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A microfluidic paper-based analytical device for the determination of zinc (II) in children's urine samples and food zinc supplement solutions

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ABSTRACT

In this work, an innovative microfluidic paper-based analytical device (µPAD) was developed for the quantification of zinc(II) in biological samples of non-invasive collection, namely children's urine. Zinc(II) in urine can be considered a biomarker of children's psychological development and mental conditions, as well as indicative of urinary tract diseases. The developed device is based on the colourimetric reaction between zinc and dithizone in an alkaline medium and is described as a simple, low-cost method with potential on-site applications. The colour reaction takes about 5 min, and the resulting signal remains stable for 30 min. It enables the quantification of zinc(II) within the dynamic range of 50.0–750 µg/L, with a limit of detection (LOD) of 10 µg/L and a limit of quantification (LOQ) of 34 µg/L. After the device's assembly, it remains stable for use for up to one week when stored at room temperature, either in air or vacuum conditions. The method validation was performed by comparing the results obtained using the µPAD with those from atomic absorption spectrometry (AAS), where urine samples were analysed directly, or diluted (1:2) when necessary. The developed µPAD offers a user-friendly, portable, and cost-effective approach for the monitoring of zinc(II) levels in children's urine, providing point-of-care analysis in clinical diagnostics and public health monitoring.

1. Introduction

Frequent monitoring can be a strategy to anticipate health issues that rely on analytical devices. These devices are user-friendly and provide real-time and accurate analytical results. Self-readable devices, together with biological fluids of non-invasive collection, allow earlier diagnoses and immediate responses. This method enables easy, off-laboratory assessments with minimal discomfort during sample collection, while being particularly advantageous for monitoring infants and children. In this context, paper sensor devices combined with the easy collection of biological fluids like urine are well-suited for both frequent monitoring and point-of-care analysis [1,2].

Urine is a biological fluid rich in biomarkers that provide valuable insights into a variety of health conditions. Compared to blood sampling, urine collection is non-invasive, minimising risks and infections and enabling a cost-effective analysis. This type of analysis is advantageous for evaluating children and the elderly, or conducting large-scale population screenings, due to its simplicity [3,4].

Zinc is an essential trace element, abundant in the body, mainly stored in muscles and bones, with vital roles in various biological processes, including enzyme function, immune responses, DNA repair, and wound healing. It is found in food, including meat, cereals, and dairy products, and stimulates bone growth and mineralization, as it influences osteoclast activity. Its multifunctionality enhances its importance in maintaining overall health and cellular functions [5,6]. It is also a crucial element for the development and growth of human beings, as well as their cognitive behaviour, particularly in the case of newborn babies and children [7,8]. Zinc deficiency is a serious problem that can affect nearly 17 % of the global population [5] and poses significant risks to children's growth, such as weakened immunity, stunting, and cognitive and motor development delays. Also, excessive zinc loss can lead to gastrointestinal or urinary tract disorders and can also be related to Attention-Deficit/Hyperactivity Disorder (ADHD) [8–10]. This can result from undernourishment and malnutrition, usually occurring in poor environments, often leading to long-term impacts [11]. Regarding urinary zinc levels, according to Zhao et al., they may vary from 0.150 to 0.850 mg/L [12] and in the work of Błażewicz et al., the reported values were from 0.420 to 0.715 mg/L [13]. Zinc urine levels can be related to

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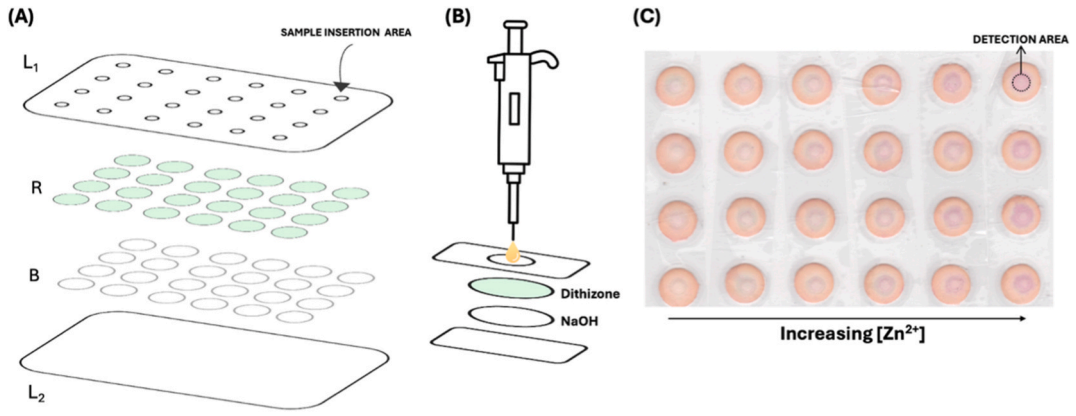

Fig. 1. Schematic representation of the μPAD for zinc(II) determination. (A) paper layers alignment; L1, top layer of the laminating pouch containing sampling holes; R, colour reagent layer; B, buffer layer; L2, bottom layer of the laminating pouch; (B) sample loading (15 μL); (C) scanned image with the developed coloured complexes and detection area. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mental conditions, such as autism spectrum disorders, and its deficiency can increase the prevalence of urinary tract diseases [6,13]. Preventive zinc supplementation has been studied as a therapeutic measure to address the deficiency associated with several health conditions and, therefore, improve health outcomes in children, such as its positive effect on growth. For this reason, monitoring the supplementation is crucial to ensure the adequate development of children worldwide [14,15].

The most common methods for quantifying zinc(II), as well as other metal ions, are classic spectroscopy techniques, namely atomic absorption spectrometry (AAS) [16,17] and spectrophotometry [18], which usually require advanced equipment and skilled professionals [19].

In recent years, microfluidic paper-based analytical devices (μPADs) have attracted significant attention, as these devices are low-cost, portable, use small volumes of sample and reagent, and are usually associated with effective point-of-care diagnostics [2,20], while following the World Health Organization (WHO) REASSURED guidelines [21].

These devices consist of defined hydrophilic zones, typically composed of filter paper, as well as hydrophobic zones/barriers. The hydrophilic area represents the reaction zone with paper as a platform, where the capillary action of the cellulose microchannels enables fluid flow without external power. Due to this paper platform, μPADs are equipment-free and lightweight, which results in easy storage and transportation [22].

There are various fabrication techniques, as is the case of wax and inkjet printing, photolithography, and cutting, with two-dimensional (2D) or three-dimensional (3D) structures. While the 2D-μPADs offer a simpler fabrication and are characterized by having a lateral flow, 3D-μPADs (obtained by stacking or folding different layers) offer increased flow control and allow different chemical reactions in the same device [1,22].

Regarding detection techniques, μPADs are often used with colourimetric analysis. A quantitative analysis can be obtained if combined with a colour image analysis software, such as ImageJ or Photoshop®, where the product colour intensities are obtained through RGB channels (Red, Green, or Blue) [23].

Therefore, this work had the main goal of devising a μPAD for zinc (II) determination in supplementation solutions and biological fluids of non-invasive collection, in this case, urine. The quantification was based on the reaction of zinc(II) and dithizone in an alkaline medium, adjusted with NaOH. The development of this device was based on stacking different filter paper layers inside a plastic pouch, without complex techniques, providing a more environmentally friendly analysis, while also being portable for in-situ measurements and a reliable point-of-care technique. To the best of our knowledge, only one μPAD was reported for the zinc(II) determination in urine samples, carried out simultaneously with copper determination, being this device based on a lateral flow approach, while also requiring a sample pretreatment [24].

2. Material and methods

2.1. Reagents and solutions

All solutions were prepared with analytical grade chemicals and Milli-Q® Water (Resistivity >18.2 MΩ cm, Millipore, Bedford, MA, USA).

The dithizone reagent solution was prepared daily by dissolving 1.0 mg of dithizone (Merck, Darmstadt, Germany) for 2 h in 10.0 mL of 96 % ethanol, achieving a final concentration of 0.10 g/L.

A 4.0 M sodium hydroxide stock solution was prepared, weighing 8.0 g of sodium hydroxide (PanReac, Barcelona, Spain) in 50 mL of water. The 0.30 M hydroxide working solution was prepared by dilution of the stock solution.

The zinc(II) stock solution of 5.00 mg/L was prepared from the 1000 mg/L AAS standard solution (Merck, Darmstadt, Germany), and the working standards within a range of 0.0500 mg/L to 0.750 mg/L were prepared by different dilutions of the stock solution.

The synthetic urine solution was prepared based on Brooks et al. [25], with the following composition: 10 g/L urea, 0.07 g/L uric acid, 0.8 g/L creatinine, 5.2 g/L sodium chloride, 0.1 g/L lactic acid, 0.4 g/L citric acid, 0.37 g/L calcium chloride dehydrated, 0.49 g/L magnesium sulphate heptahydrated, 1.41 g/L sodium sulphate, 0.95 g/L potassium dihydrogen phosphate, 1.2 g/L potassium hydrogen phosphate, and 0.49 g/L glucose.

2.2. μPAD assembly

Each μPAD was assembled by stacking two layers of filter paper discs (hydrophilic zone) aligned into a laminated plastic pouch (125 μm, 75 mic polyester, 25 mics low-density polyethene, 25 ethylene vinyl acetate), which, after lamination, consisted of the hydrophobic zone. This design provides a vertical flow approach as the sample/standard is inserted in one layer, flows through to a second layer. This approach allows a higher sample volume to be used and less loss of this volume by lateral dispersion.

There were 24 holes, sampling holes, perforated into the top layer of the plastic pouch in a 6x4 arrangement with a 3 mm of diameter (laser cutting machine, FDA, Model 3040), and the layers of filter paper discs aligned with the sampling holes resulted in 24 reading units (Fig. 1A).

For the calibration, #4 readings were performed per standard, and, in the case of sample placement, #6 readings units were utilised for

Table 1
Features of the devised μPAD for zinc(II) determination in urine samples; SD, standard deviation; RSD, relative standard deviation.

Feature	Values
Dynamic range	0.0500–0.750 mg/L
Typical calibration curve[a]	A = 0.0873 ± 0.0056 x [Zinc(II)] mg/L + 0.009 ± 0.001
A = slope ± SD × [Zinc(II)] + intercept ± SD	R^2 = 0.996 ± 0.003
LOD[a]	0.0100 mg/L
LOQ[a]	0.0340 mg/L
μPAD Reproducibility[a]	RSD = 6.4 %
Relative standard deviation of calibration curve slope (RSD)	
Precision[b]	RSD = 9.7 % (0.256 mg/L)
Relative standard deviation (RSD) of one sample (zinc(II) concentration)	
Time to scan (TTS)	5 min (up to 30 min)
Reagent consumption/μPAD	Dithizone: 24 μg NaOH: 2.88 mg
Sample consumption[c]	90 μL

[a] n = 5 calibration curves.
[b] n = 10 measurements.
[c] Per analysis (six reading units).

analysis. Both layers consisted of qualitative filter paper discs with a 9.5 mm diameter, cut with a puncher of 3/8" (EK tools, Woodridge, IL, USA). The top layer consisted of a Whatman® 1 (W1) filter paper, loaded with 10 μL of dithizone reagent and left air-drying for about 15 min before alignment.

As for the bottom layer, the paper used was Whatman® 4 (W4), impregnated with 10 μL of NaOH and followed by drying at 60 °C for 10 min. The two layers were then staked and aligned with the sample hole of the plastic pouch (Fig. 1A) and the plastic pouch laminated (A3 – 330c) to ensure the different hydrophilic and hydrophobic separation areas (Fig. 1C).

2.3. Zinc(II) determination

For the zinc(II) determination, 15 μL of sample/standard solution was loaded through the sampling hole (Fig. 1B), promoting a vertical flow through the two reagent layers (Dithizone and NaOH in Fig. 1B). For the colour development (observed in Fig. 1C), there was 5 min waiting period, at room temperature, as the time elapsed between the loading the sample/standard and scanning for image capture (named time to scan – TTS). The top layer of the μPAD was covered with tape to avoid contamination from handling biological fluids, namely urine samples. The scanning (Canon LIDE 120, 600 dpi resolution) was made on the colour reagent side (top layer), and the image was processed in the ImageJ software (version 1.53t), where the colour intensities of the different reading units were measured. The intensity values were measured at an 8-bit scale and by using an RGB (red, green, or blue) filter, and the green filter was chosen since it was the closest complementary colour of the reaction-coloured product (pink). These values were then converted to pseudo-absorbance values, using the equation $A = log_{10}\frac{I_0}{I}$, where A is the calculated absorbance, I_0 is the average intensity of the blank signal (four readings), and I is the intensity of each replica of standard/sample solution.

A calibration curve was established between the concentration values of the standards and the respective average absorbance values (four measurements), and a sample concentration can be calculated by the interpolation of average absorbance values (six measurements) in the established calibration curve.

2.4. Sample collection and/or preparation

All urine samples were collected from an age group of 5 to 10-year-olds, with informed consent, and used directly. When the zinc concentration value detected was over the dynamic range, a dilution of 1:2 with water was performed and the sample reanalysed. All samples can be analysed immediately after collecting or, if not possible, after storing at room temperature for 8 h, in the fridge for 24h, or by freezing (at −20 °C) for 1 week.

In the case of supplement samples, four zinc food supplements were purchased from local stores to be employed as reference materials and solutions were prepared for each one (ESM Table 1).

2.5. Accuracy assessment method

For the accuracy assessment of the zinc(II) determination in urine samples, the results obtained with the developed μPAD device were compared with the results obtained with atomic absorption spectrometry (AAS) for the same samples. A 2-fold dilution was made to all samples: in water for application in the developed device, and with 0.02 M nitric acid for the AAS procedure (final concentration of 0.01 M).

3. Results and discussion

3.1. Preliminary studies

The chosen colourimetric reaction for zinc(II) determination was the reaction between zinc(II) and dithizone [26]. This reaction occurs at an interval of pH 4–11, but to ensure that there were no interferences from other metals, the best reaction pH is alkaline [27]. For the batch studies, two sets of zinc(II) standards in the range 0.250–0.750 mg/L were

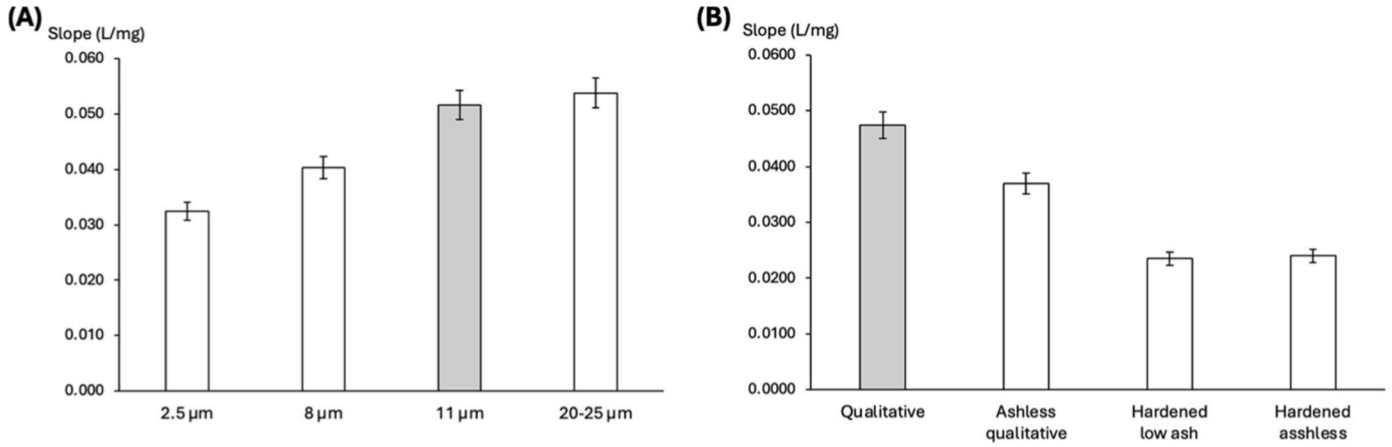

Fig. 2. Studies of the top layer filter paper influence on calibration curve slope (sensitivity); (A) papers with different pore sizes; (B) different types of papers; error bars represent a 10 % deviation; grey bars represent the chosen condition.

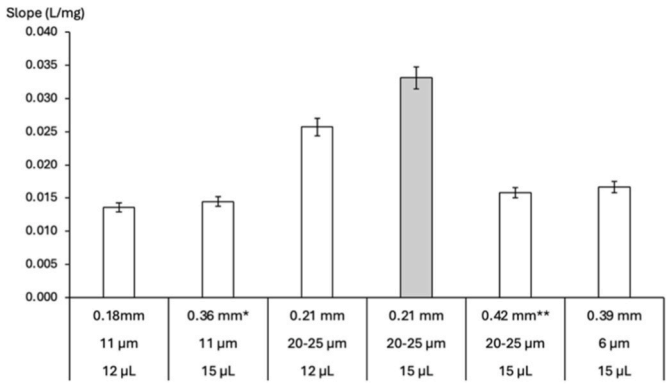

Fig. 3. Influence of bottom layer filter paper pore and thickness on sensitivity; error bars represent a 10 % deviation; grey bar represents the chosen condition; * 0.36 mm when using two stacked layers of 0.18 mm (W1); ** 0. 42 mm when using two stacked layers of 0.21 mm (W4).

prepared, one set in water and one set in synthetic urine. Then, 200 µL of standard was mixed with 50 µL of dithizone 0.1 g/L in ethanol 96 % (saturated solution), and 50 µL of borate buffer (pH 9) with a final volume of water of 1 mL. The results obtained in this batchwise procedure showed that dithizone reacts with zinc(II) in both matrices, as a linear calibration curve was obtained (ESM Fig. 1). The results also confirmed the need of having a buffered reaction at alkaline pH.

Following this requirement, a study of the µPAD basic design was carried out, first testing different paper disc diameters (ESM Fig. 2A) using both dithizone colour reagent and sodium hydroxide to ensure alkaline pH.

The best approach was using a top layer with dithizone, paper disc of 9.5 mm of diameter, and a bottom layer with sodium hydroxide, paper disc of disc 9.5 mm of diameter (assembly C4 in ESM Fig. 2A). Having the two discs of the same diameter, facilitated the assembly of the device and it enabled a higher standard volume of 12 µL. Then, calibration curves were established with zinc(II) standards in the range 0.250–1.50 mg/L reinforcing the chosen assembly of 9.5 mm of diameter for both paper discs layers with 12 µL standard volume (ESM Fig. 2B). All designs were made with dithizone on the top layer and NaOH on the bottom, as the reverse configuration did not work (no colour product was observed).

3.2. µPAD design

The paper disc of the top layer containing the dithizone colour reagent was studied in terms of paper type and pore size. The pore size was studied using qualitative paper type, and different pore sizes were tested: 2.5, 8, 11, and 20–25 µm (papers Whatman 5, Whatman 2, Whatman 1, and Whatman 4, respectively). Calibration curves were established and the slopes (sensitivity) compared (Fig. 2A). The sensitivity increased with the increase of the pore size (Fig. 2A). However, for the two higher pore sizes (11 µm and 20–25 µm) the intervals of 10 % relative deviation overlapped, so, the paper with 11 µm pore size (Whatman 1) was chosen as it is more economical.

Regarding the paper type, four paper types were tested: qualitative, ashless qualitative, hardened low ash, and hardened ashless. Once again, calibration curves were established and the slopes (sensitivity) compared (Fig. 2B). The qualitative type was chosen for being also the condition that provided the highest sensitivity. As so, the paper chosen for the reagent layer was Whatman 1 (qualitative with 11 µm).

For the bottom layer, a study with different paper pores and different thicknesses was made simultaneously. The papers Whatman 1 (11 µm pore and 0.18 mm thickness), Whatman 4 (20–25 µm pore and 0.21 mm thickness), and Whatman 3 (6 µm pore and 0.39 mm thickness) were compared, inserting different volumes of sample (12 µL and 15 µL). In all papers, 10 µL of NaOH 0.4 M was loaded into the discs, except when using the thickest paper (Whatman 3), where 20 µL of the same solution was inserted. The studies were made with one layer of Whatman 1, Whatman 4, and Whatman 3, and also with two stacked layers of Whatman 1 and Whatman 4. Observing the results shown in Fig. 3, the best sensitivities were achieved when using only one layer of Whatman 4 (20–25 µm and 0. 21 mm thickness). Two volumes were tested in this condition, and the best results were obtained when inserting 15 µL of standard/sample.

3.3. Reagents concentration

Using a solution with 0.1 g/L of dithizone, three different calibration curves were established using 0.2 M, 0.3 M, and 0.4 M of NaOH on the bottom paper disc layer. The concentration chosen was 0.3 M as it provided the highest sensitivity (ESM Fig. 3A).

Having set the concentration of NaOH (0.3 M), three different concentrations of dithizone were tested: 0.05 g/L, 0.08 g/L, and 0.1 g/L,

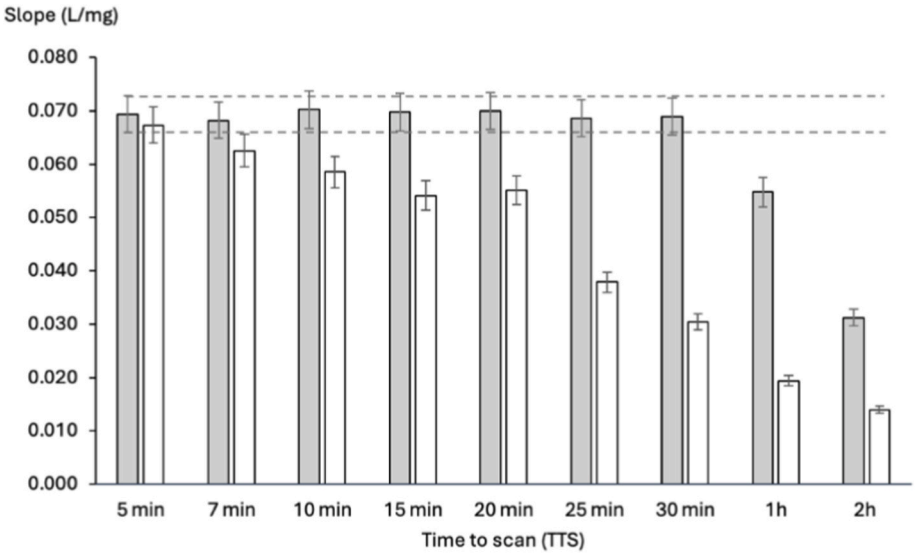

Fig. 4. Influence on the calibration curve slope (sensitivity) of the time to scan; grey bars, calibration curve slopes with 15 µL of standard/sample volume; white bars, calibration curve slopes with 10 µL of standard/sample volume; error bars represent a 10 % deviation; horizontal dashed lines represent the 10 % deviation of the calibration curve made with 15 µL of standard and 5 min time to scan time.

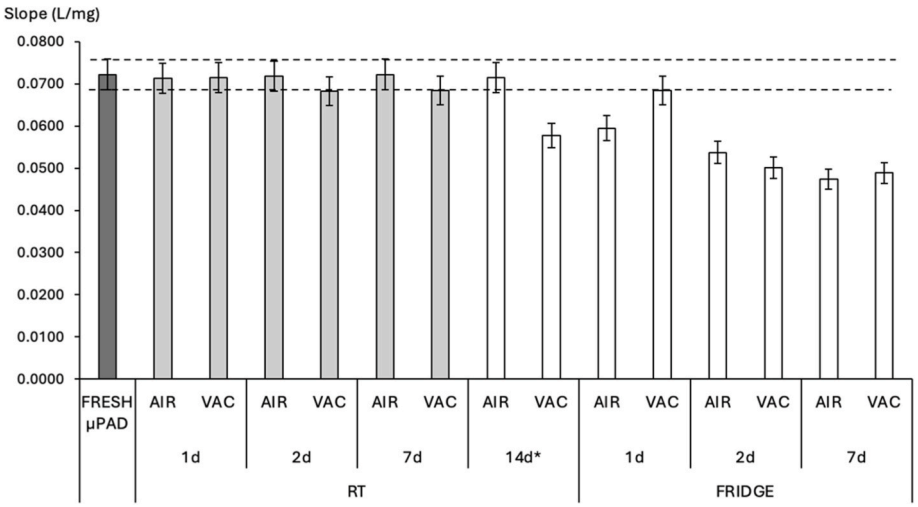

Fig. 5. Evaluation of the μPAD stability under different storage conditions by comparing calibration curves slopes; the dark grey bar represents the average of five calibration curve slopes obtained from a μPAD assembled and loaded in the same day (FRESH); light grey bars represent stored μPADs in which the device is stable; white bars represent stored μPADs in which the device is no longer stable; the error bars represent 10 % deviation; horizontal dashed lines represent the 10 % deviation from FRESH; "*" represents the calibration curves established with only three standards.

corresponding to a saturated solution.

The results obtained showed that the increase in concentration resulted in an increase in sensitivity, but from 0.08 g/L to 0.1 g/L the increase was only 3 % (ESM Fig. 3B); however, to ensure a reagent excess, 0.1 g/L was the chosen concentration.

Regarding the colour reagent stability, calibration curves were made with the same reagent preparation over 4 days, stored at room temperature (21 °C) and in the fridge (4 °C). With the obtained results (ESM Fig. 4A), it was possible to conclude that the dithizone reagent is stable for up to 3 days if stored in the fridge. However, not to compromise the following optimization studies, an approach was made for daily preparation. The dithizone dissolution time was also assessed, and the ideal time to dissolve the solid in ethanol is 2 h, as shown in ESM Fig. 4B.

3.4. Sample volume and time to scan

After setting the μPAD design and reagent concentrations, the influence of the sample volume was also studied. Then, calibration curves were established, with standard solutions prepared in MQW, with different loading volumes into the sampling hole, to determine the best time to scan with the highest sensitivity (calibration curve slope). This study also enabled to assess for how long this highest sensitivity was maintained. The device was scanned over time, from 5 min to 2 h, for the volumes of 10 μL and 15 μL (Fig. 4).

A volume of 20 μL was also tested; however, the sensitivity obtained was lower, and the loaded solution took 45 min to absorb completely. The results showed the highest sensitivity at 5 min both for 10 μL and 15 μL of sample/standard volume, so it was the time chosen. However, the highest sensitivity was maintained up to 30 min when using a volume of 15 μL (longer time span), and, for that reason, this was the volume chosen.

3.5. Matrix interference assessment

The potential chemical interference from the urine matrix was assessed by establishing two different calibration curves, one with standards prepared with Milli-Q water (MQW) and the other prepared with a solution of synthetic urine. Two μPADs were assembled, and each set of standards was applied.

The slopes of the two calibration curves were compared (ESM Fig. 5), and it was concluded that no urine matrix interference in zinc(II) determination was observed, as the difference between the calibration curve slopes was <10 %. Moreover, after performing a linear regression with a 95 % confidence interval and residual analysis, the obtained p-value was lower than 0.05, also supporting that both calibration curves don't show differences in the sensitivity. As so, the standards were subsequently prepared in MQW for the analysis.

As the compounds present in the synthetic matrix did not interfere, two urine biological samples were tested, and the results compared with those of atomic absorption spectrometry. The values obtained in the μPAD were 0.382 and 0.419 mg/L, and in the AAS were 0.412 and 0.447 mg/L, respectively, indicating that there was, in fact, no matrix interference (−7 % and −6 % relative differences). In the AAS, the samples were previously diluted to half, and the values were assessed with the dilution factor taken into account.

3.6. μPAD storage stability

To assess the device's stability, different storage conditions were tested after assembly and before the sample loading. Different μPADs were prepared according to the description above, loading the top layer paper with 10 μL of dithizone reagent (air-dried for about 15 min), and the bottom layer with 10 μL of sodium hydroxide (dried in the oven at 50 °C for 10 min). The two layers of filter paper were aligned inside the plastic pouch, followed by a lamination process. All the μPADs were stored and protected from light by covering with aluminium paper. One set was stored at room temperature (RT), and one set was placed in the fridge (Fridge). From each set, one subset was stored under atmospheric conditions (AIR) and another subset under vacuum conditions in clear zip-locked bags (VAC).

After each storage period, calibration curves within the dynamic range of 0.130–0.750 mg/L prepared in MQW were established. For that, the slope of the calibration curves obtained with the stored μPADs were compared with the average calibration curve slope (#5) obtained from μPAD assembled and loaded in the same day (FRESH), observed in Fig. 5.

All the devices stored at room temperature displayed no variation throughout 7 days (calibration curve slopes within the 10 % relative deviation interval of the average FRESH calibration curve slope). After 14 days, the calibration curves were only linear up to 0.500 mg/L (condition indicated as 14d* in Fig. 5) and the calibration curve of the devices stored in vacuum resulted in a significant slope decrease. From the devices stored in the fridge, only the ones stored under vacuum conditions for 1 day resulted in a calibration curve slope within the 10 %

Table 2
Analysis of children's urine samples with a comparative method ($[Zn(II)]_{AAS}$) and with the developed μPAD ($[Zn(II)]_{\mu PAD}$); standard deviation (SD) and relative error percentage (%RE).

Urine sample ID	$[Zn(II)]_{AAS}$	SD	$Zn[(II)]_{\mu PAD}$	SD	RE(%)
U1	0.460	0.001	0.451	0.044	−2.0
U2	0.649	0.001	0.629	0.015	−3.1
U3	0.412	0.001	0.401	0.066	−2.7
U4	0.597	0.004	0.548	0.025	−8.2
U5	0.447	0.002	0.431	0.015	−3.6
U6	0.621	0.001	0.585	0.058	−5.8
U7	0.559	0.005	0.587	0.024	5.0
U8	0.115	0.006	0.121	0.026	5.2
U9	1.419	0.010	1.375	0.030	−3.1
U11	0.431	0.002	0.461	0.025	7.0
U13	0.290	0.030	0.270	0.001	−6.7
U14	0.724	0.025	0.675	0.014	−6.8

variation of the FRESH calibration curve slope. Therefore, it was concluded that the device for zinc(II) determination is stable for up to 7 days if stored at room temperature.

3.7. Features of the μPAD

The analytical features of the devised μPAD for zinc(II) determination are presented in Table 1. The typical calibration curve was obtained from 5 calibration curves, made on different days and with standards prepared in MQW. The set dynamic range is from 0.0500 to 0.750 mg/L of zinc(II), as higher concentration no longer followed a linear response.

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to 1995 IUPAC recommendations [28]. LOD was calculated as the concentration corresponding to three times the standard deviation of the intercept, according to the formula: $LOD\left(\frac{mg}{L}\right) = \frac{3\ x\ SD_{intercept}}{Slope}$; and the LOQ as the concentration corresponding to ten times the standard deviation of the intercept, according to the formula: $LOQ\left(\frac{mg}{L}\right) = \frac{10\ x\ SD_{intercept}}{Slope}$.

The paper sensor's reproducibility was evaluated as the relative standard deviation (RSD) of five calibration curve slopes made on different days (interday). As for the precision of the method (9.7 %), this was assessed by calculating the RSD of ten measurements of the same sample. The reagent consumptions were calculated per μPAD (twenty-four reading units), 24 μg of dithizone, and 2.88 mg of NaOH, and the sample consumption was calculated per determination (six reading-units), 90 μL (15 μL per reading unit).

3.8. Accuracy assessment - application to samples

To evaluate the accuracy of the developed μPAD for zinc(II) determination, two types of samples were analysed directly in the developed device: urine samples and zinc supplement solutions.

3.8.1. Urine samples

To assess the accuracy of the developed μPAD for zinc(II) determination in urine samples, several samples (#12) were analysed directly in the μPAD and with a comparative method, atomic absorption spectrometry (AAS). The results are shown in Table 2.

All urine samples were collected from children with ages from 5 to 10 years old. The zinc(II) concentrations obtained in both methods were compared and the relative error between them was calculated. The analysed samples were diluted 1:2 to ensure they would fit the dynamic range proposed in the developed μPAD, as well as to follow the manufacture's guidelines of the atomic absorption spectrometry instrument, due to the sample matrix used. The values presented in Table 2 are the zinc(II) values considering the dilution factor. A linear correlation between the two methods was established (ESM Fig. 6B), with the equation $[Zinc(II)]_{\mu PAD} = 0.957\ (\pm 0.0515)\ [Zinc(II)]_{AAS} + 0.0086\ (\pm 0.0328)$, where the values in brackets represent a 95 % confidence interval. This showed that the devised paper sensor is not significantly different from the comparative procedure (AAS), as the slope of this equation was not statistically different from 1 and the intercept was not statistically different from 0 (t-student analysis) [29].

While carrying out studies of sample application in the zinc(II) developed device, the stability related to sample storage was assessed. The same sample was studied throughout different periods of time (0 h–1 week) and under three different conditions: stored at room temperature (21 °C), in the fridge (4 °C), and in the freezer (−4 °C).

The zinc(II) concentration values were compared always with the value obtained at the initial time (0h) (ESM Fig. 7). It was possible to conclude that, after taking a sample, if stored at room temperature, it is stable for up to 8 h, if stored in the fridge, stable up to 24 h, and, if stored in the freezer, stable up to 1 week.

3.8.2. Supplement samples

Aiming to enable the assessment of the intake and outtake of zinc(II) in children, supplement samples were also tested. Four different supplements were tested: S1, *Oligomax zinco* (Nutergia); S2, *Oligoéléments* (Bioligo); S3, *BioActivo Selénio + Zinco* (Pharma Nord); S4, *Naturmil* (Dietmed). Solutions of each supplement were prepared with different dilutions and were then loaded into the device and compared with the labelled calculated values (ESM Fig. 6A). The zinc(II) concentration obtained was compared with the concentration described in the supplements' package: S1, reported 5 mg/5 mL (per dose) and we obtained 5.1 mg per dose (RD = 1.8 %); S2, reported 0.312 mg/2 mL (per ampoule) and we obtained 0.330 mg per ampoule (RD = 5.6 %); S3, reported 15 mg/tablet and we obtained 15.5 mg per tablet (RD = 3.3 %); S4, reported 20 mg/tablet and we obtained 18.2 mg per tablet (RD = −9.3 %). This indicates that the device works for quantifying zinc(II) in aqueous samples, specifically, in zinc food supplements.

4. Conclusion

In this work, a microfluidic paper-based device was developed for zinc(II) determination in children's urine samples. With a reaction time of 5 min, this device provides a fast, inexpensive (1.50 € of consumables cost per μPAD) and in-situ monitorization of zinc(II) levels in children and, therefore, of the possible psychological development and mental conditions, as well as the occurrence of urinary tract diseases. The zinc quantification was carried out within the dynamic range of 0.0500–0.750 mg/L, which contains the expected values of zinc in urine, including values of zinc deficiency. Even when urine is concentrated above 750 μg/L (e.g., first urine of the day), a 1:2 dilution in water can be easily performed. This makes the devised paper sensor suitable for point-of-care analysis, especially for people in remote areas who do not have access to basic healthcare as it does not require specialised operators. The developed sensor is also suitable for analysing food supplement samples, to choose the right one for each child according to the zinc quantity of each supplement.

Moreover, the μPAD is environmentally friendly as it uses reduced amounts of reagent and sample, while also being easy to dispose via incineration.

After loading the samples in the device, a simple scan (after 5 min) is needed to analyse the coloured product intensities using a free image processing software (ImageJ). The image acquisition with a scanner ensures a quantitative measurement of zinc(II) with accuracy, while being non-dependent on operators that can manage devices in a different way. The zinc(II) quantification is performed with an RSD of 9.7 %, with samples being directly applied (non-diluted), or diluted (1:2 urine:water). The developed device is portable, as after the μPAD's assembly, zinc(II) can be accurately determined for up to one week, when the sensor is stored at room temperature, under atmospheric or vacuum conditions.

Table 3
Comparison of the main features of the developed method and other paper-based methods for zinc(II) determination; CD, colourimetric detection; ED, electrochemical detection; RT, reaction time.

Year	Sample matrix	Hydrophilic/Hydrophobic zones assembly	[Zinc(II)] range (mg/L)	LOD (mg/L)	LOQ (mg/L)	Sample volume (μL)	Observations	Reference
2025	Urine and food supplement solutions	Cut paper discs layered vertically and inserted inside a laminated plastic pouch	0.0500–0.750	0.0100	0.0340	90	CD; Vertical flow; 24 quantification zones in each device; RT 5 min; samples can be applied directly or diluted	This work
2024	Water (artificial)	Wax-printed patterned paper and heated at 115 °C for 60 s; cold bonding of μPAD elements on a laminating pouch	0–65	0.5	0.80	36	CD; RT 15 min; multiparametric μPAD (#3 analytes); different pH for each reaction	[31]
2023	Drinking water (spiked), dietary supplements and fertilizer samples	Wax printed channels in filter paper and heated at 150 °C for 90 s	327–10460	173	not reported	0.5	CD; fluorescence detection; quantity calculation based on the sample/standard volume (nmol)	[32]
2023	Urine	Cut printed pattern of chromatography paper and sticking it on a black cardboard	0.1–10	0.0359	0.118	15 μL	CD; RT 20 min; Need of pre-treatment with masking agents to overcome interferences; recovery studies using urine samples with zinc addition	[24]
2019	Water (spiked)	Wax-printed patterned paper placed under a screen stencil and brushed with paraffin; heating at 100 °C for 60 s	0.05–24	0.04	not reported	130 μL	CD; RT 15 min; sample filtration is needed	[33]
2018	Water	Wax-printed patterned central paper with an infrared oven at 150 °C for 4 min; inkjet printed reagents; card inserted into a lamination pouch	0.1–20	0.1	not reported	100 μL	CD; RT 3 min; No image processing step, diameter of the colour formation circular area analysed; interferences with other metals	[34]
2017	Sweat (spiked) and serum (spiked)	Wax-printed patterned paper and heated at 100 °C, for 2–4 min; three-electrodes screen-printed; addition of an insulating ink layer	0.08–2	0.025	not reported	100 μL	ED; Only recovery studies were performed as accuracy assessment	[37]
2017	Water (spiked)	Wax-printed patterned paper incorporated with three screen-printed electrodes; device folded and clamped	0.065×10^{-3} – 4.58×10^{-3}	0.020×10^{-3}	not reported	not reported	ED; connection of the electrodes into a single electrochemical system; proof-of-concept with recovery studies	[36]
2017	Water (spiked)	Wax-printed paper and melted through the paper with a hot laminator; inkjet printed reagent solutions	0.035–0.65	0.035	not reported	1 mL	CD; RT 15 min; Ca(II) interference is observed;	[35]

The proposed device in this work was compared to other paper sensors (Table 3) previously reported. All these methods used wax-printing techniques to establish hydrophilic and hydrophobic zones, as well as requiring an additional high-temperature heating step. This represents a higher cost of the fabrication process of these devices, as well as a challenge, since wax printers are becoming increasingly difficult to find due to their discontinuation [30]. These μPADs for zinc(II) determination are applied mainly to water samples, with colourimetric detection [31–35], and electrochemical detection [36]. There is also one device applied to biological samples, namely sweat and serum, with also an electrochemical response [37].

In the literature, there is only other μPAD for zinc(II) determination in urine samples [24] but it uses a lateral flow approach and has a higher limit of quantification. Having a vertical flow approach, our device provides a better control of the flow through the paper layers, as well as less loss of sample. Furthermore, in the reported paper, urine samples were used with zinc addition for recovery studies. In our work, the urine samples, without any zinc addition, were tested with the developed μPAD and the atomic absorption method.

To conclude, the devised microfluidic paper sensor provides an accurate zinc(II) determination in two matrix types, namely supplement solutions and urine samples, making it an excellent alternative to classical techniques, since besides offering an on-site application, portability, and low-cost analysis, it is also lab equipment-free and provides a simple biological sample handling.

CRediT authorship contribution statement

Maria M.P. Melo: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **António O.S.S. Rangel:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Raquel B.R. Mesquita:** Writing – review & editing, Supervision, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2025.128865.

Data availability

Data will be made available on request.

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