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Water-Soluble Humic Acid Quantification Using a Flow-Injection System with and without Sample Pretreatment

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Supporting Information

ABSTRACT: Two automated methods for the determination of humic acids (HAs) in natural water samples are presented. Flow injection systems were developed based on the formation of a complex between humic acids and toluidine blue dye. When the complex is formed, the dye absorbance signal decreases at selected operating conditions. Method A, without sample pretreatment, presents a linear calibration curve over HA concentration range 1.14−35.0 μg mL^{-1}, with a limit of detection (LOD) of 0.4 μg mL^{-1} (3 Sy/x) and a sample throughput of 80 h^{-1}. To determine lower HA concentration levels, a preceding separation/preconcentration step was included (method B). This method has a linear range of 1.5−20.0 μg mL^{-1} and a sample throughput of >8 h^{-1} and could detect humic substances at a concentration of 0.05 μg mL^{-1} after a 4-min retention time. The RSDs for methods A and B calculated using the slopes of seven independent calibration graphs obtained on different days and with different conditions (standard solution, reagent solution, etc.) were 4.5% and 4.2% respectively. Both methods were satisfactorily applied to natural water samples, with recovery percentages in the 95−107% range, and compare very well with other methods.

1. INTRODUCTION

Humic substances (HSs) formed mainly by fulvic (FA) and humic acids (HAs) are ubiquitous components of the natural organic matter present in soil and aquatic environments.[1−3] They represent about 25% of the total organic carbon on Earth and comprise 50−75% of the dissolved organic carbon (DOC) in waters,[4,5] and as such, they play an important role in the riverine ecosystem. They are known to form complexes with various metals[6−8] of importance in groundwater, freshwater, and seawater. The concentration of HAs in natural waters is not known exactly, because of the variability in their composition and the lack of a convenient analytical method.

Several analytical techniques have been used for HA identification and quantification, including electrochemical techniques,[9−13] infrared spectroscopy,[14,15] fluorescence spectroscopy,[16,17] nuclear magnetic resonance spectroscopy,[18] reverse-phase high-performance liquid chromatography (RP-HPLC) using stepwise gradients of dimethylformamide (DMF) in buffered aqueous mobile phase and a wide-pore (30-nm) octadecylsilica column,[19−22] high-performance size-exclusion chromatography,[23] high-performance capillary electrophoresis,[24] and mass spectrometry.[25] Chemiluminescence analysis has also been explored for the quantification of HAs in natural waters.[26−29] Also, a densitometric method with UV detection has been proposed.[30] However, these methods are time- and cost-consuming and/or require complex instruments, and the interferences of coexisting species are significant. Some ultraviolet−visible (UV−vis) absorption methods have also been proposed. For example, a preconcentration and determination method for humic and fulvic acids at trace levels in natural water samples by cloud-point extraction was employed for HA and FA preconcentration prior to their determination using a flow injection (FI) system coupled to a spectrophotometric UV−vis detector.[31] Another simple and rapid method was proposed for humic acid determination at milligram levels in natural waters based on the binding of toluidine blue (TB) dye to HA molecules to produce a dye−HA complex that causes a decrease in absorbance at 630 nm.[32] This method was successfully applied to the determination of HAs in natural water samples (river water). However, rapid and sensitive methods for a quantitative analysis of HAs are still highly desirable.

On the other hand, many analytical procedures have been automated to manage a significant increase in the number of laboratory samples to be analyzed and to satisfy a demand for fast and reliable techniques that can operate at all times, as is often required in process control. Flow techniques, in general, are recognized as powerful and useful methodologies for the automation of many analytical procedures. Their application to the online pretreatment of complex matrixes and subsequent detection of different parameters by spectrometric techniques is well-documented in the literature.[33] The main advantages of the flow injection analysis (FIA) technique are the low consumption of reagents and samples, better repeatability, high sample throughput, ease of medium exchange after analyte accumulation, reduction of the risk of contamination during the

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analysis step, good precision, high sensitivity, good selectivity, and a relatively low cost of the instrumentation.[34]

In this work, two continuous flow systems, one with and one without a separation and preconcentration step, were developed for HA quantification, based on the method proposed by Sheng et al.[32] Both methods are robust, simple, and fast and compare very well with the standard technique.

2. EXPERIMENTAL SECTION

2.1. Apparatus for Methods A and B. Spectrophotometric determinations were performed on a UV−vis spectrophotometer (Shimadzu UV−vis 1700) with a Hellma 178-010 QS flow cell with a 32 μL inner volume.

The continuous flow system consisted of a Gilson Minipuls 3 peristaltic pump for the propulsion system and a Rheodyne 5041 injection valve. All reaction coils were made of polytetrafluoroethylene (PTFE) tubing (0.5-mm i.d.).

The minicolumn consisted of a piece of tygon tubing (6.0-cm length, 2-mm i.d.) filled with XAD-8 resin.

2.2. Reagents and Solutions. Analytical-grade reagents were always used, as well as ultrapure water of Milli-Q quality (18.0 MΩ cm^{-1}) that was free of DOC.

A 1×10^{-3} mol L^{-1} stock solution of toluidine blue was prepared by dissolving a proper amount of $C_{15}H_{16}ClN_3S$ (Merck) in water, placed in an amber bottle, and stored in a refrigerator at 4 °C.

Humic acid (HA) stock solution (Sigma-Aldrich) was prepared after purification by the method proposed in ref 32. An amount of the standard HA solution was dissolved in 0.1 mol L^{-1} NaOH. Then, it was filtered, and the pH was adjusted to 1.0 with HCl (35−37%). Once again, the solution was filtered and washed three times using 0.1 mol L^{-1} HCl. The precipitate was dried and used as the standard. A 0.3 g L^{-1} stock solution was prepared and stored at 4 °C. Calibration solutions were prepared by dilution with water.

Three different samples of real river water with humic contents reported by an external laboratory were provided by the Geochemistry research group from Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Córdoba, Argentina, for analysis in this work. Moreover, two samples from a small lake in Sarmiento Park in Córdoba city were analyzed. All samples were previously filtered with a 0.45-μm filter to remove particulate matter.

The following solutions were used in Method A: A 0.5 mol L^{-1} EDTA solution was prepared from $C_{10}H_{14}N_2Na_2O_8{\cdot}2H_2O$ (Anedra) dissolved in water. To prepare a 0.05 mol L^{-1} sodium citrate−phosphate buffer solution, $C_6H_5Na_3O_7{\cdot}2H_2O$ (Anedra) was dissolved in water, H_3PO_4 (Carlo Erba) was added to obtain pH 7.0, and the solution was brought to volume with water. Reagent solution A was prepared by mixing 17.0 mL of 1×10^{-3} mol L^{-1} toluidine blue solution and 20.0 mL of 0.5 mol L^{-1} EDTA solution and making up the volume to 250 mL with buffer solution.

The following solutions were used in Method B: To prepare 0.1 mol L^{-1} sodium hydroxide solution, the proper amount of NaOH (Merck) was weighed and dissolved in water. Reagent solution B was prepared by mixing 15.00 mL of 1×10^{-3} mol L^{-1} toluidine blue solution in 250 mL of 0.03 mol L^{-1} phosphoric acid. For the separation/preconcentration step in this method, Amberlite XAD-8 resin was used.

Analyte calibration solutions were prepared by appropriate dilution of stock solution in 0.1 mol L^{-1} NaOH.

2.3. Procedures. *Method A.* The developed double-channel FIA system is presented in Figure 1. The sample

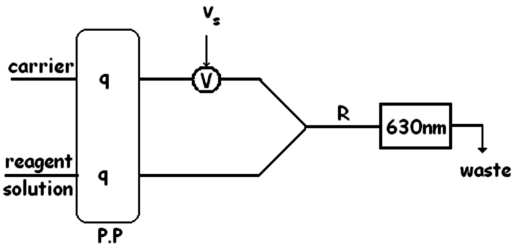

Figure 1. Schematic of the developed double-channel FIA system. PP, peristaltic pump; V_s, sample volume (150 μL). Method A: q, flow rate (both at 1.79 mL min^{-1}); reagent solution A, toluidine blue (1.3×10^{-4} mol L^{-1}) + EDTA (0.04 M) + buffer citrate/H_3PO_4 (pH 7); R, reactor (500 mm). Method B: q, flow rate 1.65 mL min^{-1}; reagent solution B, toluidine blue (0.6×10^{-4} mol L^{-1}) + H_3PO_4 (0.03 mol L^{-1}); R, reactor (600 mm).

without pretreatment was injected into a carrier solution (buffer, pH 7) and merged with reagent solution A (toluidine blue solution + EDTA solution + buffer solution) in the reactor (R). A baseline decrease as the sample passed through the flow cell was observed, and the difference in absorbance (ΔA) measured at 630 nm was recorded. The concentration of humic acids was then obtained using a calibration curve.

Method B. This method was developed to separate and preconcentrate the analyte before performing measurements. It was carried out in two separate steps, one for preconcentration and the other for quantification.

First Step: Sample Pretreatment. Figure 2 shows the system used to carry out sample preconcentration. This system

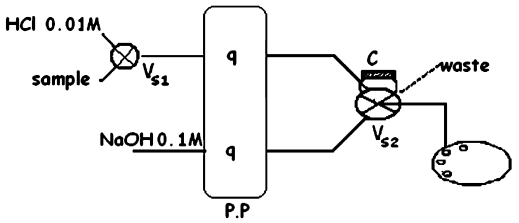

Figure 2. Separation/preconcentration system. PP, peristaltic pump; q, flow rate (1.07 mL min^{-1}); V_{s1} and V_{s2}, selection valves; C, column packed with XAD-8 resin (length, 60 mm; i.d., 2 mm).

incorporated a minicolumn filled with XAD-8 resin preceding the valve sample loop (C in Figure 2). This resin separates humic acids from other components.[34]

Samples were prepared in 0.1 mol L^{-1} HCl to favor the adsorption on the resin.

Resin regeneration was performed by passing a 0.01 mol L^{-1} HCl stream for 50 s (regeneration time). Then, the sample was loaded by switching valve V_{s1} for a fixed time (sample loading time), and the sample was discarded through valve V_{s2} after passing through the column. After this time, valve V_{s1} was switched again, and a stream of 0.01 mol L^{-1} HCl rinsed the system for 30 s (rinse time). Afterward, valves V_{s2} and V_{s1} were switched simultaneously, and the eluent (0.1 mol L^{-1} NaOH) passed with back-elution through the column washing the humic acids that had collected into graduated vials (40 s) (elution time). Finally, valve V_{S2} was switched, and a NaOH

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stream was passed through the column for 30 s (cleaning time) before the cycle was started again.

Second Step: Quantification. The second step was performed on the same FIA manifold as used in method A (Figure 1) but with other reagents (see Method B in section 3.1). Therefore, preconcentrated samples were injected into carrier solution (0.1 mol L^{-1} NaOH) and merged with reagent solution B (0.6×10^{-4} mol L^{-1} toluidine blue and 0.03 mol L^{-1} H_3PO_4) in the reactor (R). Measurements were carried out in the same way as for method A. The HA concentration was obtained using the same calibration curve and considering the sample loading time.

3. RESULTS AND DISCUSSION

3.1. Optimization of Chemical and FIA Variables and Analytical Parameters. The developed method is based on complex formation between humic acids and toluidine blue dye. The formation of the dye−humic substance complex diminishes the dye absorbance at selected operating conditions, as reported elsewhere[36] (Figure 3).

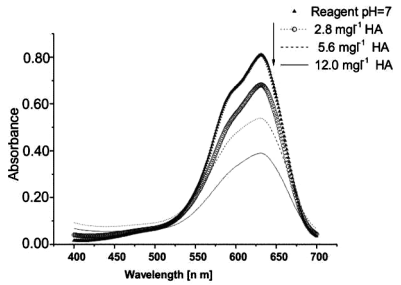

Figure 3. Interaction between toluidine blue dye and humic acids in aqueous solutions.

The performances of methods A and B were optimized by using the univariant method for all variables.

Method A. Method A was developed to improve sensitivity, decrease the detection limit, and obtain a higher sample throughput compared to the batch process.[32] Based on previously studied characteristics of dye−HA complex formation, different FIA manifolds were tested. The system that presented the best reproducibility and peak formation and height is depicted in Figure 1.

Formation of the dye−HA complex results in the decrease of the dye absorbance at different pH values. This absorbance difference (ΔA) is significant starting at pH 7.0 and increases with increasing pH up to 10. However, bearing in mind that proteins and polysaccharides present higher interference at higher pH values and considering that this interference is at a minimum at pH 7.0,[32] this pH value was used in method A (method without pretreatment). To control the pH, a carrier solution of 0.05 mol L^{-1} sodium citrate−phosphate buffer was used.

The reagent solution was prepared by mixing toluidine blue, EDTA, and buffer solutions. To establish the optimal toluidine blue concentration, different HA calibration curves were obtained at different toluidine blue levels in the concentration range from 5.0×10^{-5} to 5.0×10^{-4} mol L^{-1}. The analysis was carried out comparing the slopes of the calibration curves, and a concentration of 1.3×10^{-4} mol L^{-1} toluidine blue was selected as the optimum, as it presented the best analytical sensitivity. To maintain the optimum pH value (approximately 7.0), toluidine blue solution was prepared in buffer solution.

EDTA was used to eliminate interference from cations, which are commonly present in natural water samples. It was tested in the concentration range of 0−0.08 mol L^{-1}, and 0.04 mol L^{-1} was selected as the optimum value, as this concentration did not promote signal interference and was high enough to eliminate possible interferences.

The FIA variables were optimized, and these values and their ranges are listed in Table S1 of the Supporting Information. The calibration curve was linear over the humic acid concentration range of 1.14−35.0 μg mL^{-1}, represented by $\Delta A = [0.0140\ (\pm 0.0002)]C + 0.0071\ (\pm 0.0021)$, where ΔA is the absorbance difference and C is the concentration of humic acids (μg mL^{-1}), with $R^2 = 0.9994$; the limit of detection (LOD) was found to be 0.4 μg mL^{-1} (3 Sy/x), and limit of quantification (LOQ) was 1.33 μg mL^{-1} (10 Sy/x). The interday reproducibility (RSD, %) calculated using the slopes of seven independent calibration graphs obtained on different days and with different conditions (standard solution, reagent solution, etc.) was 4.5%, and the sample throughput was 80 h^{-1}.

Method B. To reach lower concentration levels of humic acids than determined by the proposed method A, a continuous flow injection system with an online XAD-8 packed column as a separation/preconcentration reactor was developed (Figure 2). Use of XAD-8 resin is one of the most frequently applied methods for humic acid isolation and purification, and it was adopted as the standard method for isolating and fractionating humic acids by the International Humic Substances Society.[37,38] Humic acids are retained by XAD-8 resin at a pH of ca. 2 with hydrochloric acid, whereas 0.1 mol L^{-1} NaOH is an effective eluent. A clean resin aliquot[34] in its hydrogen form was packed inside a PTFE tube (2-mm i.d.) with a 6.0-cm length of resin. All variables and ranges tested are listed in Table S2 (Supporting Information).

As already mentioned, 0.1 mol L^{-1} NaOH solution was used as the eluent. It is worth noting that it was not possible to place the resin column in the injection loop in the quantification system (Figure 1), as doing so caused serious signal disturbances. For this reason, this step was performed separately.

The eluated solution was injected into the FIA system shown in Figure 1. As the eluate had an alkaline pH value, it was necessary to change the operating conditions. For that reason, 0.1 mol L^{-1} NaOH was selected as the carrier, and standard solutions were prepared in this medium. Other reagents and their concentration values were optimized in the same way as described before for method A.

In method B, a different reactive solution was used. Specifically, EDTA solution was discarded, as humic acids were separated before being determined, so cations did not interfere. Toluidine blue was prepared in an acid medium to decrease the final pH, as the pH must be close to 7 to diminish interferences from probable polysaccharides and proteins. Therefore, to obtain an appropriate pH value in the confluence point after mixing with the carrier solution (NaOH), phosphoric acid addition was tested. Different acid concentration values were studied in the range of 0.01−0.1 mol L^{-1}, and 0.03 mol L^{-1} phosphoric acid was selected. Under these conditions, the obtained final pH value was 7.5 ± 0.3, producing peaks with appropriate shapes and heights. The toluidine blue concentration was varied in the range from 5.0×10^{-5} to 5.0×10^{-3} mol L^{-1}, and the selected concentration was

6.0×10^{-5} mol L^{-1}, as it presented the best analytical sensitivity.

Again, the FIA variables were tested, and their optimum values are listed in Table S2 (Supporting Information). The calibration graph obtained by injecting standard solutions without retention was linear over the HA concentration range of 1.5−20.0 μg mL^{-1} (see Figure 4). The LOD was 0.98 μg

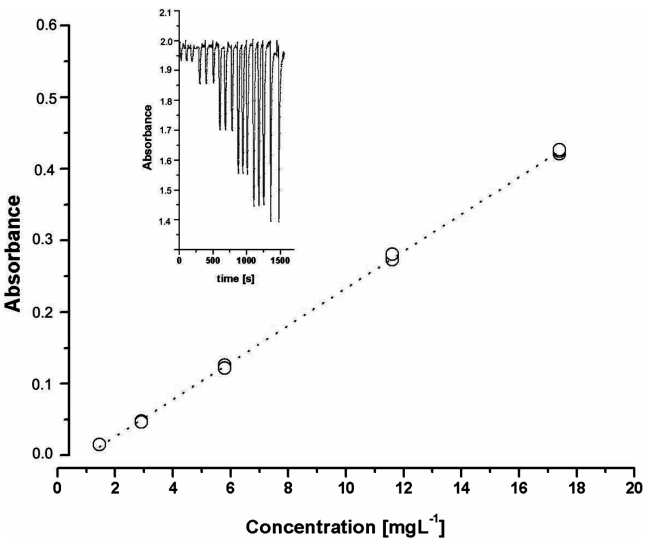

Figure 4. Calibration curve for method B: ΔA = [0.0259 (±0.0001)] C_{HA} μg mL^{-1} − [0.0255 (±0.0013)], R^2 = 0.9996. Inset: FIA peaks.

mL^{-1} (3 Sy/x), and the LOQ was 1.8 μg mL^{-1} (10 Sy/x). The RSD was 4.2% (reproducibility obtained from the slopes of seven calibration graphs obtained on different days with freshly prepared solutions). The sample throughput varied between 8 and 20 h^{-1} depending on the sample retention time used. Figure 5 shows the signal increase for different HA concentrations at different retention times. As can be observed, the relationship was always linear. Furthermore, experimentally, it was found that, after a retention time of 4 min, humic substance could be detected at a concentration of 0.05 μg mL^{-1} with an RSD of 4.5% (n = 5). However, if necessary, to

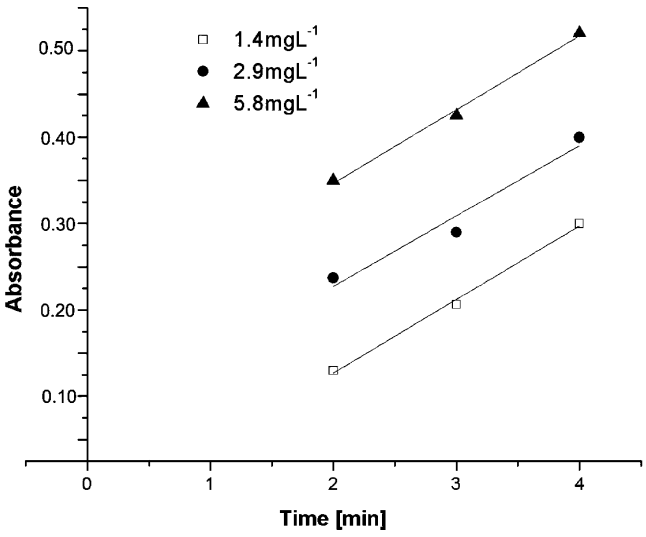

Figure 5. Increasing signals for different HA concentrations at different retention times.

determine lower HA concentrations, longer retention times or column lengths could be used.

3.2. Interferences. The effects of foreign ions commonly present in natural water samples were evaluated for 16.8 μg mL^{-1} humic acids determination (method A). A signal relative error of >5% was considered as interference (see Table S3, Supporting Information). The same species in the same proportions as used previously were tested by applying the separation/preconcentration step over 2.90 μg mL^{-1} (method B, 2-min retention time), and relative errors of less than 5% were obtained.

3.3. Application to Real Samples. To assess the quality of the obtained results with the proposed methods for humic acids determination in natural water, the procedure suggested by Massart et al.[39] was applied. Several samples were analyzed by both proposed procedures (methods A and B). Table 1 reports

Table 1. Recovery Percentages from Humic Acids Added to Natural Water Samples and Comparison of Results Obtained with Reported Values

	amount (mg L^{-1})				
sample[a]	added	found (value ± SD[b])	recovery (%)	reported[c] (value ± SD[b])	error
		Small Lake I			
method A	0	2.6 ± 0.2	–	2.8 ± 0.4	−7.1
	8.7	11.2 ± 0.8	99	–	–
method B[d]	0	2.9 ± 0.4	–	3.0 ± 0.3	−3.3
	2.9	5.9 ± 0.6	103		–
		Small Lake II			
method A	0	2.8 ± 0.2	–	2.7 ± 0.3	3.7
	8.7	11.1 ± 0.1	95	–	–
method B[d]	0	3.0 ± 0.3	–	2.8 ± 0.5	7.1
	2.9	5.8 ± 0.6	97	–	–
		Suquía River (Santa Fe Bridge)			
method A	0	1.2 ± 0.4	–	<LOD[e]	–
	8.7	10.5 ± 0.8	107	–	
method B[f]	0	1.1 ± 0.3	–	<LOD[e]	–
	1.4	2.5 ± 0.6	95		
		Suquía River (Glen)			
method A	0	1.8 ± 0.2	–	1.4 ± 1.0	>10
	8.7	10.9 ± 1.0	105	–	–
method B[f]	0	1.7 ± 0.7	–	1.6 ± 0.9	5.9
	1.4	3.3 ± 0.6	107	–	–
		Primero River			
method A	0	5.5 ± 0.3	–	5.9 ± 0.3	−6.8
	8.7	14.4 ± 0.8	102	–	–
method B[d]	0	5.1 ± 0.6	–	4.7 ± 0.8	8.5
	1.4	6.5 ± 0.4	97	–	–

[a]Córdoba province, Argentina. [b]SD = standard deviation (n = 5). [c]Using standard methods.[35] [d]Loading time = 2 min. [e]LOD = limit of detection. [f]Loading time = 4 min.

the humic acids contents from natural water samples and the recovery percentages after sample spiking. In addition, results obtained for the river samples by an external laboratory are also presented. As can be observed in Table 1, the results from both proposed methods are in good agreement with those provided by the external research group (3−10% error). Furthermore, the recovery percentages obtained by both proposed methods

Table 2. Comparison with Other Methods

LOD	linear range	RSD (%)	ref (year)	detection
1.3 μg	$\leq$80 μg	3.2 (42.6 μg)	30 (1991)	densitometry on Amberlsyt A-27[a]
5 μg L^{-1}	$\leq$1 mg L^{-1}	3.1 (0.2 mg L^{-1})	31 (2003)	FI–UV[a]
800 μg L^{-1}	$\leq$40 mg L^{-1}	3.5 (20 mg L^{-1})	32 (2007)	spectrophotometric[b]
270 μg L^{-1}	60 and 600 μg L^{-1}	not reported	13 (2007)	stripping voltammetry
240 μg L^{-1}	0.5–20.0 mg L^{-1}	1.4–8.1 (10 mg L^{-1})	27 (2005)	chemiluminescence[b]
500 μg L^{-1}	0.5–20 mg L^{-1} 20–50 mg L^{-1}	0.294 (0.5 mg L^{-1})	40 (2010)	spectrophotometric (FI–UV)
400 μg L^{-1} (method A)	1.14–35.0 mg L^{-1}	4.5[c]	this work (2013)	spectrophotometric (FI–UV), method A[b]
50 μg L^{-1} (method B)	1.5–20 mg L^{-1}	4.2[c]	this work (2013)	spectrophotometric (FI–UV), method B[a]

[a]With preconcentration step. [b]Without preconcentration step. [c]RSD obtained using seven calibration graphs.

were in the range of 95–107%, indicating that these methods can be used for humic acids determination in natural waters.

4. CONCLUSIONS

The developed methods with spectrophotometric detection proposed for HA determination in natural waters are an interesting alternative to other methodologies. They provide high-quality results in terms of accuracy and precision (RSD $\approx$ 4%). Method B, with a separation/preconcentration step, is based on the same reaction and allows a considerable decrease in detection limit.

The analytical parameters provided by the developed methods are comparable to those of other proposed methods (Table 2). However, they have the advantage that both the reagents and equipment used are relatively inexpensive and readily available. Moreover, the spectrometric technique is always available in routine laboratories.

Both methods were also satisfactorily applied to natural water samples (lake and river water).

■ ASSOCIATED CONTENT

Supporting Information

Additional supporting data tables. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

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