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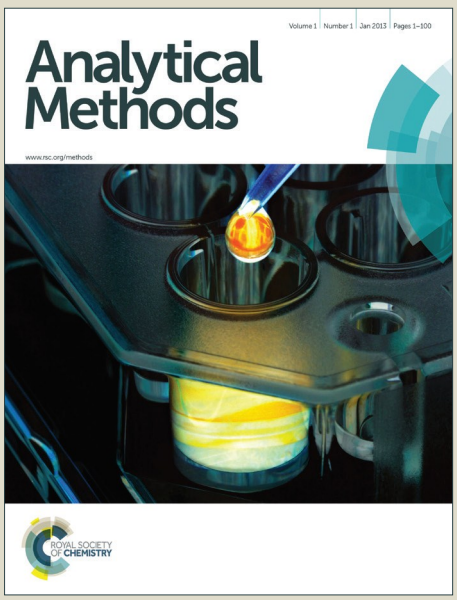

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ARTICLE

Colorimetric determination of nitrite in clinical, food and environmental samples using microfluidic devices stamped in paper platform

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This study reports the use of microfluidic paper-based analytical devices (μPADs) associated with colorimetric detection for the determination of nitrite in clinical, food and environmental samples. μPADs were fabricated by simple and fast stamping process in a geometry containing eight circular detection zones and one central zone to sample inlet interconnected by microfluidic channels. The colorimetric determination of nitrite was performed through the modified Griess reaction. Detection zones were spotted with a 0.75 μL aliquot of a solution containing 50 mmol L^{-1} sulfanilamide, 1.2 mol L^{-1} hydrochloric acid and 4 mmol L^{-1} N-(1-napthyl)ethylenediamine. The monitoring of the background colorimetric response revealed good stability over 12h for devices stored in the absence of light. After the addition of standard or real samples, the resulting images were captured with a scanner, converted to a color scale and analyzed in the magenta channel. The analytical sensitivity and the limit of detection achieved after a preconcentration stage were 0.56 (AU/μM) and 5.6 μM, respectively. The preconcentration provided an enrichment factor of *ca.* 3.2 times. The concentration levels of nitrite were successfully determined in saliva, preservative water, ham, sausage and river water samples. The concentration levels attained for each sample using μPADs were compared to the values found by spectrophotometry and there was no significative difference from one another at a confidence level of 95%.

Introduction

In the last years, microfluidic paper-based analytical devices (μPADs) have appeared as a very promising platform for the point-of-care testing (POCT)[1-6]. The fabrication of μPADs often involves well established techniques including photolithography[7,8], wax printing[9,10] and laser cutting[11] or alternative approaches with low instrumental requirements like wax dipping[12] and stamping-based methods[13-19]. μPADs have been used for different applications in association with electrochemical[20, 21], mass spectrometry[22, 23] and colorimetric detection[8, 24]. The latter is one of the most popular detectors on μPADs due to its global affordability and capability of capturing digital images with benchtop and handheld electronic devices[6, 25, 26]. Furthermore, the portability of electronic devices like cell phone camera, webcam, digital cameras, or handheld scanners allows their use for on-site applications. Among all portable electronic devices, scanner has been the most widely used to provide colorimetric measurements. Besides the advantages mentioned above, scanner allows auto-focus control and minimizes external interferences related to the light[25, 27]. Other remarkable advantage of colorimetric detection mode is regarded to its easiness of data analysis, once quantitative information can be extracted based on color intensity. Although most papers reported in literature make use of graphic softwares to individually analyze pixel intensity inside each detection zone, portable instruments based on light reflectance principle have been reported to allow the colorimetric automatic readout[28, 29].

The determination of nitrite is usually performed by conventional techniques including spectrophotometry[30,31], amperometry[32], chemiluminescence[33] and fluorescence[34]. Different authors have also used these detectors coupled with electrophoretic[35,36] or chromatographic[34,37] separation methods or flow injection analysis[38]. Most of these techniques require large sample volume and expensive instrumentation, which are not available in low resource settings. On the other hand, one of the simplest methods for the determination of nitrite is through the Griess reaction, which promotes the formation of a magenta azo compound[39,40]. The colored product of this reaction can be then determined based on the digital-image analysis, as recently reported by different groups[15,40-43].

The colorimetric assay for nitrite on μPADs has been reported by different authors. However, the disclosure of this technology using real samples is still in an early stage. Klasner *et al.* reported the colorimetric detection of nitrite in urine and salivary samples on μPADs fabricated by photolithography[42]. He and colleagues described the determination of nitrite in food samples with μPADs fabricated by alkylsilane self-assembling and UV/O_3 patterning[43]. Bhakta and co-workers reported recently the use of μPADs produced by wax printing for the quantification of nitrite in saliva samples[40]. In all examples cited above, digital images were captured with benchtop scanner. Most recently, Li and coworkers developed a portable instrumentation based on light reflectance principle for colorimetric

assays on paper-based devices[29]. The authors successfully reported the detection of nitrite in a local tap water sample.

In this current study, we describe the use of μPADs prepared by stamping of paraffin barriers for the colorimetric determination of nitrite levels in saliva, preservative water, ham, sausage and river water samples. Nitrite is an important compound for clinical, food and environmental sciences. In the clinical field, the monitoring of nitrite concentrations levels plays a key role to obtain helpful information about different diseases according to the biological fluid. In saliva samples, for example, the concentration levels of nitrite may be associated with periodontitis[40]. Saliva has been studied as a biological fluid to be used on POCT devices specially because its collection is a simple and noninvasive method[44]. As described by Blicharz and colleagues[45], the presence of salivary nitrite can be a potential biomarker for different functions in human body. The monitoring of salivary nitrite may be useful to indicate the need for dialysis treatment or to measure indirectly the production of nitric oxide (NO) by kidneys[45]. Nitrite is also commonly used in food science as additive due to its capability of inhibiting growth of microorganisms and lipid oxidation that leads to rancidity[46]. In the environmental field, the presence of nitrite in river samples is derived from biological denitrification, acid rain, and industrial waste[47]. As pointed out by different authors, nitrite can react readily with secondary and tertiary amines producing carcinogenic nitrosamines compounds[48]. For this reason, the monitoring of nitrite levels in clinical, food and environmental samples is of paramount importance. Regarding the clinical and food samples, the nitrite levels were directly determined based on the standard analytical curve. On the other hand, a preconcentration step was required to reach the nitrite levels in environmental samples. The values achieved on stamped μPADs were compared to those found by spectrophotometry.

Materials and methods

Chemicals and instrumentation

Hydrochloric acid, sulfanilamide, N-(1-napthyl) ethylenediamine (NED) and sodium nitrite were acquired from Sigma Aldrich Co. (Saint Louis, MO, USA). Filter paper (model JP 40, 12.5 cm diameter and 25 μm pore size) was purchased from JProlab (São José dos Pinhais, PR, Brazil). Nylon-type syringe filters (0.22 μm pore size and 25 mm diameter) was received from Allcrom (São Paulo, SP, Brazil). Paraffin (code 140º/145ºF) was supplied by Petrobras (Rio de Janeiro, RJ, Brazil). A scanner (model Scanjet G4050) was purchased from Hewlett-Packard (Palo Alto, CA, USA) to perform colorimetric measurements. All reagents were analytical grade and used as received.

Sample preparation

Saliva samples

The studies with saliva samples were carried out in accordance with the ethical principles for medical research involving human subjects described by the World Medical Association. In order to demonstrate the capabilities of the proposed system, saliva from three human volunteers was collected. All subjects received informed consent forms before inclusion in the study and voluntarily agreed to participate. The donors were also requested to fill an additional form providing information about their age, gender, diet and general health questionnaire. The donation of saliva involves no risks to the donors and samples were numerically identified to preserve their anonymity. Aliquots of 500 μL were collected into 1-mL-eppendorf tubes in the morning 30 minutes after the oral hygiene. Afterwards, samples were filtered through pore size of 0.22 μm.

Food samples

Aliquots of preservative water were directly collected from Vienna sausage bottle and filtered using sequentially quantitative filter paper and syringe filter. The preparation of ham and sausage samples was performed by boiling of 50 g of each sample in 100 mL of water at 100 °C during 1 hour. Prior to colorimetric analysis, samples were then filtered similarly to the procedure described for the preservative water sample.

Environmental samples

Two water samples were collected at the Meia Ponte river (Goiânia, Goiás, Brazil) using an empty polymeric bottle. Samples A and B were collected in the geographic coordinates 16.628291"S, 49.270308"W and 16.642225"S, 49.256722"W, respectively. The samples were filtered similarly to the procedure for food sample preparation.

Fabrication of μPADs

μPADs were fabricated by a stamping-based method recently reported by our group[15] and it is schematically shown in Figure 1A. Briefly, a filter paper piece was immersed into liquid paraffin (at 90ºC) during 60 seconds. The paper was then removed from the paraffin, allowed to solidify at room temperature and then placed on a native paper piece. A metal stamp containing the microfluidic network was preheated and brought in contact with the paraffined paper during 2 s. This step enables the quick transference of the paraffin from the paraffined to native paper, thus forming the hydrophobic barriers. μPADs were designed in a geometry containing eight circular detection zones for bioassays interconnected by microfluidic channels and one central zone to sample inlet. All channels were nominally fabricated with a 10-mm length and 3-mm width. The diameter values for detection and central zones were 5 and 10 mm, respectively. The final dimensions of μPADs were 45 mm × 45 mm. An optical micrograph of the stamped μPADs is depicted in Figure 1B.

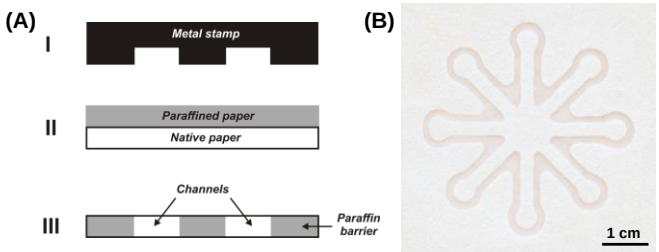

Figure 1. Presentation of (A) steps involved in the stamping-based fabrication technique and (B) a final stamped μPAD. In (I), a metal stamp containing the microfluidic network was designed in high-relief; In (II), the paraffined paper was placed in contact with a native paper piece; In (III), after a pressure-assisted 2-s contact, hydrophilic channels are formed during the creation of paraffin barriers.

Nitrite assays

The nitrite assay was performed on stamped μPADs designed with eight detection zones. In this configuration, three zones were selected as control zones (labelled as zones 1-3) and five zones were selected to obtain the colorimetric response for the nitrite assay (labelled as zones 4-8). Figure 2 displays an optical micrograph of the μPAD after the nitrite assay showing the zones used for control and colorimetric measurements. Each zone for nitrite assay was spotted with 0.75 μL aliquots of color solution containing a aqueous mixture of 50 mmol L^{-1} sulfanilamide, 1.2 mol L^{-1} hydrochloric acidand 4 mmol L^{-1} N-(1-napthyl)ethylenediamine. Afterwards, the zones were allowed to dry

at room temperature during 10 min. Then, aliquots of 50 µL containing standard solution or real sample were added in the central zone of the µPADs. Due to the hydrophilic nature of paper surface, sample was distributed towards the detection zones through the microfluidic channels defined by paraffin barriers.

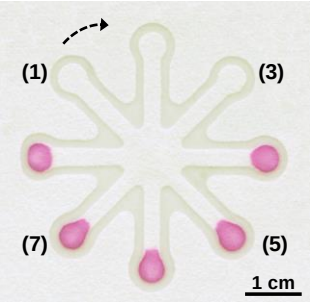

Figure 2. Representation of the stamped µPADs used for the determination of nitrite through Griess reaction. The labels 1-3 and 4-8 indicate the zones for control and nitrite detection, respectively. For illustrative purpose, the concentration of nitrite was 300 µM.

Preconcentration step

A preconcentration step was performed on µPADs aiming to attain the nitrite concentration levels in river samples. For this purpose, ten 40-µL aliquots of standard solution or river samples were sequentially added in the central zone. Between each new pipetting, the µPAD was allowed to dry at room temperature during 10 min. After the preconcentration, aliquots of 0.75 µL of color solution previously described were added inside the detection zones for the colorimetric measurements of nitrite levels.

Colorimetric detection

Colorimetric detection was performed with an office scanner (Hewlett-Packard, model Scanjet G4050) using a 600-dpi resolution. Images were captured 15 min after the reaction initialized. The recorded images were first converted to a 32-bits color scale (CMYK dimension) and then analyzed into the magenta channel in Corel Photo-Paint™ software. The arithmetic mean of the pixel intensity within each test zone was used to quantify the nitrite concentration in different samples. In order to compare the data achieved by the use of µPADs, quantitative analyzes were also performed with a spectrophotometer model SP1105 acquired from Bel Engineering (Monza, MB, Italy). Absorbance measurements were recorded at 520 nm using a glass cuvette with 1-cm of optical path length. The comparison was performed using the same chromogenic compound as well as the same analyte concentration range.

Results and discussion

The development of stamping-based techniques to produce paper microfluidic devices has been reported in the last years by different authors[13-19]. In comparison to conventional fabrication technologies, the use of this alternative protocol offers simplicity and requires minor instrumentation. Examples of stamps prepared in PDMS[14, 19], paper[17], polyethylene[18], iron[13], rubber[16] and stainless steel[13, 15] have been developed to create hydrophobic barriers on paper platforms using PDMS[18], indelible ink[14, 19], wax[13] and paraffin[15]. When compared to previous reports found in literature, the use of a stainless steel stamp to create paraffin barriers offers several advantages including higher mechanical and chemical resistances. Furthermore, the stamp is lightweight and its robustness allows prototyping of µPADs in a matter of seconds with great reproducibility[15].

In this study, we propose the analytical investigation of stamped paper-based devices for the determination of nitrite in three different classes of samples. As recently demonstrated by different authors, the modified Griess reaction is one of the simplest ways to determine nitrite concentration based on the formation of a magenta azo compound[39-42, 49]. We modified the original Griess reaction replacing the sulfanilic acid per sulfanilamide and hydrochloric acid. This procedure was adopted once it promotes faster kinetics and better color stability[50, 51]. In addition, Bhakta *et al.* reported that the stronger the acidic environment, the higher the analytical sensitivity[40]. The modified Griess reaction has been perfectly adapted for on-chip reaction with digital image analysis. When related to the use of µPADs, one of the major advantages is regarded to the minimal consumption of reagents. On the other hand, a common drawback associated with the colorimetric detection of nitrite refers to the Griess reagent stability[40], which may be affected depending on the storage mode prior to use. In this way, a total of twenty-four µPADs were prepared as described earlier and the background signal of each device was monitored over 96h. In this evaluation, after spotting the detection zones for nitrite assay, µPADs were stored at 5±1°C (n=12) and 25±2°C (n=12) in the absence of light. For this purpose, µPADs were placed into a polymeric box and covered with aluminium foil. Stored devices were independently evaluated over a time period between 10 min and 96h. Figure 3 displays the data recorded in different time intervals after addition of ultrapure water in the sample inlet zone and storage at 5±1°C (Figs. 3A and 3B) and 25±2°C (Figs. 3C and 3D).

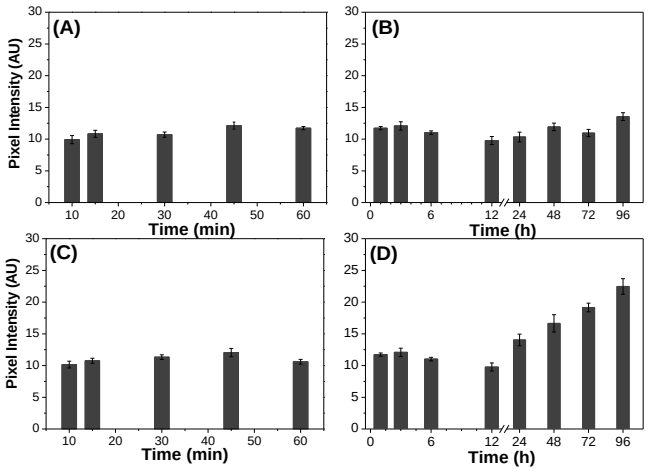

Figure 3. Monitoring of the background colorimetric response (n=5) on µPADs spotted with modified Griess reagent and stored under different times at (A, B) 5±1°C and (C, D) 25±2°C.

As it can be seen in Figures 3A and 3B, the storage of µPADs at 5±1°C between 10 and 60 min and between 1 and 96h did not exhibited noticeable changes in the pixel intensity response. The RSD values determined for the pixel intensities recorded on detection zones on µPADs were below 10%. The same behaviour was observed during 10-60 min and 1-12h for µPADs stored at 25±2°C, as shown in Figures 3C and 3D. However, the storage during longer time at room temperature induces a pronounceable background signal compromising the reliability of the colorimetric measurement. As presented in Figure 3D, the background signal for µPADs stored during 96h at room

temperature was ca. 2 times higher than that for storage time of 12h. This phenomenon may be attributed to the degradation of the Griess reagent[52, 53]. This problem can be overcome by encapsulating the Griess reagent in separated zones, as demonstrated by Bhakta and co-workers[40].

According to our results (see data shown in Figure 3), we can conclude that the storage of modified Griess reagents at 5±1°C and 25±2°C in the absence of light ensure stability for colorimetric measurements over 12h. Once no significative difference was observed for storage times between 10 and 60 min, we have fixed the storage time in 10 min at room temperature to investigate the feasibility of the stamped µPADs for colorimetric determination of nitrite in real samples.

Analytical performance

The analytical performance of the proposed device for the determination of nitrite in clinical and food analysis was investigated with standard solutions and compared to a spectrophotometric method. The colorimetric measurements on µPADs were performed using images captured 15 min after the addition of sample in the central zone. This time has been selected due to its better reproducibility for the quantitative analysis. As it can be seen in Figure 4A, the analytical response measured in terms of mean pixel intensity revealed a linear behaviour (coefficient of correlation higher than 0.99) for the concentration range between 0 and 100 µM. The data presented in Figure 4A represent the average and the standard deviation of five colorimetric measurements. The analytical sensitivity and the limit of detection (LD) achieved were 0.18 (AU/µM) and 11.3 µM, respectively. The LD value found was calculated based on the color intensity resulting from the ratio between three times the standard deviation for the control zone and the angular coefficient of the analytical curve. The analytical performance seen in Figure 4A was suitable for clinical and food analysis.

As previously determined by a reference spectrophotometric method, the levels of nitrite in the environmental samples were lower than the LD found by colorimetric measurements on µPADs. For this reason, a preconcentration step on µPADs was required to improve the detectability levels and to allow the detection of nitrite in lower concentrations. As it can be seen in Figure 4B, the analytical curve after the on-chip preconcentration has exhibited a good linear behaviour for a concentration range from 0 to 25 µM. When compared to the profile observed prior to preconcentration, it can be inferred that the enrichment factor was *ca.* 3.2 times. This value was calculated based on the ratio between the slopes of the analytical curves after and before preconcentration. Consequently, the LD value calculated was *ca.* 5.6 µM. Taking into account the enhanced analytical performance, the nitrite concentration was determined in one sample from the Meia Ponte river (Sample A).

The LD value and the linear concentration range reported were compared to the data recently described by other authors who used the modified Griess reaction for the colorimetric detection of nitrite on µPADs. As it can be seen in Table 1, the LD found after preconcentration is comparable to the value reported by Klasner and co-workers[42]. The acidic media chosen by different authors to modify the Griess reaction is also presented in Table 1.

The preconcentration strategy adopted in this study required the addition of 0.4 mL of sample on the µPAD. This volume has been optimized according to the dimensions of the channels created with paraffin barriers. In addition, it is important to highlight this volume allows the analysis of eight simultaneous assays. In this case, the volume required per assay would be around 50 µL. Once the channels were *ca.* 3 mm wide and 10 mm long, the use of shorter and narrower channels would demand smaller amount of sample to assess similar enrichment factor. When compared to conventional preconcentration techniques[54], the strategy used in this study to promote a preconcentration step on paper devices is simpler and cheaper. Furthermore, it is less laborious and it only requires consecutive additions of sample.

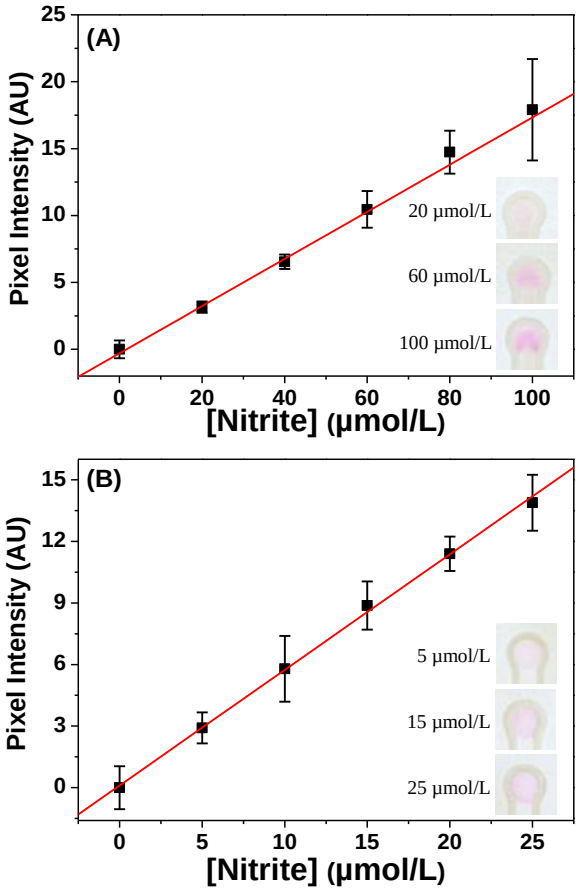

Figure 4. Analytical curves for nitrite (A) before and (B) after preconcentration. The mean color intensity (n=5) was recorded in the magenta channel using the Corel Photo-Paint™ software. The analytical response was normalized according to the mean intensity captured inside the control zones. The equations for the analytical curves exhibited in (A) and (B) were $y_{nitrite}$= -0.2985 + 0.1763×[nitrite] and $y_{nitrite}$= 0.1040 + 0.5636×[nitrite], respectively. The optical images shown inside both analytical curves represent the detection zones for three concentration levels of nitrite in the working range.

Table 1. Comparison of the acidic media and the analytical parameters found for the nitrite determination through the modified Griess reaction.

References	Reagent	Range (µM)	LD (µM)	R^2
Bhakta *et al.*[40]	H_3PO_4	0 – 1000	10	0.988
Xiao *et al.*[41]	$C_6H_8O_7$	0 – 300	*ca.* 0.5	0.999
Weng *et al.*[55]	$C_6H_8O_7$	0 – 250	*	0.991
Jayawardane *et al.*[26]	$C_6H_8O_7$	0 – 150	1	0.999
Klasner *et al.*[42]	$C_6H_8O_7$	0 – 250	5	0.996
Li *et al.*[29]	H_3PO_4	0 – 326	11.5	0.991
Lopez-Ruiz *et al.*[19]	$C_6H_8O_7$	0 – 1848	11.3	0.994
This study	HCl	0 – 100[a] 0 – 25[b]	11.3 5.6	0.997 0.999

*not informed; [a]without preconcentration; [b]with preconcentration

Real samples

The nitrite levels were determined in saliva samples donated by three volunteers. The achieved results according to the analytical performance displayed in Figure 4 are shown in Table 2. As it can be seen, the nitrite levels ranged from 26 to 176 μmol L^{-1}. These values are in agreement with the data found by the reference method. Also, it was observed a noticeable discrepancy for the salivary nitrite collected from the donators. As pointed recently by Bhakta and co-workers, the amount of nitrite in saliva is affected by a large number of parameters including gender, age, diet and genetics[40]. Furthermore, the salivary nitrite is a marker for the NO concentration in blood. NO is produced by the kidney due to the metabolism of L-arginine[45]. Differently from other volunteers, the donator from saliva #2 made use of dietary supplements containing L-arginine. For this reason, the nitrite concentration level was higher than other samples.

Table 2. Comparison of the nitrite concentration levels in clinical, food and environmental samples achieved by spectrophotometry and colorimetric measurements on stamped μPADs (n=5).

Samples	Reference method (μmol L^{-1})	Stamped μPADs (μmol L^{-1})	Error (%)
Saliva #1	83.1 ± 0.1	83 ± 11	–0.1
Saliva #2	171.5 ± 0.1	176 ± 22	2.6
Saliva #3	21.2 ± 0.1	26 ± 2	22.6
Preservative water	105.9 ± 0.1	100 ± 6	–5.6
Sausage	185.7 ± 0.1	186 ± 25	0.2
Ham	150.0 ± 0.2	149 ± 11	–0.7
River water - Sample #A	8.3 ± 0.1	8 ± 1	–3.6
River water -Sample #B	2.8 ± 0.2	ND*	---

*ND= not detected

Regarding food applications, nitrite concentration was determined in ham, sausage and conservative water. Likewise clinical samples, nitrite concentrations achieved with μPADs also exhibited a good correlation with the values found with the reference method (see Table 2). According to the Food and Drug Administration (FDA) agency from USA[56] and the Ministry of Agriculture, Livestock, and Supply (MAPA) from Brazil[57], the maximum amount of nitrite in foods is limited to 200 mg/kg and 150 mg/kg, respectively. Taking into account sample dilution, the amount of sodium nitrite in food samples ranged from 14 to 25 mg/kg. Based on these results, the methodology used on stamped μPADs is able to be used in food quality monitoring.

Lastly, the nitrite concentration was determined in two environmental samples. Basically both samples were collected in two designed regions in the greatest river supplier of water for the population of Goiania (Goias State, Brazil). Even with the preconcentration step, the performance of the stamped μPADs was able to reach the nitrite concentration in just one of the samples, as displayed in Table 2. The concentration of sample #B was not determined on the proposed device due to the fact that its concentration was lower than the found LD. As it can be seen in Table 1, the value achieved for sample #A on μPADs is in agreement with the concentration determined by the reference method. According to the Environmental Protection Agency (EPA) from USA[58] and Ministry of the Environment (MMA) from Brazil[59], the maximum amount of nitrite in drinking water samples is *ca.* 22 μM. Levels above this value may be an indicative of pollution.

It is important to note that the concentration levels attained to each sample (presented in Table 2) using μPADs and spectrophotometric method were statistically compared based on a paired t-test. We have not found significative difference from each other at the confidence level of 95%.

Conclusions

Overall, we have described the use of stamped μPADs for the determination of nitrite in clinical, food and environmental samples. The study on the modified Griess reagent stability exhibited reproducible background colorimetric response for μPADs stored under the absence of light over 12h. For storage during longer period, devices should be kept refrigerated. Despite the lower sensitivity of colorimetric measurements when compared to spectrophotometric method, the analysis of digital images on stamped μPADs has provided suitable performance for the quantitative assays. The on-chip preconcentration step has allowed the determination of nitrite in one of the environmental samples using μPADs. The strategy adopted in this current study required a sample volume of 0.4 mL. However, this total volume allows the analysis of eight simultaneous assays. Once this aliquot has been optimized according to the channel width defined with paraffin (*ca.* 3 mm), the use of narrower and shorter channels would require smaller amount of sample. The data achieved with the proposed method did not show statistical difference related to the data found with the reference method. Lastly, the LD reported herein (5.6 μM) represents one of the lowest values already reported in literature, in which was achieved with an enrichment factor of 3.2. The sensitivity of the proposed method can still be enhanced by selecting thicker paper substrates[60], for example. Based on the analytical performance reported herein, we believe the stamped μPADs are powerful tools to be used in the POCT, especially in the determination of NO markers in blood, food quality control[61] and environmental monitoring[62].

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