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Development of a microfluidic paper-based analytical device for magnesium determination in saliva samples

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ABSTRACT

In this work, a microfluidic paper-based analytical device (μPAD) was developed for magnesium determination in saliva samples. The idea was to develop a fast and simple method for biological magnesium quantification targeting saliva as an easy to collect and non-invasive alternative to blood or urine samples. The μPAD approach was chosen due to its advantages, namely ideally suited to conduct on-location determinations, and not requiring trained operators or specialized laboratory equipment. The developed μPAD was based on the colorimetric reaction between eriochrome cyanine and magnesium to form an intense orange/reddish colour product. The colour intensity was determined by image processing after digital scanning, made within 10 to 90 min after sample loading. Under optimal conditions, the dynamic concentration range was 82–247 μM, with detection and quantification limits of 62 μM and 81 μM, respectively. The device is stable for up to 3 months when stored in vacuum or in a modified nitrogen atmosphere. An accuracy assessment was made by comparing the results obtained using the developed μPAD with those from atomic absorption spectrometry (AAS). The relative difference between the two sets of results was below 5%.

Introduction

Of all the cations in the body, magnesium is the fourth most prevalent and the second most abundant intracellularly. This metal is associated with several important physiological roles in many body functions such as synthesis of nucleic acids and proteins, regulatory systems (e.g. oxidative phosphorylation, replication, glycolysis, etc.) and acts as a cofactor for more than 300 enzymes [1]. Disorders involving magnesium are characterised in two groups: hypomagnesemia (magnesium deficiency) and hypermagnesemia [2]. Very low magnesium concentrations are commonly associated with certain diseases (endocrine and metabolic disorders), specially *Diabetes Mellitus* [3]. Recently, some studies associate high plasma and cellular magnesium concentrations with the development of malignant tumors in the parotid gland [4,5].

Magnesium monitoring in the organism is very important because there is still no simple and rapid laboratory test to determine total body magnesium. To date, the serum magnesium concentration is the predominant test applied to blood samples [6]. However, this type of method requires high volumes samples, as well as complex equipment and technically trained staff to perform the analysis. To overcome these disadvantages, other biological samples, such urine and saliva, could potentially be used. When compared with blood sampling, saliva collection is non-invasive, does not involve some potential risk (e.g. bruises, discomfort or infections) and the methodology is simple, stress-free and cheap [7–9]. These type of samples are useful if children and the elderly need to be evaluated, or for large-scale population screening, offering an economic approach [10]. However, saliva collection has some disadvantages, such the fact that the sample volume is limited, and salivary biomarkers are still mostly unknown [11,12]. Saliva samples are stable for 24 h at a room temperature and, if the evaluation of the samples is desired within 3-6 days, they can be stored at $\leq$ 4°C [9]. The magnesium concentration in human oral fluid is usually about 0.2 mmol/L [13]. The most frequently methods used for quantifying metals ions are methods that require high profile equipment and qualified professionals, such atomic absorption spectrometry (AAS), high performance liquid chromatography (HPLC), ion chromatography (IC), colorimetric/ spectrophotometric or gas chromatography (GC)

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[14]. Although these techniques offer high analytical performance (sensitivity, accuracy, and precision), the limitations mentioned above contribute to a significant cost of analysis. In recent years, the advances in technologies there has been a great interest in the development of diagnosis methodologies more simple that ensure the characteristics suggested by the World Health Organization: devices must be affordable, easy-to-use, user-friendly, equipment-free, rapid and robust [15].

The concept of microfluidic paper-based analytical devices was introduced and described by a research group from Harvard University in 2007 [15], as devices that allow for microscale laboratory operations and use a low-cost miniaturized equipment. The μPADs use small amounts of sample volume, which has become quite beneficial if the amount of sample to be used are from biological samples [16,17]. There are two different zones that are characteristic of these devices: the hydrophilic area and the hydrophobic area. The hydrophilic area is where the chemical reaction takes place, and the hydrophobic area delimits the reaction zone. Paper is a widely used material as a chemical platform because it presents the advantage of being available in a range of thicknesses, porosities, and is easy to use, store and transport. As the μPADs are mainly made of paper (and sometimes plastic), they can be disposed by incineration quite quickly and safely [18,19].

Colorimetry is the most common method for μPAD analysis [20]. With colorimetric detection it is possible to get qualitative (visible to the naked eye – higher or lower colour intensity) or quantitative (using analysis software) analysis [21]. Usually, the detection zones of the μPAD are scanned to the computer using a flatbed scanner, camera or even mobile phone, depending on the image quality and lighting conditions [22]. The absorbance values can be obtained from the colour intensities measured for each detection zone by image processing (e.g. Image J or Photoshop). To obtain a higher colour intensity and contrast of detection zone, a RGB format is applied, and the filter colour is chosen depending on the colour of the reaction product. Comparing conventional methodologies and microfluidic paper-based analytical devices, the μPAD presents several advantages allowing performing bioassays faster with low manufacturing cost, no requirement of complex equipment or specialized operators [23]. They are affordable, portable and can be used anywhere, including in less industrialized areas or in developing countries, where the analytical and medical infrastructure is limited [24].

In this context, the aim of this work was to develop a new microfluidic paper-based analytical device for the magnesium quantification in human saliva samples. This μPAD uses an innovative assembly concept consisting of the alignment of paper discs within plastic pouches to create the hydrophilic and hydrophobic areas, respectively. The detection was based upon the colorimetric reaction between eriochrome cyanine reagent and magnesium ions present in saliva to form a strongly coloured complex. The device was studied to be used as a simpler and faster alternative with a non-invasive collection in a context of clinical diagnosis for diseases associated with magnesium concentration.

Materials and methods

Reagents and solutions

All solutions used in this work were prepared with analytical grade chemicals and Milli-Q® water (resistivity > 18 MΩ ·cm, Millipore, Bedford, MA, USA).

The eriochrome cyanine (EC) colour reagent solution was obtained by dissolving 0.50 g of the solid Eriochrome Cyanine R (Chromoxane Cyanine R Sigma, Germany) in 1 mL of 8 M HNO_3 and then adding 0.80 g of sodium chloride (Panreac, Germany) and 0.80 g of ammonium nitrate (Sigma, Germany) in 100 mL of water to final concentrations of 0.01 M EC, 0.14 M NaCl and 0.10 M NH_4NO_3 [25]. The 8 M HNO_3 solution was prepared from dilution of the 14 M concentrated acid (d = 1.39; 65%; Supelco, Merck, Germany).

The buffer solution was prepared by dissolving 0.67 g of NH_4Cl (Merck, Germany) in 6 mL of concentrated NH_4OH (d = 0.90; Merck, Germany) and diluted to 100 mL of water [26].

Magnesium stock solution of 2.0 mM (50 mg/L) was prepared by dissolving 19.5 mg of $MgCl_2$ (Sigma, Germany) in 100 mL of water and from this solution, an intermediate standard of 0.4 mM (10 mg/L) was obtained. Magnesium working standards in the range 0.082 – 0.247 mM (2.0 – 6.0 mg/L) were prepared from the intermediate Mg standard to final volume of 10 mL completed with synthetic saliva solution with BSA.

Synthetic saliva solution was prepared by dissolving 2.25 g of KCl (Merck, Germany), 0.55 g of KH_2PO_4 (Merck, Germany), 4.80 g of HEPES (Sigma, Germany) and 2.70 g of BSA, Bovine Serum Albumin, (Sigma, Germany) in a 1 L standard flask of water [27].

μPAD design and assembly

The developed μPAD (Fig. 1a) consisted in an array of 4 rows x 6 columns hydrophilic units composed of 3 layers of paper discs. Each paper unit has a detection layer (R_1), consisting of papers discs 9.5 mm (Whatman 50 filter paper) impregnated with the EC reagent solution. The middle layer (B_1), consisting of paper discs 9.5 mm impregnated with buffer solution (Whatman 1 filter paper) and the top layer (E_1) contained of empty 9.5 mm paper discs (Whatman 1 filter paper). The colour reagent discs were prepared by adding 10 μL of eriochrome cyanine reagent to each paper disc and oven dry at 50°C for 10 min. The buffer discs were prepared adding 5 μL of buffer solution and allow drying at ambient temperature (25°C) for 10 min.

The hydrophobic zones of the μPAD resulted from the use of laminating plastic pouches (Q-Connect). To add the sample/standard in the assembled μPAD, a sample hole (3.5 mm of diameter) must be made in one of the sheets of the lamination pouch (L_1).

The three hydrophilic layers were stacked into a paper unit (Fig. 1c) so that all layers overlapped, then the units placed between the two sheets of the plastic pouch and aligned with the sample hole of the top plastic sheet. After that, the lamination process takes place (Fellowes L125) and the hydrophilic areas (paper discs) are effectively delimited by the hydrophobic zones (plastic pouch).

Analytical procedure for magnesium determination

After assembly of the μPAD, 15 μL of sample/standard was loaded through the sample hole. Two minutes after loading the sample, the μPAD sample holes were covered with adhesive tape. The sample/standard flows vertically through the E_1 layer following B_1 layer and react with eriochrome cyanine in the layer R_1. This reaction forms an orange/reddish coloured product. The intensity of orange/reddish colour is directly proportional to the concentration of magnesium in the sample, as can be seen in the schematic bottom view of the μPAD (Fig. 1b).

The time between the sample/standard addition and the scanning of the μPAD was named time-to-scan (TTS) and was set to 5 min.

To measure the intensity of orange/reddish the colour, the detection zone of the μPAD (bottom view) was scanned using a flatbed scanner (Canon LIDE 120) to obtain the intensity readings. The high-resolution image of the μPAD was obtained in JPG format and the images processed using an image software (Image J, National Institutes of Health, USA). The RGB format was applied, and the colour intensity profile plots were obtained using the blue filter (as complementary colour). The selected area for intensity counts was 9.5 mm in diameter in the centre of each paper unit and, for each sample/standard, #8 units were measured. The measured intensities were imported into Excel (Microsoft Office Excel, version 16.16.4) and the colour intensity of each unit was subsequently converted to absorbance values (A) according to the equation: $A = log_{10}\ (I_0/I_S)$, when I_0 is the average of the #8 measured blank intensities, and I_S the measured sample/standard intensity. The blank signal, made for each μPAD, was attained loading synthetic saliva

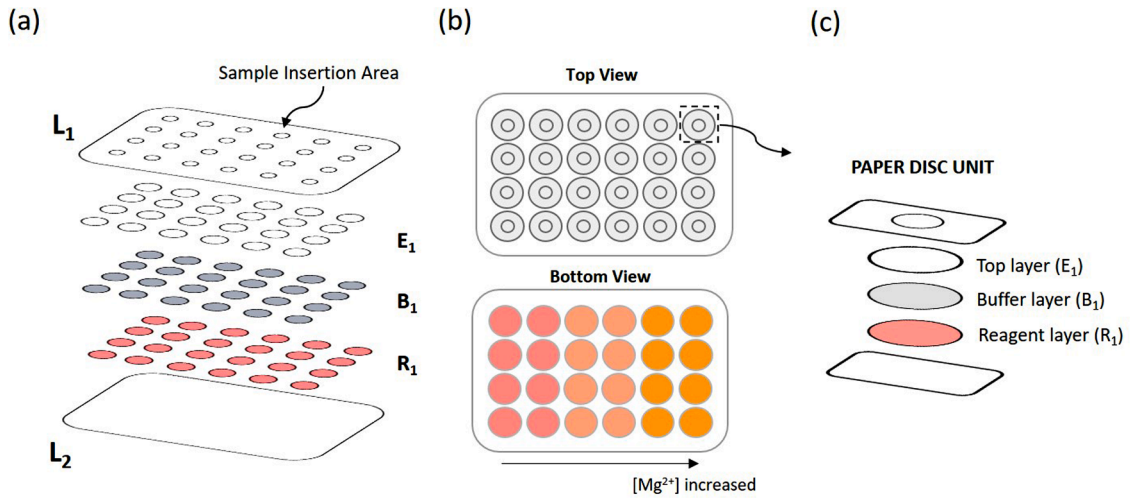

Fig. 1. Schematic representation of the μPAD assembly for magnesium determination. **(a)** the paper discs alignment and the respective layers of the μPAD: L_1, layer of the laminating pouch containing the holes for loading the sample; E_1, top layer; B_1, buffer layer; R_1, reagent layer; L_2, bottom layer of the laminating pouch. **(b)** Schematic representation of the top and bottom view of the μPAD after placing the standard. **(c)** Schematic representation of one paper unit of detection.

solution with BSA. Although it can be considered as a pseudo absorbance, it was named as absorbance values and a correlation with the magnesium concentrations was established through a calibration curve.

Samples collection

The saliva samples used in this work were collected from healthy volunteers, aged between 18 and 45 years old, recruited for the IMPULSE study, a one-group comparison, quasi-experimental dietary intervention, designed to assess the impact of a daily ovo-lacto-vegetarian pulse-based meal on health. This study (IMPULSE: Impact of a PULSE-based partial replacement diet on metabolome and health) intended to study the global need to adopt more sustainable food production and consumption systems. For this, several volunteers were submitted to a specific diet to evaluate the benefits of leguminous consumption. In this IMPULSE study, a fasting venous blood sample of each participant was collected in Vacutainer® serum tubes (BD, USA). The samples of this study were not handled directly in our work, only the values of the magnesium serum content were provided.

At the time each volunteer was giving the blood sample to the IMPULSE study, a saliva sample collection was also performed. The saliva sample collection procedure entailed placing a sterilized gauze (4 × 4 cm) in the oral cavity for approximately 2 min. After that, the gauze was filtered through a 0.2 μm syringe filter to Eppendorf tube. These samples were diluted in a solution of synthetic saliva with a 1:2 dilution factor and were used immediately. All saliva samples were stored in a freezer at -20°C.

Human and animal rights

As the saliva samples involved in this work were obtained directly from informed voluntary participants and no identification nor any extra information required, all rights were respected.

Reference procedure – accuracy assessment

To assess the accuracy of the μPAD measurements and to validate the developed paper-device for magnesium determination, a comparison was made between the μPAD measurements and the results obtained by Flame Atomic Absorption Spectroscopy (FAAS) reference procedure (see Electronic Supplementary Material ESM Table 1) for water analysis [28] since there are no reference methods for saliva analysis.

Results and discussion

Preliminary studies

Batch approach

In order to choose the most appropriate reagent for the magnesium determination, several colorimetric reactions were tested, namely the titan yellow [29], eriochrome black T [26], 4-(2-Pyridylazo) resorcinol (PAR) [30] and eriochrome cyanine (EC) [25]. The results obtained in a batchwise procedure showed a higher sensitivity for the reaction with EC (see Electronic Supplementary Material ESM Fig. 1), so it was the reagent chosen.

Having chosen EC solution as coloured reagent, some parameters of this reaction were tested using batchwise approach. According to Elenkova N. et al. [31], this reaction is quantitative and forms a well-defined coloured complex in the pH range 10.0 – 12.0. Therefore, these pH values were tested by adjusting the buffer solution to 10, 11 and 12 with sodium hydroxide (see Electronic Supplementary Material ESM Fig. 2). As the sensitivity was similar (RD < 5%) for all the tested pH it was chosen to use the buffer at pH 10 to avoid the potential precipitation of the magnesium ions. The use of a basic pH for the reaction with EC also avoids potential interference from other metal ions reported to react with EC in acidic conditions, namely Al(III) [25] and V (IV) [32].

μPAD approach

Having set the reagent and buffer solution, the colorimetric reaction was performed on a paper approach using a single paper layer (Whatman 42 filter paper). In this case, the EC and buffer solutions were mix and added to the same paper disc. However, after adding the magnesium standards, it was possible to observe lack of colour uniformity for the same magnesium concentration. Thus, a μPAD design with two layers (Whatman 42 filter paper) was tested and the EC and buffer solutions were added in different layers, R_1 and B_1 respectively and there was an improvement in the colour uniformity.

As the EC reagent has a reddish colour and in the presence of magnesium ions it forms an orange-coloured product (Fig. 1b), it was necessary to study the appropriate filter (RGB format) for image processing. Two possible complementary colour filter (blue and green) was studied, and the blue filter was chosen because the sensitivity increased 65% and allowed to obtain an image of μPAD detection zone with higher contrast and brightness.

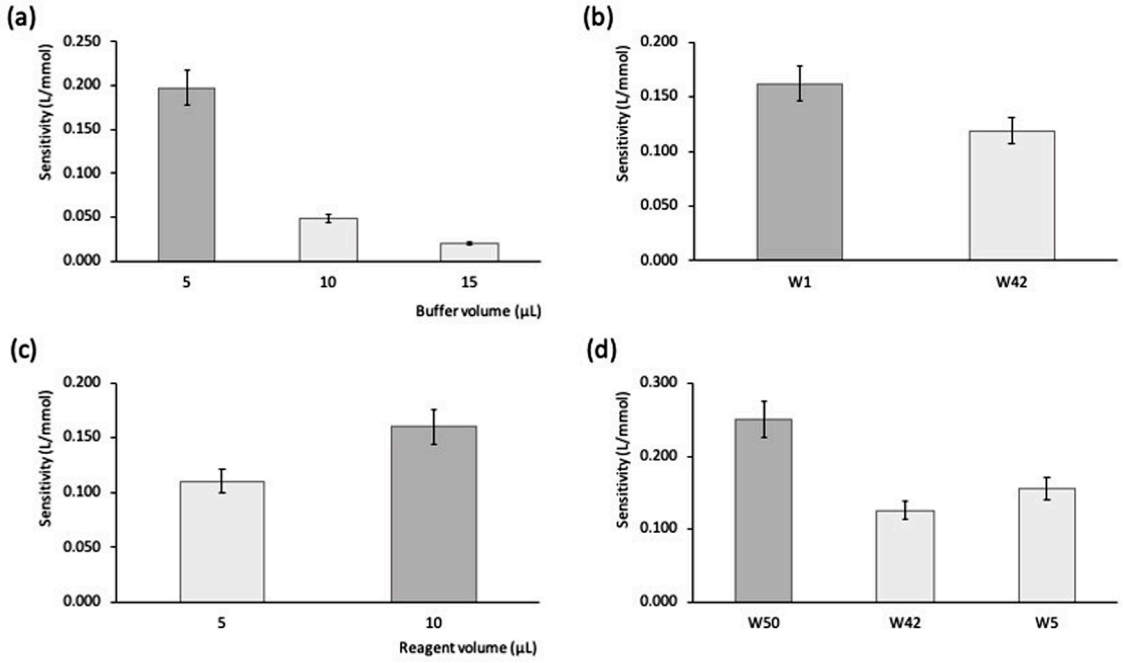

Fig. 2. Study of the influence in the calibration curve slope of the working volumes and different paper filters for the buffer and reagent layer. **(a)** The buffer volumes; **(b)** Different filter papers for buffer layer; **(c)** The reagent volumes; **(d)** Different paper treatments for reagent layer; the dark grey bars represent the chosen option; the error bars represent 10% deviation of the measurements.

Magnesium determination

Having set the main physical structure of the µPAD: top layer for the buffer solution and a bottom layer (detection zone) for the colour reagent, other determination parameters were studied. For the next studies, calibration curves were established to evaluate the highest sensitivity (calibration curve slope) obtained. Magnesium standard solutions in the range 0.082 – 0.247 mM and prepared in synthetic saliva with BSA (bovine serum albumin) were used.

Physical and chemical parameters

Initially, to optimise the buffer layer (B_1), different buffer volumes and filter papers grades were tested. The buffer volume was studied from 5 to 15 µL and represent the minimum and maximum volume possible, respectively. 5 µL was the volume chosen because it presented higher sensitivity (Fig. 2a). Two different types of filter paper (Whatman 1 and Whatman 42) were studied to evaluate the increase the rate reaction (Fig. 2b). The particle reaction of Whatman 1 (11 µm) was higher than Whatman 42 (2.5 µm). Compared the sensitivity of both calibration curves, it was possible conclude that the Whatman 1 would be the best option (higher sensitivity).

For the reagent layer, the influence of the eriochrome cyanine volume was also studied in the range of 5 – 15 µL (Fig. 2c). The 15 µL of EC solution was excluded because it saturated the 9.5 mm diameter paper and therefore the same reaction conditions were not guaranteed. The volume 10 µL was chosen as it provided the highest sensitivity. Different paper grades with similar porosity (Whatman 50, Whatman 42 and Whatman 5) were studied (Fig. 2d). The results obtained showed a higher sensitivity when the W50 was used, so it was the chosen one.

Sample volume

The influence of sample/standard volume in the calibration curve was assessed testing 10, 15 and 20 µL of sample/standard. However, when applying 20 µL, it took over 40 min for the µPAD to dry which would impair a fast determination and increase potential contamination and inaccuracy (possible evaporation over absorption) so that volume was excluded. For the 10 and 15 µL of sample/standard, the µPAD takes 10 to 20 min to dry, respectively. As sensitivity of the calibration curve did not change up to 1 h, it is possible to conclude that all the sample/standard was absorbed (see Electronic Supplementary Materials ESM Fig. 3). Still, when comparing performance associated to the two volumes, the sensitivity increased 67% using 15 µL of sample/standard, so this was the chosen volume.

Handling biological fluids

To reduce potential µPAD contamination and protect the operator from direct contact with biological samples, adhesive tape was used to cover all sample holes in the plastic laminating pouch after the sample loading. Using the adhesive tape did not interfere with the calibration curve slope (see Electronic Supplementary Material ESM Fig. 4). To reduce the contact time with biological samples, the insertion of a third layer was studied to reduce the absorption time of 20 min. The idea was to insert a new paper layer to promote the vertical flow and force the sample/standard through the layers (E_1-B_1-R_1). The Whatman 1 filter paper was chosen for this layer because it had a higher porosity (promote a greater flow rate) and it was the simplest and most economical paper. The calibration curves slope of 2 -ayer and 3-layer design were compared (see Electronic Supplementary Materials ESM Fig. 5) and the results obtained showed that there were no significant differences in the sensitivities (RD $<$ 10%). However, the 3-layer design was chosen as it had a drying time of approximately 2 min enabling a "time-to-scan" TTS of 5 min, while the µPAD with two layers took over 10 minu, resulting in a TTS of 20 min. The difference between the drying time and the TTS is due to the needed action of placing the adhesive tape.

Stability studies

Colour product stability

Considering the portability of the µPAD card device and its potential use in a "out of the lab" diagnosis tool used by non-specialized personnel, it is important to assess the time interval between the sample/standard loading and µPAD scanning. This time interval corresponds to the colour product stability. To study the stability of coloured reaction product of the developed µPAD, the calibration curve sensitivity was

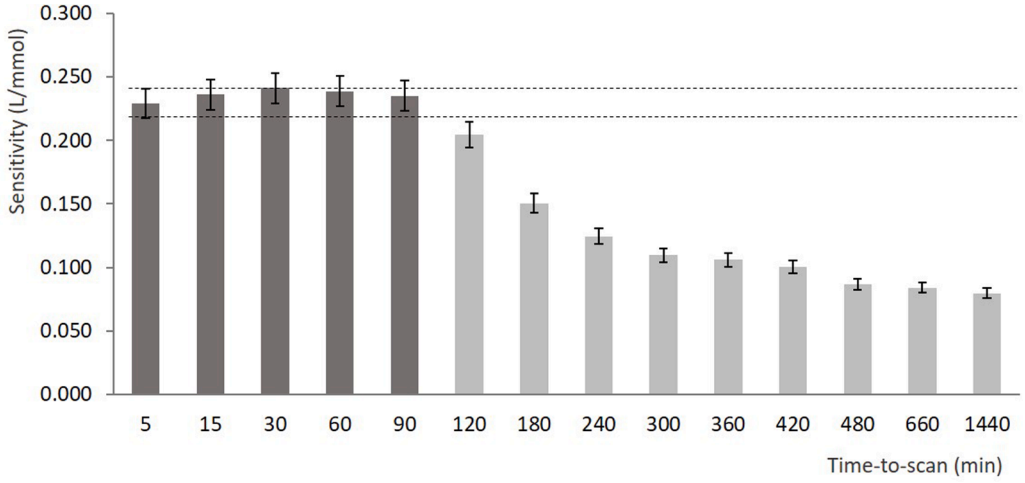

Fig. 3. Study of coloured reaction product stability on the μPAD. The recommended reaction times for the magnesium determination are represented by the dark grey bars (Time-to-scan (TTS) between 5 and 90 min); the black horizontal lines represent the range of the error bars with 5% deviation of the measurements at 5 min.

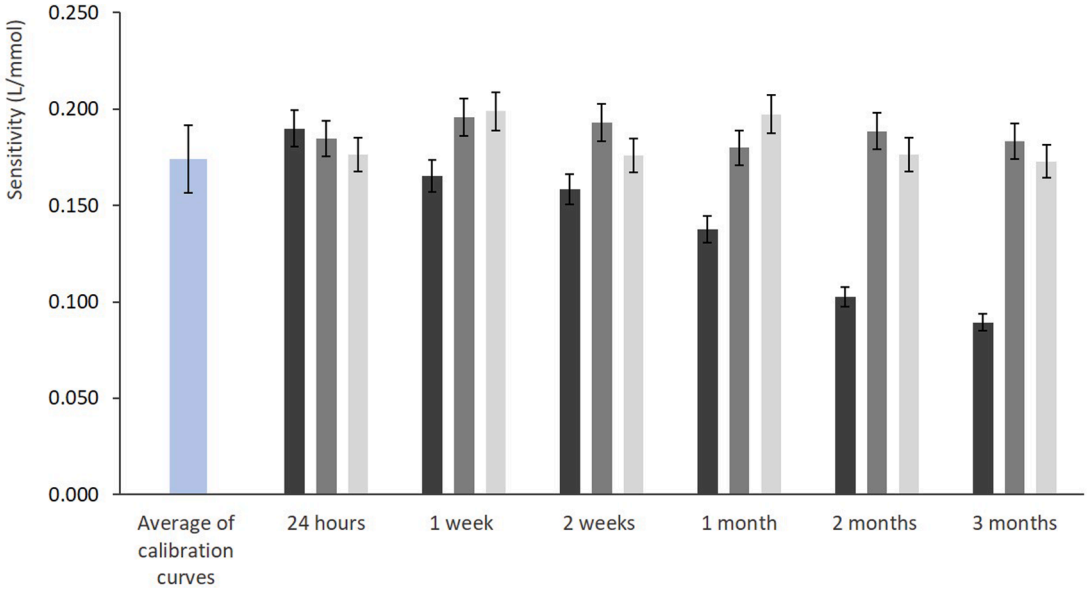

Fig. 4. Study of the stability of the developed μPAD under different storage conditions. The grey bars represent different storage conditions: dark grey bars – μPAD in contact with air; medium grey bars – μPAD in vacuum atmosphere; light grey bars - μPAD in nitrogen atmosphere. The blue bar indicated the average of the six calibration curves and the blue horizontal lines represent the range of the error bars with a 10% deviation from average of the calibration curves.

assessed with different scanning times starting at the established TTS of 5 min up to 24 h (Fig. 3).

The sensitivity of the calibration curve remained relatively constant during the first 90 min. However, after 2 h, there was a decrease in sensitivity. The sensitivity of the magnesium determination was stable if the detection reading was performed up to 90 min.

μPAD stability

To analyse the stability of the developed device, the μPADs were stored protect from light under different atmospheric conditions: in contact with air, in vacuum and under a modified atmosphere (nitrogen). The μPADs in air atmosphere were wrapped in aluminium foil; the devices in nitrogen atmosphere were stored in a closed zip bag (filled with nitrogen gas); and the μPADs stored in a vacuum, were closed in a sealed zip bag (air removed using a vacuum pump). All the devices were stored for periods ranging from 24 h to 3 months. For each calibration curve of all storage periods and conditions, a calibration curve obtained from a freshly assembled device was made and the average slope of six calibration curves were compared to the slope of each storage period and condition (Fig. 4).

Up to the two-week period all tested conditions showed sensitivity values within limits (a variation under 10% of the average calibration curves). However, after two weeks, a variation was considered non-significant only in the μPADs in vacuum and nitrogen atmosphere. A summary of the studied parameters and the chosen conditions are presented in Table 1.

Features of the developed μPAD for magnesium determination

The main features of the developed device are summarized in Table 2. The limits of detection (LOD) and the limit of quantification (LOQ) were calculated according to IUPAC recommendations [33] as the concentration equivalent to three times and ten times, respectively, the standard deviation of the intercept ($n=3$).

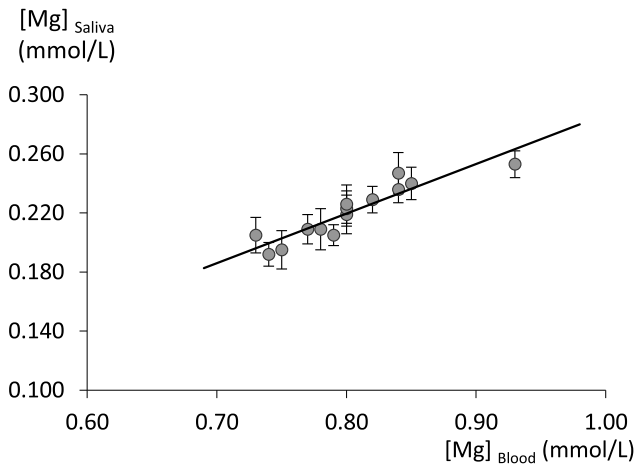

Fig. 5. Plotting of the results obtained with the developed μPAD for magnesium determination in saliva ($[Mg]_{saliva}$) with the magnesium concentration in blood ($[Mg]_{blood}$); the error bars represent the standard deviation of the measurements; the full line represents the linear correlation between the two sets of results.

Table 1
Summary of the optimized parameters of the μPAD developed.

Parameters	Tested conditions	Chosen condition
Reagents	Eriochrome black T, titan yellow, PAR and EC	EC, Eriochrome cyanine
RGB filter	Blue and green	Blue
Buffer volume (μL)	5 - 15	5
Buffer filter paper	Whatman 42 and 1	Whatman 1
Reagent volume (μL)	5 – 15	10
Reagent filter paper	Whatman 42, 50 and 5	Whatman 50
Sample volume (μL)	5 - 20	15
Sample hole size (mm)	3, 3.5, 4 and 5	3.5
Number of layers	1, 2 and 3	3

Table 2
Summary of the features of the developed μPAD for magnesium determination in saliva samples; Limit of Detection (LOD); Limit of Quantification (LOQ); Relative standard deviation (RSD);

Dynamic range (mmol/L)		0.082 - 0.247
Typical calibration curve [a] (A = slope x [Mg] + b)		A = 0.212 ± 0.008 $[Mg^{2+}]$ + 0.009 ± 0.001
LOD (mmol/L)		0.024
LOQ (mmol/L)		0.081
Repeatability, RSD (%)	Intraday [b]	1%
	Interday [b]	3%
Reagents consumption [c] (mg)		EC = 0.043 NH_4Cl = 1.62 NH_4OH = 13.0
Sample consumption/ determination (μL)		120
Time to scan (min)		5 (up to 90)

[a] n = 5
[b] n = 4
[c] per calibration curve

The μPAD repeatability was assessed by calculating the relative standard deviation of four calibration slopes in the same day (intraday RSD) and four calibration curves in the same week (interday RSD). The reagents consumption values were also calculated per calibration curve (each calibration curve = two μPADs). The sample consumption value is the volume required for each determination, i.e. the sample volume to fill eights detection units.

Interferences assessment – application to saliva

To evaluate the possible interference related to the salivary matrix, several μPADs were assembled and used with standards prepared in different compositions of saliva: synthetic saliva with BSA protein (as described in Section 2.2.); synthetic saliva matrix with mucin protein (replacing the BSA in the same concentration); and commercial artificial saliva (*Pickering laboratories*).

For this study, magnesium standards in the range 0.082–0.247 mmol/L were used and calibration curve established to compare the calculated slopes. The standards prepared in saliva with mucin was excluded because it was not a homogeneous solution, in fact suspended solids were observed. Between the synthetic saliva with BSA and the commercial artificial saliva no significant differences were observed in the calibration curve slope (RD < 10%) so the synthetic saliva prepared with BSA was chosen as a more economic choice.

Based upon the salivary matrix composition and the chosen reaction at basic medium, the only potential interference would be the presence of calcium, as both analytes can complex with eriochrome cyanine [34]. There are reported calcium concentrations in saliva ranging from 1 to 4 mmol/L, higher that the magnesium concentration of about 0.2 mmol/L, being 4 mmol/L the highest amount ever reported [13].

To assess the potential interference, three sets of Mg standards were prepared, one set with no calcium, one set with 1 mM of Ca^{2+}, and another set with 4 mM of Ca^{2+} (worst case scenario approach). Then, calibration curves were obtained with these sets of standards and the calculated slopes compared (see Electronic Supplementary Materials ESM Fig. 6). There was no difference between Mg standards and Mg standards with 1 mM of Ca^{2+} (RD < 5%) but with 4 mM of Ca^{2+} in the Mg standards a significant slope increase was observed (RD = 36%) indicating a positive interference.

As 4 mM of Ca^{2+} is not expected to be the usual (healthy) amount in saliva, it was important to assess the maximum calcium concentration that could be present in salivary samples without interfering. For this study, two Mg standards were prepared: *P1* with $[Mg^{2+}]$ = 0.082 mM and *P4* with $[Mg^{2+}]$ = 0.245 mM. These Mg standards were prepared without calcium and with increasing calcium over a concentration range 0.5 – 4.0 mM of Ca^{2+} (see Electronic Supplementary Materials ESM Fig. 7). The results showed that only the one with 4 mM Ca^{2+} interfered in the absorbance signal (RD >10%) for a TTS of 5 min, indicating that for the expected usual amounts calcium (about 2 mM), the magnesium determination was not affected. It was important to ensure setting the TTS at 5 min as the study also showed that the interference increased with increasing contact time.

Application of the developed μPAD to saliva samples

Accuracy assessment

To assess the accuracy of the developed μPAD for magnesium determination in saliva samples, six saliva samples were analysed and the results obtained by the μPAD method were compared with the results obtained by the Flame Atomic Absorption Spectrophotometry (FAAS) reference procedure. The magnesium concentration values for each sample were obtained through interpolation in the calibration curves for each method (see Electronic Supplementary Materials ESM Fig. 8). A linear regression was established between the two sets of values: $[Mg^{2+}]_{\mu PAD}$ = 1.00 (± 0.07) $[Mg^{2+}]_{FAAS}$ + 0.034 (± 0.077), R^2 = 0.997, where $[Mg^{2+}]_{\mu PAD}$ was the concentration obtained by the developed method and $[Mg^{2+}]_{FAAS}$ was the concentration of reference procedure (FAAS). It was possible to conclude that the slope was not statistically different from 1 and the intercept is very close to 0 with a 95% confidence interval. The results allowed to conclude that there is no differences between the two methods.

Assessment of potential correlation between salivary and serum magnesium

For this assessment, the salivary magnesium content of several sample was plotted against the magnesium level in the blood (values provided) and a linear relationship was established between the two sets of results (Fig. 5), illustrating a correlation between the salivary and the serum magnesium content.

The relative difference between the two sets of results was calculated and an average of -73% was obtained indicating that the salivary magnesium corresponds to about 30% of the magnesium in serum (see Electronic Supplementary Materials ESM Table 2). To test this hypothesis, a comparison was made between the calculated serum magnesium ($[Mg]_{blood\ calculated}$), from the salivary magnesium value, and the assessed serum magnesium ($[Mg]_{blood\ assessed}$) and no differences were observed (see Electronic Supplementary Materials ESM Fig. 9). In the end, it was possible to conclude that, considering that the salivary magnesium about 30% of the magnesium content in serum, the developed μPAD can be an accurate tool to monitor the magnesium concentration.

Conclusion

This work presents the development of a new microfluidic paper-based analytical device for the magnesium quantification in saliva samples. This device proved to be a simple, rapid, and affordable method, which could be an alternative for clinical diagnosis of conditions associated with Mg concentration in the body. The developed μPAD showed to be suitable for on-site analysis and point of care testing (POCT) due to its simplicity and it does not require trained operators or specialized laboratory equipment. An additional advantage is the fact that the device is disposable by incineration, which makes this it more "environmentally friendly" compared to other analysis methods/equipment and is an asset for biological samples.

The described device in this work was successfully applied to saliva samples in a range of 82 to 247 μM with limit of detection and limit of quantification of 24 and 81 μM, respectively. It important to emphasize that it only use 120 μL of sample and small amounts of reagents, which is very beneficial compared to other types of biological sample, namely blood and urine samples.

Ethics approval

The saliva samples involved in this work were blind samples, with no identification nor any information required or register, obtained from voluntary participants with informed consent. There was no association to a clinical trial.

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Authors' contributions

Conceptualization - Raquel B. R. Mesquita; Methodology – Juliana I. S. Aguiar, Mafalda T. S. Silva, Helena A. G. Ferreira; Validation - Juliana I. S. Aguiar; Formal analysis - Juliana I. S. Aguiar, Mafalda T. S. Silva, Raquel B. R. Mesquita; Investigation - Juliana I. S. Aguiar, Mafalda T. S. Silva; Resources - António O. S. S. Rangel, Marta W. Vasconcelos; Writing - Original Draf Juliana I. S. Aguiar; Writing - Review & Editing - Raquel B. R. Mesquita, Elisabete C. B. Pinto, António O. S. S. Rangel; Project administration - Raquel B. R. Mesquita, António O. S. S. Rangel; Funding acquisition António O. S. S. Rangel; Marta W. Vasconcelos.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Supplementary materials

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