Micropaper-based analytical device (μPAD) for the simultaneous determination of nitrite and fluoride using a smartphone

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A B S T R A C T

The design, optimization and evaluation of a micro paper-based analytical device (μPAD) for the simultaneous determination of fluoride and nitrite in real water samples was developed. In order to create hydrophilic and hydrophobic zones on the laboratory filter paper device, a wax ink printer was used. The μPAD has four reaction zones for each analyte where the respective colorimetric reactions took place using the SPADNS method and Griess method for fluoride and nitrite respectively. A Smartphone was used to capture the corresponding images which were then converted to the RGB colour space by the software ImageJ using a personal computer. Under the optimal conditions, the linear range was 0.23–2.26 mg L^{-1} for fluoride with limit of detection (LOD) and limit of quantification (LOQ) of 0.13 mg L^{-1} and 0.25 mg L^{-1}. The working range for nitrite was 0.05–10.0 mg L^{-1} with LOD of 0.03 mg L^{-1} and LOQ 0.13 mg L^{-1}. The μPAD was used to determine the analytes in water samples obtaining satisfactory results in both cases. The validation of fluoride determination was performed comparing the μPAD with the reference method and for nitrite determination, a recovery study was carried out. In both cases, the obtained results demonstrated a good agreement.

1. Introduction

Due to the increasing impact of industry, growing population, urbanization and many other human activities, water pollution is becoming a serious concern affecting human beings.

Nitrite (NO$_2^-$) and fluoride (F$^-$) are two classes of inorganic contaminants present in water sources. The first is widely used as an additive in nitrogen based fertilizers [1] and food preservatives [2]. High levels of nitrite in drinking water can trigger numerous human diseases such as metahemoglobinemia [3,4] or stomach cancer through the formation of N-nitrosamines. The European Commission and the Código Alimentario Argentino have regulated the maximum value for this ion in drinking water as 0.5 and 0.1 mg L^{-1} respectively [5,6]. Concentrations above these indicate contamination. On the other hand, fluoride ion is one of the most important anions because of its role in the prevention of osteoporosis and dental cavities [7]. However, the excess of fluoride intake, over a long period of time, may result in serious diseases such as gastric and kidney disorders and fluorosis [8,9]. Fluoride is naturally incorporated in water sources through the weathering and dissolution of rocks and soil [10]. In Argentine and other regions of the world, there are some typical fluoride-rich regions affecting millions of people [11]. According to the European Commission, fluoride ion (F$^-$) is detrimental to human health when its concentration in drinking water is higher than 1.5 mg L^{-1}, however, for the Código Alimentario Argentino it is already dangerous if it is in a range of 0.7 to 1.2 mg L^{-1} [5,6].

Some of the most common methods developed for determining nitrite in water samples are spectrophotometric [12,13], chemiluminescent [14,15], chromatographic [16], spectrofluorimetric [17–19], electrochemiluminescent [20] and electrochemical ones [21,22]. In the case of fluoride, predominantly non-spectroscopic techniques have been used, such as Fluoride Ion Selective Electrode (F-ISE) [23] or ion chromatography (IC) [24]. Many others, including UV–Vis spectrophotometry [25], fluorimetry [26] and capillary zone electrophoresis [27] have been also reported.

Since water quality has become a global concern, the quality control of drinking water is crucial. Therefore, it is necessary to develop simple, low cost, portable and user-friendly methods for this purpose [28,29]. Analytical methods that use paper-based analytical devices (μPAD) satisfy these characteristics. The μPADs are devices developed with a cellulose substrate on which hydrophobic barriers are created, generating hydrophilic channels. They are essentially microfluidic tools made from different types and qualities of paper substrate. A wide variety of methods such as photolithography [30], polymer screen printing [31], cutting [32], inkjet etching [33] and wax printing [34] have been referred to its manufacture. The μPADs are a promising

alternative to traditional laboratory tests because they allow the collection of analytical information through a rapid and inexpensive analysis [35]. μPADs offer many advantages since they are simple and portable platforms which require just a drop of sample for detecting various analytes such as: proteins, environmental contaminants, pathogens, chemicals, heavy metals and drugs [36–38].

The detection on a μPAD can be carried out visually by comparing the colour intensity in each reaction zone. In order to obtain this colorimetric information, apart from the human eye [39,40], various devices such as cameras [41,42], scanners [43–45] and smartphones [46] can be used. By using a suitable software application it is possible to get the parameters of any colour space from digital picture of μPADs. These parameters can be used to obtain the colour intensity vs. concentration regression line. For example, the RGB colour space applied to digital images gives three channels of information that can be used to generate a calibration curve [47]. Thus working in this way, the digital picture can be sent and analyzed elsewhere around the world, being this, another significant advantage in favor of these kinds of devices.

Many years ago, analytical methods usually required dangerous reagents and generated toxic residues. Nowadays, the trend is the redesign of chemistry, especially analytical chemistry, with the aim to achieve the principles of green chemistry [48]. By using non-toxic solvents, biodegradable raw materials and putting greater emphasis on on-site analysis, the lab-on-paper becomes a perfectly adjustable option, being able to project in the near future, a totally sustainable chemistry that generates the least possible impact on the environment.

According to previous literature on the topic, there are previous studies on the use of paper-based devices for nitrite determination utilizing the same reagent (NED) [49,50]. However, the employment of a system for simultaneous detection of fluoride and nitrite has not been reported, until now.

The aim of this work is the development of a new paper-based analytical device printed with wax ink and composed by 8 reaction zones (4 for each analyte) and one sampling zone. The new μPAD was used for the simultaneous determination of nitrite and fluoride in real water samples employing a mobile phone for detection. The determinations were performed using the traditional SPADNS [51] and Griess [52] reagents for fluoride and nitrite respectively. The analysis of four water sample replicates took only 12 min. This newly developed, inexpensive and user-friendly μPAD allowed an analytical process miniaturization with such inherent advantages as in situ monitoring and transportability.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared from analytical grade chemicals and using ultra pure water (18.0 MΩ) provided by Milli-Q system (Millipore, Bedford, USA). Stock solutions of sodium fluoride and sodium nitrite (Sigma, Buenos Aires, Argentine) were prepared dissolving a suitable amount in water in order to obtain concentrations of $1\,mg\,L^{-1}$. These solutions were stored at 2 °C until their use. Standard working solutions of fluoride and nitrite were prepared daily diluting the above mentioned stock solutions with ultrapure water. Phosphoric acid 5%, sulfanilamide 2% and N-(1-naphthyl) ethylenediamine (NED) 0.2% were acquired from Sigma Aldrich Co. (Saint Louis, MO, USA). SPADNS reagent (4,5-Dihydroxy-3-(*p*-sulfophenylazo)-2,7-naphthalene disulfonic acid, trisodium salt) was purchased from Hach (USA). Xerox solid ink 8400 (black, yellow, magenta and cyan) was acquired from Xerox Argentine and filter paper CLT 597 Whatman S&S (Ref. 10311887, basis weight 85 g/m²; thickness 190 μm; retention 4–7 μm) was used.

2.2. Instrumentation

μPADs were designed using the software Inkscape from Free Software Foundation, Inc. After that, a Xerox Phaser 8400 wax ink printer was used for printing them, and a Smartphone (Motorola Moto G5 Plus) was selected to capture the photos. Then, the free software ImageJ 1.50i was used to analyze them. An Agilent 8453 UV–visible spectrophotometer, a fluoride selective electrode (Van London Co.), Orion pH/ISE meter 710A and San Jor SE60A laboratory furnace were also used.

2.3. Image capture

In order to assess the change of colour after the reaction with the nitrite and fluoride solution in the μPAD, a photo in JPEG format was taken using a Smartphone. The device was photographed under the following optimized conditions: ISO 64, shutter speed 1/187, aperture value f/1.7 and focal length of 4.28 mm.

The PAD was placed into a 14 × 14 × 30 cm homemade square prism made of plied wood (3 mm of width) to isolate it from external light and for keeping the same lighting conditions. The box was painted black on the inside and 16 led lamps were placed on the bottom of the box, in two rows of 8 lamps each. The rows were separated by a distance of 2 cm. The distance between the lamps on each of the two rows was 1 cm. The Smartphone was placed on top of the box and an orifice for the lens was prepared in the centre to provide a perfect shot of the μPAD. The μPAD was located halfway between the base of the box and the hole made for the camera lens.

2.4. Image processing

As to complete the colour evaluation, RGB colour space was utilized. Using ImageJ 1.51q software, the images were processed to obtain the values of the three different RGB colour space channels. A single circular selection (2400 pixels) in every reaction zone was used and, the distribution of the results of each pixel was included into a histogram graphic.

The obtained RGB values were used for the analytical study: in the case of Fluoride, the G parameter showed a linear response to concentration. On the other hand, in the case of nitrite, the difference between the G and B channels was taken as the analytical response.

2.5. Samples

Six samples, from different places, were analyzed: three from Coronel Dorrego, Buenos Aires province (groundwater (A), tap water (B) and tap water treated with inverse osmosis (C)), other two from Bahía Blanca, Buenos Aires province (tap water (D) and filtered tap water (E)), and the last one was taken from Abramo (F), La Pampa province. All samples were from Argentina and they were collected in 2017. The samples from Coronel Dorrego were strategically selected due to their high content of fluoride.

2.6. Procedure

The traditional colorimetric reactions of Griess for NO_2^- and SPADNS for F^- were selected for this study (Fluoride: from Pale Violet Red (R 219G 112 B 147) to Old Orchid (R 227G 173 B193); Nitrite from Beige Grey (R180 G173 B 169) to Old Lilac (R 176 G 119 B 140). Fig. 1 shows the device used to carry out the simultaneous determinations. First of all, 0.5 μL of each reagent were placed in the corresponding reaction areas, selecting four zones for NO_2^- and the remaining four for F^-. Then, a 20 μL water sample was introduced into the centre of the sampling zone. The fluid began to flow along the channels towards the reaction areas and after two minutes, the whole sample reached the zone where the reagents were confined. Ten minutes later, photographs
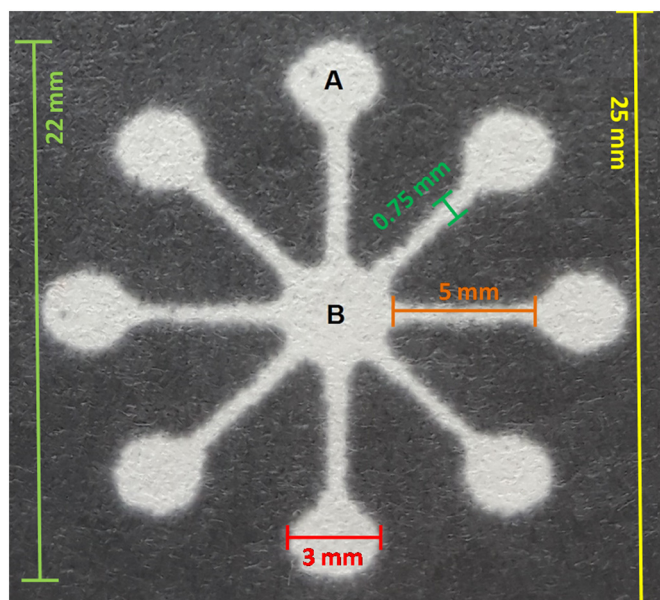
Fig. 1. Real photography and detail of the dimensions of the selected device. A: reaction area; B: sampling area.
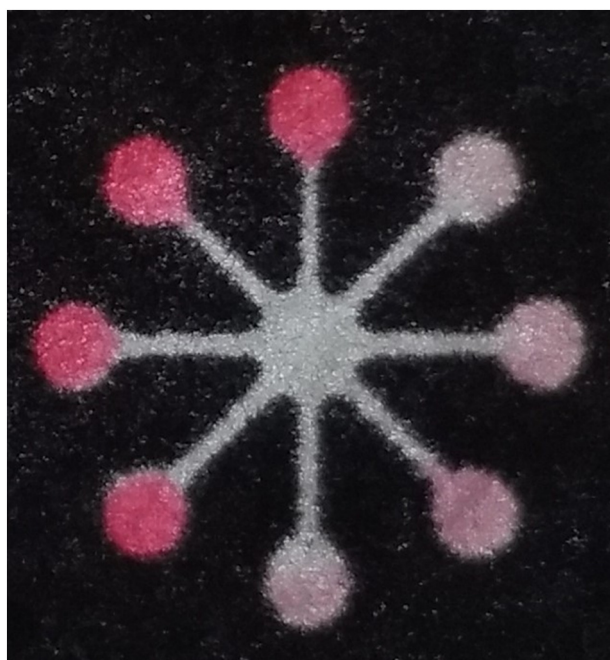

Fig. 2. Real photography of a µPAD after reaction.

were taken using a Smartphone camera (see Fig. 2) and sent to a computer, first to obtain the corresponding values for each channel of the RGB colour space, and then to process the data.

3. Results and discussion

3.1. Fabrication of micropads

3.1.1. Design

Paper-based devices were designed using the Inkscape program and printed on laboratory filter paper. The reaction and sampling areas of the µPAD are in the shaped of an eight spike asterisk and present four reaction zones for each analyte where the respective colorimetric reactions took place. Several designs were analyzed to obtain the optimal microfluidic performance and the best illumination to be photographed.

The different models tested are shown in Fig. 3. Considering many variables, the model selected was "d", since it showed a more homogeneous distribution of the sample, identical detection times in all reaction zones and greater number of determinations per square centimeter of the device. Besides, this model also presented the simplest introduction of the sample. The dimensions shown in Fig. 1 are selected in order to generate hydrophilic channels wide enough to allow an appropriate flow of the sample without increasing the volume and the determination time. The dimensions of the reaction zones were suitable to contain the 0.5 µL of reagent required for the determinations. On the other hand, the outer sector of the device was printed to improve the parameters of the photographic capture. The final dimensions of the µPAD were 25 × 25 mm with an effective area of 221 mm^2.

3.1.2. Paper selection, printing and melting

The selection of the appropriate type of paper was made taking into account the quality of the substrate and the homogeneity of the results. Devices of various sizes and shapes were printed and then heated so that the wax would be absorbed. Tests were made with napkins, tissues, toilet paper and filter paper without any type of glazing. CLT 597 Whatman S&S filter paper showed the best characteristics to resist the complete process of fabrication and, at the same time, presented precise results.

The µPADs used in all experiments were obtained by wax ink printing on filter paper, utilizing a conventional Xerox Phaser 8400 wax ink printer (working under the manufacturer's pre-established conditions). After that, the printed paper was heated on a laboratory furnace for 2 min to allow the penetration of the wax into the cellulose fibers to generate hydrophobic barriers and hydrophilic channels. It is worth clarifying, that when heating the paper for 2 min by furnace, appreciable damage could not be seen, contrary to what happened when a hot plate was used. This is probably due to the homogeneous, controlled and fast way to apply the heat in the furnace. In order to create an impermeable barrier, adhesive tape was used on the back of the device so as to avoid liquid permeation.
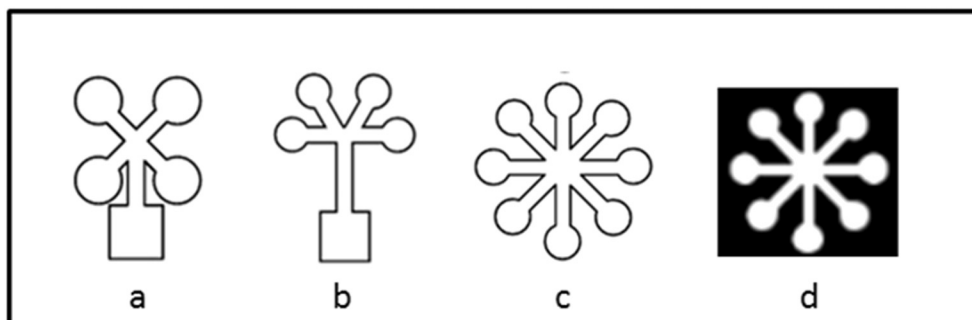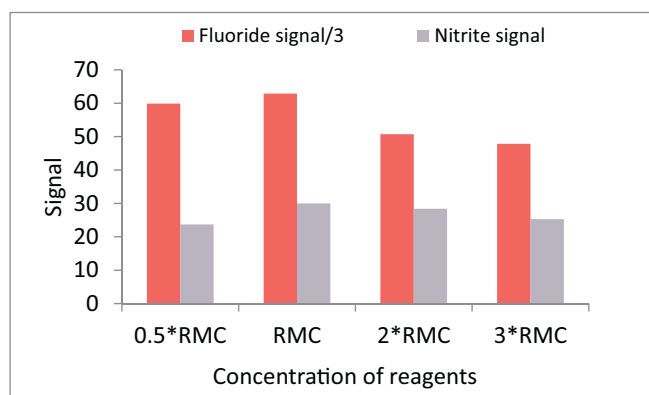

Fig. 3. Tested devices.

Fig. 4. Optimization of reagent concentrations. RMC: Reference Method Concentration.

3.2. Optimization of reagent and sample volumes

In order to assess the optimal concentration of reagents to be used, different experiments were carried out. As it can be seen in Fig. 4, the best condition for nitrite and fluoride determination was the concentration recommended by the reference methods.

The optimal volume of reagents to be deposited on the reaction area was 0.5 μL. Lower volumes were not enough to cover it, leading to an irregular distribution in the reaction zone and, higher volumes caused an advance of the reagent through the sample channel generating reactions outside the detection area. 20 μL of sample was the optimal volume to fill up a μPAD because lower volumes did not reach the reaction zones, and higher volumes caused the colored products to move towards the sampling zone through the hydrophilic channels.

3.3. Optimization of the reaction time

In order to know the most appropriate time at which the images should be captured, a study of signal intensity vs. time was carried out. In the case of fluoride, two minutes after the contact between the reagent and the sample, the signal kept constant. For nitrite, the reference method [52] establishes that spectrophotometric measurements should be taken between 10 and 120 min after adding the Griess reagent. When the study was performed using the μPAD, the optimal time for taking photographs was just 10 min (see Fig. 5a). In this way, the reaction time was in accordance with the reference method (see Fig. 5b).

3.4. Analytical performance

Working under the optimized conditions, a $Green_{int} = (12.43 \pm 0.38)$ $[F^-] + (38.52 \pm 0.52)$ regression equation for fluoride was

Table 1
Results for fluoride determination applying the μPAD and the reference method in real water samples.

Fluoride		
Sample	μPAD Found[a] (SD)[b] [mg L^{-1}]	Reference method Found[a] (SD)[b] [mg L^{-1}]
A	2.34 (0.39)	2.64 (0.03)
B	1.84 (0.10)	1.80 (0.01)
C	0.42 (0.06)	0.40 (0.01)
D	0.36 (0.08)	0.40 (0.01)
E	0.37 (0.07)	0.39 (0.01)
F	0.19 (0.16)	0.24 (0.01)

[a] Average of 8 replicates.
[b] Standard deviation.

Table 2
Results of nitrite recovery study.

Nitrite			
Sample	Added [mg L^{-1}]	Found[a] (SD)[b] [mg L^{-1}]	% Recovery[a]
A	1.00	1.01 (0.03)	100.9
	3.00	2.88 (0.07)	96.1
	7.50	7.64 (0.13)	101.9
B	1.00	1.00 (0.03)	99.7
	3.00	2.92 (0.11)	97.3
	7.50	7.69 (0.26)	102.6
C	1.00	0.94 (0.03)	93.9
	3.00	3.06 (0.14)	101.9
	7.50	7.59 (0.14)	101.2
D	1.00	0.96 (0.04)	96.0
	3.00	2.98 (0.17)	99.5
	7.50	7.54 (0.27)	100.5
E	1.00	1.06 (0.06)	106.4
	3.00	3.10 (0.07)	103.4
	7.50	7.53 (0.19)	100.4
F	1.00	0.99 (0.05)	99.4
	3.00	3.18 (0.04)	106.1
	7.50	7.55 (0.17)	100.7

[a] Average of 8 replicates.
[b] Standard deviation.

obtained ($R^2 = 0.996$), with a linear range of 0.23–2.26 mg L^{-1}. The detection limit (LOD) was 0.13 mg L^{-1}, and the corresponding quantification limit (LOQ) was 0.25 mg L^{-1}, both calculated according to IUPAC [53]. The relative standard deviation (%RSD) values were calculated from 8 independent measurement of each real sample. In terms of repeatability (intra-day, same μPAD) the obtained values were lower than 3.8% and in terms of intermediate precision (inter-day, different μPADs) they were lower than 6.3%.

In the case of nitrite determination, the working range was 0.05–10.0 mg L^{-1} and the calibration curve was exponential, working with a signal corresponding to the difference between Blue and Green
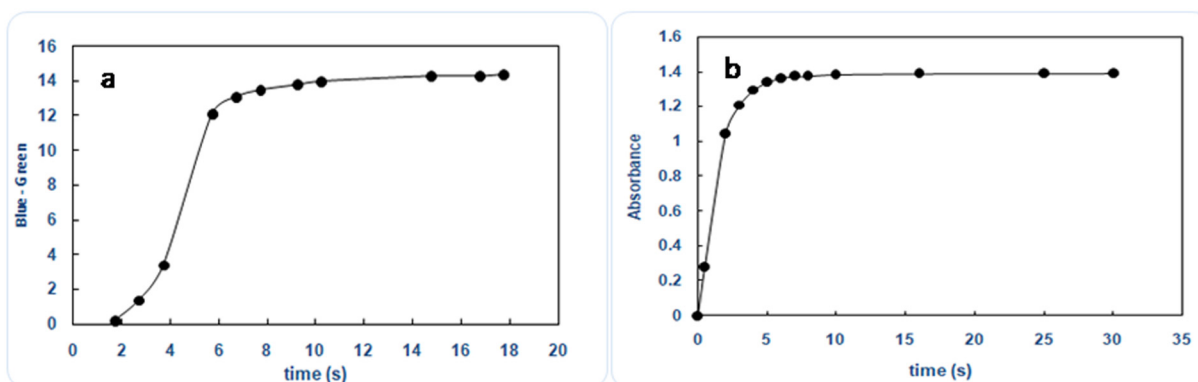

Fig. 5. Optimization of reaction time of nitrites. A: proposed μPAD; B: reference method (3.00 mg L^{-1}).

Table 3
Comparative parameters from different methods.

Samples	Preparation of PAD	Reagents	Detection	Analytical characteristics of µPAD	Reference
Natural water (20 µL)	Inkjet printing method	Griess reagent (1 µL)	Flatbed scanner (Canoscan Lide 700f)	Working range: 10–150 µM LOD 1.0 µM RSD < 2.9% ($n \le 8$)	[49]
Artificial saliva (5 µL)	Photolithography Polymer blend: 1:1 mixture of Zipcone UA, acryloxy-terminated siloxane polymer and Norland Optical Adhesive 74 (NOA 74), an acrylate based polymer.	Modified Griess reagent: (citric acid) (0.5 µL)	Flatbed scanner (Microtek Scanmaker 4800).	Working range: 5 to 2000 µM LOD: 5 µM	[50]
Natural water (10 µL)	Craft-cutting technique Reagent 3,6-bis(3,5-dimethyl-1H-pyrazol-1-yl)-1,4-dihydro-1,2,4,5-tetrazine (DHBPTz) is immobilized	Citric acid (1 µL) DHBPTz (1 µL)	Canon PowerShot G12 digital camera and iPhone 4.0 Smartphone	Working range: 5–500 (µM) LOD 1.30 µM LOD 2.20 µM RSD 3.5 (10 µM) RSD 4.5 (100 µM) RSD 4.7 (200 µM)	[55]
Pond Water Sample (1.40 µL)	Permanent marker pen	Modified Griess reagent (citric acid)	Camera-phone	Working range: 0.156–1.25 mM RSD < 5%.	[56]
Saliva, preservative water, ham, sausage and River water samples (50 µL)	Stamping	Modified Griess reagent (HCl) (0.75 µL)	Scanner (Scanjet G4050)	Working range: 0–25 µM LOD: 5.6 µM RSD < 10%.	[57]
Saliva sample (0.20 µL)	Hand-held corona generator OTS–hexane	Griess reagent (5 µL)	EPSON Perfection V300 Photo desktop scanner	Working range: 20–160 µM LOD: 7.8 µM SD: 5.1 µM	[58]
Simulated human urine (2/ 3.5 µL)	µTAD	Modified Griess reagent (citric acid) (2/4.5 µL)	Ruler and iPhone 5	Working range: 0–1000 M	[59]
Wastewater (30 µL)	Stamping method 10:1 v/v ink–solvent mixture	Modified Griess reagent (citric acid) (3 µL)	Samsung Galaxy SII	Working range: 4.0–85.0 mg L^{-1} LOD: 0.52 mg L^{-1} RSD: 0.51% at 4.0 mg L^{-1}	[60]
Groundwater and tap water (20 µL)	Wax ink printing	Griess reagent (0.5 µL)	Motorola Moto G5 Plus	Working range: 0.05–10.0 mg L^{-1} LOD: 0.03 mg L^{-1}	This work

intensities. Applying the logarithm to the original curve, a linear model is obtained. The corresponding function, after applying regression analysis, was log $(Blue_{int} - Green_{int}) = (0.53 \pm 0.01)$ log $[NO_2^-] + (0.82 \pm 0.01)$, with a determination coefficient of 0.998. The LOD and LOQ were 0.03 mg L^{-1} and 0.13 mg L^{-1} respectively, again taking into account the IUPAC recommendation [53]. The nitrite content in the samples was measured by the reference method [52], obtaining no detectable values. Therefore, a recovery study was carried out, and the relative standard deviations (%RSD) were calculated from 8 replicates for each sample and each concentration level. The values were lower than 5.8% and 6.5% in terms of repeatability (intra-day, same µPAD) and intermediate precision (inter-day, different µPADs) respectively.

3.5. Analysis of real samples and validation

The µPAD was applied to six water samples of different origins. All of them did not contain nitrite when the reference method was applied so, they were fortified at three concentration levels. The simultaneous determination of fluoride and nitrite were done on the fortified samples at the second concentration level (3.00 mg L^{-1}). Sample A had to be diluted since its fluoride level was higher than the linear range of the calibration curve. Table 1 shows the obtained results for fluoride determination and those obtained applying the reference method [54]. As it can be seen, the values are similar. In order to validate the method, a comparison between them using the paired *t*-test, was carried out. Previously, the homoscedasticity was tested and the $t_{calculated}$ value was 0.024. It was lower than the $t_{tabulated}$ value (2.306) at 95% confidence level, indicating that there were not significantly statistic differences

between both methods.

The validation of nitrite determination was performed doing a recovery study shown in Table 2. The recovery and the standard deviation values demonstrated good accuracy and repeatability when the device was applied to real samples.

The obtained results showed the suitability and reliability of the proposed µPAD for the simultaneous determination of fluoride and nitrite in water samples.

As it can be seen in Table 3, it is important to highlight the novelty use of wax ink printing for nitrite determination. Moreover, the proposed device presents the following advantages: the obtained LOD is the lowest one obtained in comparison to previous works for the same analyte, and it utilized the minimal reagent volume reported until now [50].

4. Conclusions

A simple, portable, inexpensive (less than U$D 0.01 per device) and user-friendly µPAD for the simultaneous determination of fluoride and nitrite in water samples was developed. It is of paramount importance to mention that this is the first PAD developed for fluoride determination. This new device combines the advantages of biodegradable raw materials as well as minimum volumes of sample and reagents. With this device the analytical procedure was miniaturized and sample pre-treatment, as pre-concentration step, was not necessary. The device was successfully applied for the determination of both analytes in samples from different origins with lower LODs than the recommended values by the European Commission as well as the Código Alimentario Argentino. The obtained results not only demonstrate the applicability

and benefits of this very promising analytical tool but also its versatility in a wide field of application.

Acknowledgements

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