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Topics: Nitrite, Griess test and Nitrate

Microfluidic paper-based analytical device (μPAD) for the determination of nitrite and nitrate

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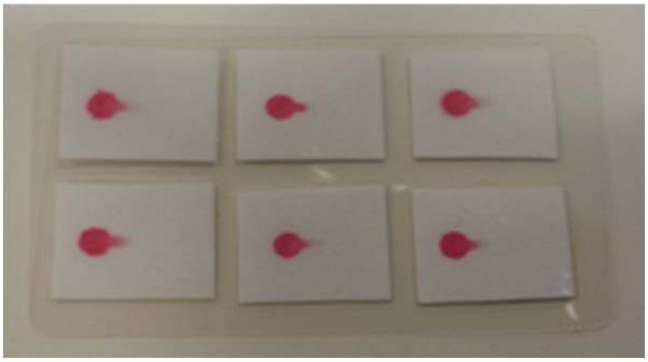
TOC Graphic



Abstract

A low-cost disposable colorimetric microfluidic paper based analytical device (μPAD) was developed for the determination of nitrite and nitrate. Nitrite is determined directly by the Griess reaction while nitrate is first reduced to nitrite in a hydrophilic channel of the μPAD with immobilized zinc microparticles. This μPAD is fabricated by a simple and inexpensive inkjet printing method. Under optimal conditions, the limits of detections and quantification for nitrite are 1.0 and 7.8 μM, respectively, while the corresponding values for nitrate are 19 and 48 μM, respectively. The repeatability, expressed as RSD, is less than 2.9% and 5.6% ($n \leq 8$) for the determination of nitrite and nitrate, respectively. This μPAD was successfully applied to the determination of nitrate and nitrite in both synthetic and natural water samples. It is user and environmentally friendly and suitable for on-site measurement of the analytes mentioned above in environmental and drinking waters.

Keywords: microfluidic paper based analytical device (μPAD); determination of nitrate and nitrate; Griess reaction

Introduction

Water supplies have a high risk of nitrate contamination from fertilizer use, animal waste, and sewage-disposal. Nitrates and nitrites have high potential to migrate in ground water due to their high water solubility and weak retention by soils[1]. Nitrates and nitrites do not volatilize and therefore are likely to remain in water until consumed by plants or other organisms[1]. The presence of nitrate and nitrite in drinking water is of considerable health concern. Nitrate can be reduced to nitrite either chemically or biologically[2]. These processes can even take place in the human digestive system where nitrate can oxidize iron in haemoglobin thus leading to methemoglobinemia (e.g. blue baby syndrome). Nitrite forms carcinogenic nitrosamines under the acidic conditions of the stomach which can cause gastric cancer[3]. The accepted Maximum Contaminant Levels (MCLs) for nitrite and nitrate in drinking water, specified by the U.S. Environmental Protection Agency, are 1.0 mg N L^{-1} (71.4 μM) and 10.0 mg N L^{-1} (714.3 μM), respectively[4]. Therefore it is essential to be able to measure these analytes reliably and at a low cost.

Nitrate and nitrite can be measured by a number of analytical methods utilizing colorimetry[5], ion chromatography[6], flow injection analysis (FIA)[7], sequential injection analysis (SIA)[8], capillary electrophoresis[9], and electrochemical techniques[10]. Some of these methods can be used to directly measure both nitrite and nitrate individually, while others measure these analytes as nitrite after nitrate has been reduced to nitrite. A variety of reagents such as zinc[11,12], cadmium[13], hydrazine-copper[14], copperised cadmium[15,16,17] and enzymes[18] have been used for the reduction of nitrate to nitrite. Colorimetric techniques have been used frequently due to their simplicity, favorable limits of detection and relatively wide linear concentration range[19]. However, most of these methods must be run by trained operators,

produce relatively large amounts of carcinogenic or toxic waste, and may not be suitable for field use.

Microfluidic paper-based analytical devices (μPADs), introduced by Whitesides et al.[20], provide a revolutionary approach for conducting inexpensive and rapid on-site analysis. This approach utilizes patterned paper as a platform for microfluidic manipulation of liquid samples and reagents which are transported in the hydrophilic channels and reacted in the detection zones of the μPADs. A variety of paper patterning techniques have been used which involve hydrophobic materials or paper-sizing chemistry. Martinez et al. showed that μPADs image based colorimetric detection can be used for telemedicine, since the colorimetric testing results can be scanned or photographed and transmitted electronically from the testing site to an analytical center[21]. The use of internal calibration curves in μPADs for sample analysis further increases the reliability of the results[22,23]. These applications demonstrate the potential of μPAD to be used in a wide range of analytical applications.

In this work we show for the first time the possibility of incorporating a flow-through solid phase reactor into a μPAD by depositing the solid material in a hydrophilic channel. In this system the nitrate is reduced to nitrite as the sample is transported through the channel mentioned above by capillary forces. This new concept allows an analyte to be chemically converted from a colorimetrically undetectable form into a colorimetrically detectable form, and subsequently detected when the sample reaches the detection zone. The determination of nitrate as nitrite using the Griess reaction can illustrate the potential of this new concept. The Griess reaction, which has been used since 1879[24], is the most popular color reaction used for nitrite detection. It is based on nitrite reacting with a primary aromatic amine (e.g. sulphanilamide) under acidic conditions forming a diazonium salt which further reacts with an aromatic compound containing an amino group (e.g. N-(1-naphthyl)-ethylenediamine

77 dihydrochloride) to form an intensely colored azo dye. Nitrate cannot be detected by the
78 Griess method and must be reduced to nitrite first using a suitable reductant, such as metallic
79 Zn or copperised Cd.

80 The present paper reports on the development of a pair of μPADs with identical fluidic
81 design which employ the Griess reaction for the determination of nitrite and nitrate in
82 environmental samples. One of the μPADs is used for the determination of nitrite only while
83 the other one allows the determination of the both nitrite and nitrate after the reduction of
84 nitrate to nitrite in a hydrophilic channel containing Zn microparticles and acting as a virtual
85 flow-through solid phase reactor.

86 # Experimental

87 ## Reagents

88 The Griess reagent was prepared by dissolving 50 mM sulfanilamide (≥ 99%, Chem-supply,
89 LR), 330 mM of citric acid (≥ 99.5%, Chem-supply, AR) and 10 mM of N-(1-naphthyl)-
90 ethylenediamine dihydrochloride (≥ 98%, Sigma) according to previously reported
91 method[25,26]. The Zn suspension was prepared by mixing 500 mg of Zn dust (< 10 μm, ≥ 98%,
92 Sigma-Aldrich) in 10.0 mL of deionized water. The 10 mM stock solutions of nitrate and
93 nitrite were prepared by dissolving 101.1 and 69.0 mg of potassium nitrate (Asia Pacific
94 Chemical Ltd, AR) and sodium nitrite (≥ 99%, Sigma-Aldrich), respectively. Working
95 solutions of nitrate and nitrite were prepared daily. Stock solutions used in the interference
96 studies contained 100 mM of one of the following salts: $NaH_2PO_4.2H_2O$ (BDH), KCl (Chem-
97 Supply), CH_3COONa (Chem-supply), and NH_4Cl (BDH). All aqueous solutions were
98 prepared in deionized water (18 MΩcm, Millipore Synergy 185, France).

99

100 **Design and fabrication of the device**

101 Two designs of μPADs, a 2D and a 3D configuration (Fig. 1), were fabricated by patterning
102 Grade 1 and 4 filter paper (Whatman) by a previously reported method [25] which produced the
103 desired hydrophilic zones (i.e. 2 channels and a detection zone). The optimization of the
104 design and operational parameters of the proposed μPADs were conducted with Grade 1 filter
105 paper. The fabrication procedure involved printing a hydrophobic coating of a 4% (v/v)
106 solution of alkyl ketene dimer (AKD, Precis 900, Hercules Chemicals Australia) in n-heptane
107 on filter paper using a Canon P4700 ink jet printer. This was followed by baking the μPADs
108 at 105 ^{o}C for 30 min to make the printed geometry permanent.

109 In the μPAD used for the determination of the combined concentration of nitrate and nitrite,
110 Zn suspension was deposited into one of its two hydrophilic channels where nitrate was
111 reduced to nitrite. For the determination of nitrite only, no Zn suspension was added. Griess
112 reagent was deposited into the detection zone. The μPADs were dried in an oven at 50 ^{o}C for
113 6 min. The dimensions of the reduction channel (13 mm x 4 mm), the detection zone (5 mm
114 diameter) and the transport channel (3 mm x 2 mm), located upstream of the detection zone,
115 were identical in both the 2D and 3D configurations. In the 3D configuration, the reduction
116 channel, transport channel and detection zone were printed on adjacent sides of the paper so
117 that they became aligned in a transverse fashion when the paper was folded, as shown in
118 Fig. 1b. A photograph of a partially folded 3D μPAD is shown in Fig. 1c. A single μPAD
119 card (credit card size) accommodated 6 individual 2D or 3D μPADs (Fig. 1d). Experiments
120 involving 3D μPADs with the same width of their transport and reduction channels have been
121 also conducted to check if a width difference between the 2 channels can affect the analytical
122 performance of the device.

123 Both the 2D and 3D μPADs were laminated (GBC HeatSeal™ H65) to prevent the

evaporation of the sample during reduction and detection and to inhibit the oxidation of Griess reagent in the reagent zone and metallic zinc in the reduction channel. Laminating the 3D µPADs also maintained the alignment of the folded 3D configuration[27]. A tissue biopsy punch was used to punch a hole of 2 mm in diameter in the plastic cover over the end of the reduction channel opposite the detection zone (Fig. 1) to allow sample introduction. To prevent evaporation of the sample through the sample introduction hole during reduction, the latter was covered with a masking tape after adding the sample. This approach improved sensitivity and repeatability.

For better control of the reduction time in the 3D configuration, an interleaving cellulose acetate sheet was inserted between the 2 paper layers of the folded µPAD (Fig. 1b). Immediately before sample introduction, one end of the laminated 3D µPAD was snipped off and subsequently the interleaving sheet was pulled out after a predetermined period of reduction time to allow the transverse transport of the reduced sample from the reduction channel to the transport channel (Fig. 1b). This approach markedly improved the sensitivity and repeatability of the determination of nitrate.

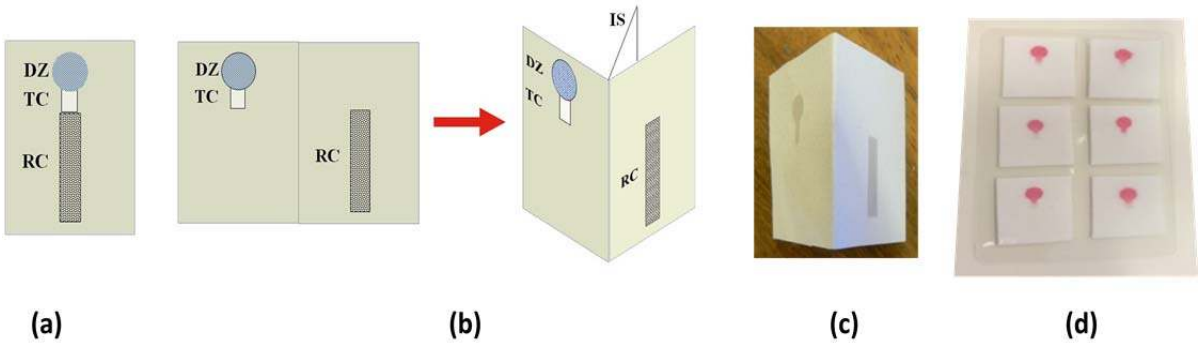


Figure 1: Schematic diagrams of the proposed 2D (a) and 3D (b) µPADs and a photographic image of an individual unused 3D µPAD (c) and a card incorporating 6 used 3D µPADs (d). (DZ: detection zone, diameter 5 mm; TC: transport channel, 3 mm x 2 mm; RC: reduction channel, 13 mm x 4 mm; IS: interleaving sheet, cellulose triacetate).

144 **Analytical procedure, its optimization and data processing**

145 For the determination of the combined nitrate and nitrite concentrations, a sample or standard
146 was deposited into the sample hole of a μPAD with a Zn reduction channel. The sample hole
147 was covered with a masking tape and after a predetermined period of reduction time the
148 interleaving sheet was removed to allow the reduced sample to reach the detection zone by
149 capillary forces where red-violet color developed at ambient temperature (20 -25 ^{o}C). After
150 predetermined duration of the color development time, the detection zone was scanned by
151 means of a flatbed scanner (Canoscan™ Lide 700f) and the image was processed by Image J
152 software (National Institutes of Health, USA, http://imagej.nih.gov./ij). The highest color
153 intensity for the centre of each detection zone, where color intensity was more uniform, was
154 obtained when the intensity of the green color was used. The average color intensity for each
155 detection zone was subsequently converted to absorbance as proposed by Birch and Stickle [28]
156 The same approach was used successfully previously by us[22]. The average absorbance of
157 each standard and sample was determined on the basis of 10 replicate measurements.
158 Absorbance values higher that the 90th or lower than the 10th percentiles of the full set of 10
159 replicate absorbance values were excluded in the calculation of the average absorbance value
160 of the standard or sample.

161 The same procedure, except for the reduction step, was used for the determination of nitrite
162 only. However, in this case the μPADs utilized did not contain Zn microparticles.

163 The concentration of nitrite was calculated on the basis of the average absorbance value
164 obtained in μPADs without Zn microparticles using a linear calibration equation. The
165 concentration of nitrate was calculated by another linear calibration equation on the basis of
166 the difference in the absorbance values produced by the sample or standard in μPADs with
167 and without Zn microparticles.

168 The proposed paper-based method was successfully applied to the determination of nitrate
169 and nitrite in synthetic samples, spiked tap water samples and original pond and mineral
170 water samples. The concentrations of nitrate and nitrite in the original pond and mineral
171 water samples were also determined by a conventional ion chromatography (IC-Dionex Dx
172 120) method with the linear working ranges up to 200 and 50 μM for nitrate and nitrite,
173 respectively[5]. The μPAD and IC data were compared by the paired t-test.

174 The main parameters of the proposed μPADs were optimized with respect to sensitivity and
175 the ranges within which each parameter was optimized are listed in Table 1.

176 **Table 1**: Summary of μPAD parameters investigated in the present study

Parameter	Range tested	Optimum value
Deposited mass of Zn (mg)	0.25-1.50	1.00
Sample/standard contact time with Zn (s)	30-150	75
Sample/standard volume (μL)	5-30	20
Deposited volume of Griess reagent (μL)	0.5-1.5	1.0
Color development time (min)	3-60	3-7

177 **Stability studies**

178 The stability of the proposed μPADs were studied under different temperature conditions
179 such as room temperature (with or without exposure to light), at temperatures $\leq$ 4 ^{o}C
180 (refrigerator) and $\leq$ -20 ^{o}C (freezer) with and without vacuum sealing. To minimize the
181 exposure to air, μPADs were placed in FoodSaver® Vacuum zipper bags and vacuum sealed.
182 The stability of μPADs was assessed by daily measurements of the absorbance of their
183 detection zones before and after the addition of nitrite (100 μM) and nitrate (500 μM)
184 standards and continued until the calculated mean concentration values decreased by more
185 than 2 σ_{n-1} from the true value.

186 **Interference studies**

187 The interference of common ions such as K^+, Na^+, Cl^-, PO_4^{3-}, NH_4^+, and CH3COO$^-$ was
188 studied by analysing standards containing 75 μM nitrite or 500 μM nitrate in the presence of
189 2,500 to 50,000 μM KCl, NaH_2PO_4, NH_4Cl, or CH_3COONa.

190 Results and discussion

191 **Design and reductant selection**

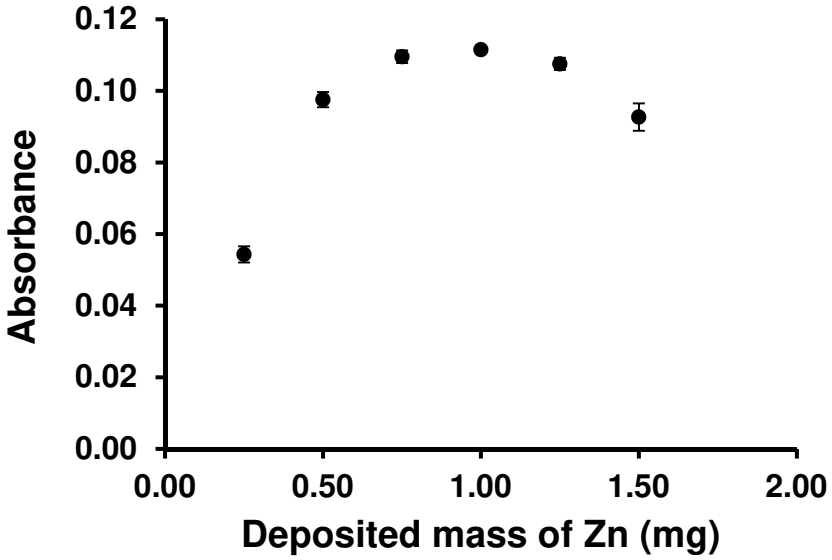
192 Cd microparticles and Zn microparticles produced similar results for nitrate and due to the
193 higher toxicity of Cd, Zn was selected for the reduction of nitrate in the subsequent
194 experiments.

195 The 2D configuration (Fig. 1a) was not adopted because the Zn microparticles were swept
196 into the detection zone which resulted in poor reproducibility. This problem was avoided in
197 the 3D configuration (Fig. 1b) where the sample was transported from the reduction channel
198 to the transport channel across the paper layer containing the transport channel and the
199 detection zone. This paper layer acted as a barrier to the movement of the Zn microparticles.
200 It should also be pointed out that the 3D configuration provides a much better flow control
201 capabilities than the 2D configuration by allowing the introduction of a switching mechanism
202 between the reduction and detection steps. In this way it is possible to accurately control the
203 duration of the reduction reaction which is expected to influence to a considerable extent the
204 sensitivity of the proposed μPAD.

205 **Optimization of the μPAD parameters**

206 The ranges in which the main μPAD parameters were studied and the corresponding
207 optimum values are listed in Table 1 in the order in which the optimization procedure was
208 carried out.

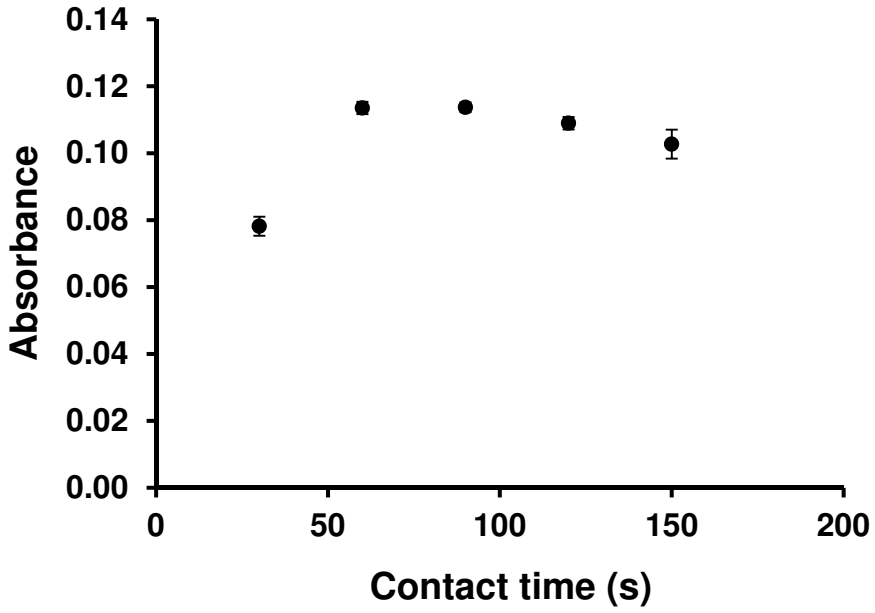
209 The mass of Zn present in the reduction channel was controlled by varying the volume of Zn
210 suspension deposited in this channel. The sensitivity increased initially with increasing the
211 mass of Zn and after levelling off in the range 0.75 to 1.25 mg started to decrease (Fig. 2).
212 This was probably due to the reduction of nitrite at heavy Zn loadings of the reduction
213 channel. The optimum mass of Zn was determined as 1.0 mg.



214

215 **Figure 2**: Absorbance versus deposited mass of Zn in the reduction channel (Fig. 1b).
216 Experimental conditions: sample/standard volume – 25 µL; sample/standard contact time –
217 60 s; Griess reagent volume - 1 µL; and color development time – 5 min. The error bars are
218 $\pm 1\sigma_{n-1}$ (n=4).

219 The sample/standard contact time with Zn microparticles in the reduction channel was varied
220 from 30 to 150 s after which the interleaving sheet was removed (Fig. 3). It was found that
221 the maximum reduction of nitrate to nitrate in the concentration range studied (50 - 1000 µM)
222 was reached after a contact time of 60 s. However, the sensitivity was found to gradually
223 decrease for contact times greater than 90 s which might be due to reduction of nitrite [29,30].
224 Therefore, 75 s was selected as the optimum duration of the sample/standard contact time.



225

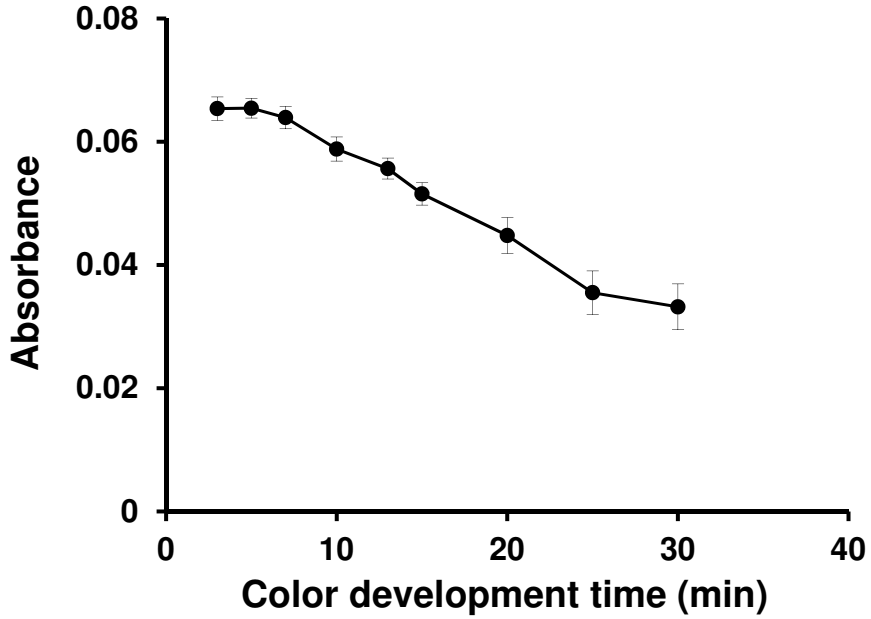
226 **Figure 3**: Absorbance versus sample/standard contact time for 1000 μM nitrate standard.
227 Experimental conditions: deposited mass of Zn – 1.0 mg; sample/standard volume – 25 μL;
228 Griess reagent volume - 1 μL; and color development time – 5 min. The error bars are $\pm 1\sigma_{n-1}$
229 (n=4).

230 To improve the sensitivity of the proposed paper-based method, the sample volume was
231 studied in the range 5 - 30 μL. The absorbance increased rapidly with increasing the sample
232 volume up to 20 μL and then levelled off. Therefore, the optimum sample volume was
233 selected as 20 μL.

234 The volume of Griess reagent deposited in the detection zone was varied between 0.5 and
235 1.5 μL. The sensitivity increased initially and levelled off at 1.0 μL, and consequently 1 μL
236 was chosen as the optimum value.

237 The colour development of the azo dye complex was monitored at ambient temperature (20 -
238 25 ^{o}C) by recording the absorbance every 2 min during the first 15 min and every 5 min
239 thereafter. As shown in Fig. 4 the absorbance gradually decreased with time indicating a

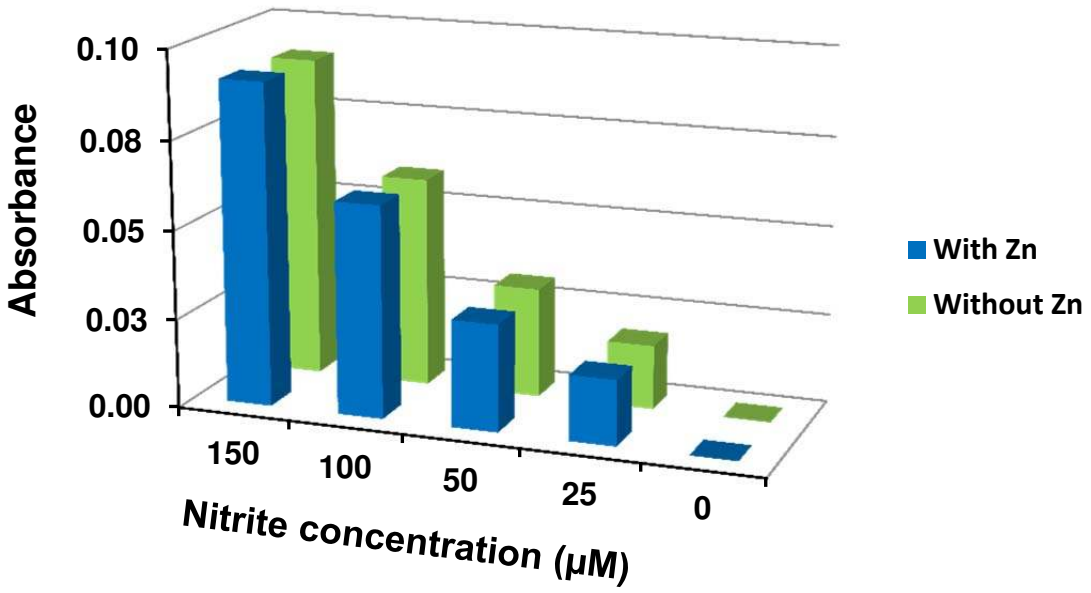
240 decomposition of the azo dye[29]. Therefore 5 min was selected as the optimum color
241 development time.



242

243 **Figure 4**: Absorbance versus color development time for 600 μM nitrate standard under
244 optimum experimental conditions (Table 1). The error bars are $\pm 1\sigma_{n-1}$ (n=5).

245 The results presented in Fig. 4 prompted a study of the effect of the Zn microparticles in the
246 reduction channel on the determination of nitrite only. The experiments were conducted
247 under the optimum conditions for the determination of the combined concentration of nitrite
248 and nitrate (Zn microparticles included) and nitrite only (no reduction step). The results,
249 presented in Fig.5, showed that under the optimum conditions for the determination of the
250 combined concentration of nitrite and nitrate there was no significant reduction of nitrite.
251 This observation was confirmed using a paired t-test which showed that there was no
252 statistically significant difference between the two sets of results (t_{stat}=0.3931, p=0.7335,
253 $t_{critical}$ (two-tail) =2.7764, df = 4).



254

255 **Figure 5**: Effect of Zn on the determination of nitrite at different concentrations (n=5) under
256 optimum experimental conditions (Table 1).

257 µPADs made of Grade 1 and Grade 4 filter papers (Whatman) were applied to the analysis of
258 synthetic samples containing 100 µM nitrite and 500 µM nitrate under optimal experimental
259 conditions (Table 1). The results obtained for both nitrite (i.e. 99.6±2.7 µM and 98.6±1.9 µM
260 for Grade 1 and Grade 4, respectively) and nitrate (500.2±6.9 µM and 500.5±5.5 µM for
261 Grade 1 and Grade 4, respectively) did not show any statistically significant difference for
262 Grade 1 and Grade 4 filter papers at the 95% confidence level when the paired t-test was used
263 (t_{stat} = 0.385, p = 0.708, t_{crit} (two-tail) = 2.201, and df = 11).

264 The potential effect of the difference in width between the transport (TC) and reduction (RC)
265 channels (Fig. 1b) on the performance of the 3D µPADs was investigated by analyzing
266 synthetic samples containing 75 µM nitrite and 500 µM nitrate with the proposed 3D µPADs

(Fig. 1b) and 3D μPADs where the widths of their 2 channels (i.e. TC and RC) were equal. Measurements were conducted under optimal experimental conditions (Table 1). The results obtained for both nitrite (i.e. 74.4 ± 1.8 μM and 75.7 ± 2.1 μM for the configurations with different and equal width of TC and RC, respectively) and nitrate (499.7±5.5 μM and 496.3±7.8 μM for the configurations with different and equal width of TC and RC, respectively) did not show any statistically significant difference between the 2 configurations at the 95% confidence level when the paired t-test was used (t_{stat} = 1.005, p = 0.336, t_{crit} (two-tail) = 2.201, and df = 11). On the basis of these results it was concluded that small differences in the width of the transport and reduction channels of the proposed 3D μPADs did not affect their analytical performance.

Analytical performance

Analytical figures of merit

Under the optimum conditions the proposed paper-based method is characterized by linear calibration ranges for nitrite and nitrate of 10-150 and 50-1000 μM, respectively, and calibration equations $Absorbance_{(nitrite)}=(5.97\pm0.07)x10^{-4}C_{(nitrite)}+(1.16\pm0.76)x10^{-3}$ (R^2=0.999) and $Absorbance_{(nitrite+nitrate)}-Absorbance_{(nitrite)}=(1.12\pm0.01)x10^{-4}C_{(nitrate)}-(6.41\pm3.49)x10^{-4}$ (R^2=1.000), respectively. The regression coefficients are shown with ± standard error. Due to non-ideal mixing of the sample in the reduction channel only 20% of nitrate is reduced to nitrite. However, this degree of reduction is sufficient to achieve acceptable sensitivity for nitrate detection. The limits of detection (LODs) for nitrite and nitrate were 1.0 and 19 μM (n≤8), respectively, and corresponding limits of quantification (LOQs) were 7.8 and 48 μM (n≤8), respectively. The linear regression method of Miller and Miller[31] was utilized in determining these values. The repeatability of the μPADs for nitrite was less than 2.9 % and for nitrate was less than 5.6 % RSD (n≤8) for all concentrations studied. These results clearly

show that the proposed paper-based method has the potential to be used for determination of nitrite and nitrate in drinking water, since the LOQ values achieved are much lower than the U.S. Environmental Protection Agency drinking water MCL values of 71.4 and 714.3 μM for nitrite and nitrate, respectively[4].

Interference studies

The interference studies showed that the percentage recoveries for 75 μM nitrite in standards containing 50,000 μM of one of the salts KCl, NaH_2PO_4 and NH_4Cl were 99.6±2.8, 98.6±3.3 and 99.7±1.9, respectively. However, CH_3COONa was found to interfere at concentrations higher than 2,500 μM (recovery - 99.6±2.5%). The recoveries at 10,000 μM and 50,000 μM concentrations were 79.1±7.4% and 58.9±10.4%, respectively. KCl, NaH_2PO_4 and NH_4Cl did not interfere with the determination of 500 μM nitrate in concentrations up to 50,000 μM.

Analytical applications for synthetic and pond water samples

Recovery experiments were conducted using synthetic samples and spiked tap water samples. All the samples were measured under ambient conditions and recovery values are reported in Table 2. In all cases, excellent percentage recoveries of 94.3-104.0 % and 95.2- 102.8 % were obtained for nitrite and nitrate, respectively. Repeatability values of less than 6.8 % were obtained.

The results obtained by the proposed paper-based method for the determination of the concentrations of nitrite and nitrate in mineral and pond water samples were compared with those obtained by the analysis of the same samples by ion chromatography (Table 3). The paired t-test showed that there was no statistically significant difference between the two sets of results for both nitrite (t_{stat} = 0.9398, p = 0.4167, $t_{critical}$ (two tail) = 3.182 and df= 3) and nitrate (t_{stat} = 0.7207, p = 0.5232, $t_{critical}$ (two tail) = 3.182 and df= 3). The paper-based method also exhibited acceptable repeatability (i.e. RSD in the range 3.1 - 7.9%).

315 **Table 2**: Percentage recovery of nitrite and nitrate in synthetic and spiked tap water samples.

316 The values in parenthesis indicate the corresponding standard deviations (n≤8).

Sample ID	Nitrite				Nitrate			
	Spiked (µM)	Measured (µM)	Recovery (%)	RSD (%)	Spiked (µM)	Measured (µM)	Recovery (%)	RSD (%)
Synthetic 1	50.0	50.7(2.8)	101.4	5.6	100.0	97.7(6.1)	97.7	6.3
Synthetic 2	25.0	26.0(1.4)	104.0	5.5	300.0	308.3(9.4)	102.8	3.0
Synthetic 3	12.5	12.4(0.7)	99.3	6.0	400.0	389.4(6.4)	97.4	1.6
Synthetic 4	12.5	11.8(0.6)	94.3	5.5	300.0	300.4(9.1)	100.1	3.0
Tap water 1	15.0	14.7(0.9)	98.1	6.3	50.0	47.6(3.3)	95.2	6.8
Tap water 2	15.0	14.6(0.9)	97.5	6.1	400.0	402.9(8.6)	100.7	2.1
Tap water 3	50.0	48.7(1.7)	97.4	3.5	75.0	75.3(3.7)	100.3	4.9
Tap water 4	20.0	19.4(0.9)	97.2	4.6	100.0	98.8(5.3)	98.8	5.3

317 **Table 3**: Analysis of nitrite and nitrate in tap water and pond water samples by the proposed

318 paper-based method (n≤8) and ion chromatography (n=3). The values in parenthesis are

319 standard deviations.

Sample Id	Nitrite			Nitrate		
	Spiked (µM)	IC (µM)	µPAD (µM)	Spiked (µM)	IC (µM)	µPAD (µM)
Pond water 1	20.0	19.2(0.1)	19.8(1.3)	60.0	61.3(0.1)	61.6(2.9)
Pond water 2	25.0	24.8(0.1)	24.8(1.1)	120.0	125.3(0.4)	124.5(3.9)
Pond water 3	40.0	39.7(0.2)	40.6(1.4)	60.0	62.7(0.4)	62.8(2.3)
Mineral water	10.0	10.1(0.2)	9.7(0.8)	40.0	75.0(0.3)	74.7(3.0)

320 **Life time of the μPADs**

321 The response of the μPADs was constant for a period of 7 days if the μPADs were stored in a
322 freezer at -20 ^{o}C, for 5 days if stored in a refrigerator at ≤ 4 ^{o}C and for 2 days if stored in the
323 dark at room temperature. The stability was 1 day if the μPADs were exposed to daylight at
324 room temperature. The lifetime of μPADs stored in vacuum sealed zipper bags in a
325 refrigerator at ≤ 4 ^{o}C doubled. This observation agrees with the findings of Bhakta et al. [32]
326 that limiting the exposure of a nitrite μPAD by manufacturing and storing it under nitrogen
327 can suppress the background signal for at least 13 h. These authors did not report the stability
328 of their μPAD for longer storage times. The lifetime of the proposed μPAD stored in vacuum
329 sealed zipper bags was extended another 2 fold by freezing the bags at -20 ^{o}C instead of
330 refrigeration at ≤ 4 ^{o}C. These results show that when appropriately stored (e.g. in a freezer
331 and in vacuum sealed zipper bags) the μPADs are stable for 30 days.

332 # Conclusions

333 3D paper-based μPADs for the determination of nitrate and nitrite were developed. To the
334 best of our knowledge, this is the first use of solid-phase chemistry in a μPAD, i.e. for
335 reduction of nitrate to nitrite in a Zn reduction channel. This concept is expected to expand
336 the analytical capabilities of such devices.

337 On the basis of the results obtained it can be concluded that the proposed paper-based method
338 is characterized by high sensitivity and acceptable repeatability, a high degree of portability,
339 and very low cost of analysis (i.e. a few cents per μPAD),. The sensitivity allows the use of
340 the method for low–cost monitoring of nitrite and nitrate in environmental and drinking
341 waters and therefore it is expected to be of particular interest to developing countries.

342 It should also be pointed out that the proposed method is also environmentally friendly due to
343 the use of very small amounts of reagents, and the replacement of toxic Cd with Zn.

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