulphamide and NED were 2.3% and 0.12%, respectively, and the regression coefficient was 0.9989, showing the good mathematical model adjustment (Figure 4).

Table 3. Correlation between the measurements performed using the webcam and absorbance values for different concentrations of red, green and blue solutions with regression parameters ($Y = mC + b$)

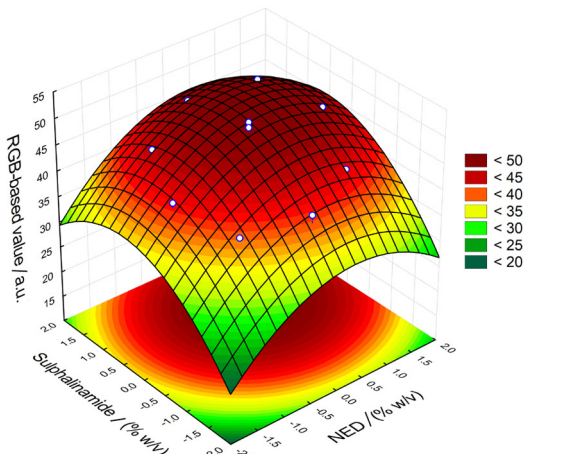
Solution	Webcam			Absorbance		
	m^a	b^b	r^c	m^a	b^b	r^c
Red	0.33466	0.06906	0.9986	0.00216	-0.00149	0.9999
Green	0.12916	0.09243	0.9997	0.00101	-0.04723	0.9993
Blue	0.46874	0.04649	0.9997	0.00154	0.06323	0.9988

^a m = angular coefficient; ^b b = linear coefficient; ^c r = linear regression coefficient.

Table 4. Optimized parameters of the multicommuted flow system for nitrite and nitrate determination

Parameter	Interval	Selected value
Flow rate-peristaltic pump / (mL min ⁻¹)	3.1-6.6	4.3
B2 reactor length / cm	50-100	90
Sampling cycles	3-7	5
Sample volume / μ L	35-70	70
Reagent volume / μ L	35-70	35
pH of the buffer solution	6.0-8.5	7.2
Sulphalamide / (% m/v)	1.3-2.7	2.3
NED ^a / (% m/v)	0.03-0.17	0.12
Phosphoric acid / (mol L ⁻¹)	0.3-1.5	0.5

^aN-1-Naphthylethylenediamine dihydrochloride (NED).

**Figure 4.** Central composite design of sulphalamide and NED concentration. $RGB\text{-based value} = 49.67 + 2.19NED - 2.46(NED)^2 + 2.86(\text{sulphalamide}) - 2.71(\text{sulphalamide})^2 + 0.25(NED)(\text{sulphalamide})$.

The reagent volume *per* determination was 175 μ L, and the total waste generation was about 2.2 and 3.2 mL for nitrite and nitrate, respectively. The sampling rate was 103 h⁻¹ for nitrite and 80 h⁻¹ for nitrate.

Analytical application

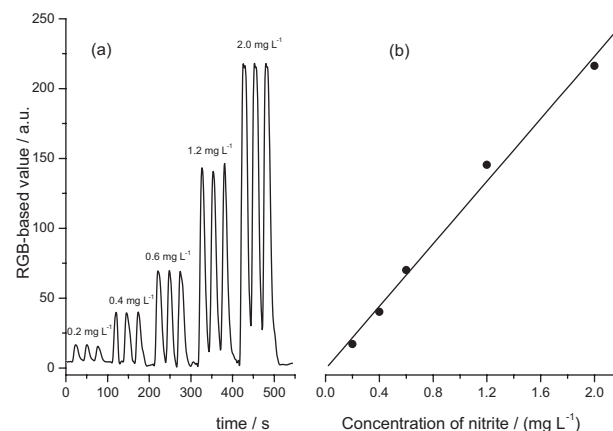
The transient signals and linear calibration graphics are shown in Figures 5 and 6, respectively, for nitrite and

nitrate. The optimized conditions are presented in Table 4. Under the proposed conditions, the dynamic ranges were 0.2 to 2.0 mg L⁻¹ NO₂⁻ and 1.0 to 10.0 mg L⁻¹ NO₃⁻. The linear behavior was described by the equations 3 and 4.

$$RGB\text{-based value} = -0.647 + 112 [NO_2^-] \quad (R^2 = 0.9956) \quad (3)$$

$$RGB\text{-based value} = -16.2 + 20.0 [NO_3^-] \quad (R^2 = 0.9944) \quad (4)$$

The coefficients of variation (relative standard deviation, RSD) were estimated at 0.62% and 1.34% for 10 measures corresponding to solutions of 0.6 mg L⁻¹ NO₂⁻ and 6.0 mg L⁻¹ NO₃⁻, respectively. At a 99.5% confidence level, the detection limits were estimated as 0.01 mg L⁻¹ NO₂⁻ and 0.04 mg L⁻¹ NO₃⁻.

**Figure 5.** (a) Transient signals; (b) calibration curve for determination of the 0.2-2.0 mg L⁻¹ NO₂⁻, using detection by webcam.

Groundwater samples were spiked with 0.6, 1.2 and 1.6 mg L⁻¹ NO₂⁻ and 3.0, 6.0 and 8.0 mg L⁻¹ NO₃⁻ and analyzed by the proposed method. The results are shown in Table 5.

Recoveries ranged from 96.9% to 109.2% for nitrite and 100.0% to 103.2% for nitrate, similar values to those obtained by reference methods NBR 12619²⁷ and APHA 4500-NO₃ I.²¹

Figure 7 shows the results of the determination for nitrite and nitrate in groundwater samples employing both the proposed procedure and the reference. The excellent

Table 5. Mean values and uncertainties (n = 3) for recovery of nitrite and nitrate in groundwater samples by the proposed and reference procedures

Compound added in samples	Fortification / (mg L ⁻¹)	Reference procedure ^{a,b}		Proposed Procedure		Error / %
		Found / (mg L ⁻¹)	Recovery / %	Found / (mg L ⁻¹)	Recovery / %	
Nitrite	1.60	1.56 ± 0.01	97.5	1.55 ± 0.03	96.9	-0.6
	1.20	1.24 ± 0.01	103.3	1.31 ± 0.03	109.2	5.6
	0.60	0.60 ± 0.01	99.5	0.60 ± 0.01	100.0	0
Nitrate	8.00	8.32 ± 0.12	104.0	8.25 ± 0.14	103.1	-0.8
	6.00	6.00 ± 0.18	100.0	6.11 ± 0.11	101.8	1.8
	3.00	2.98 ± 0.07	99.5	3.00 ± 0.16	100.0	0.7

^aNBR 12619 method (for nitrite); ^bAPHA 4500-NO₃-I method (for nitrate).

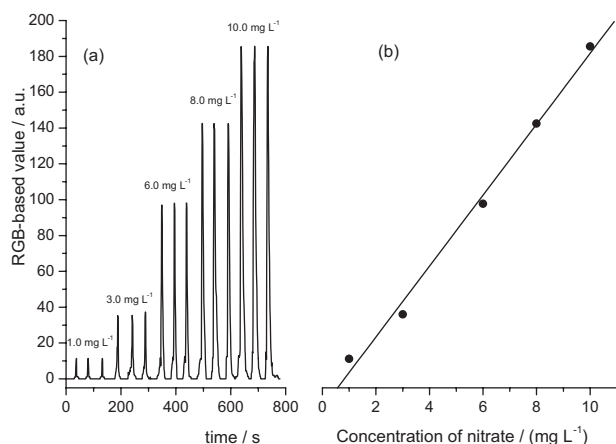


Figure 6. (a) Transient signals; (b) calibration curve for determination of the 1.0-10.0 mg L⁻¹ NO₃⁻, using detection by webcam.

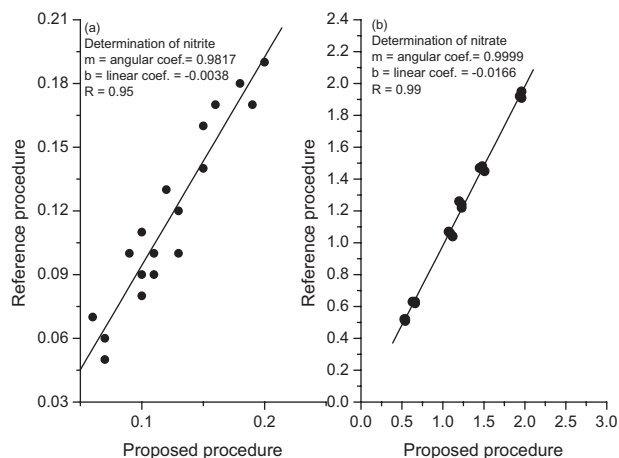


Figure 7. Comparison of webcam detector and conventional method in groundwater samples; (a) determination of nitrite; (b) determination of nitrate.

correlation demonstrates the accuracy of the new procedure and proves the viability the webcam detector.

Conclusions

The proposed flow procedure is robust and very easy to operate, and it has a great potential in analysis to

detect the visible region of the electromagnetic spectrum. In addition, since this characteristic can dispense a wavelength selector, it could be exploited to reduce cost and simplify instrumentation methods for measurements in the visible region. The procedure was implemented with inexpensive instrumentation by exploiting the multicommutation approach and using a webcam as an analytical detector. Low reagent consumption and minimal waste generation offer additional advantages. The method is simple, rapid and inexpensive, and it was successfully applied for the determination of nitrite and nitrate in water samples.

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