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Salivary calcium determination with a specially developed microfluidic paper-based device for point-of-care analysis

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ABSTRACT

The calcium monitoring in the body not only anticipates several potential diseases (osteoporosis, kidney stones or high blood pressure) but also helps to improve target therapies and follow-up the patient's health status. Calcium monitoring is essential for the diagnosis of one of the most common endocrine disorders worldwide, namely hyperparathyroidism. So, in this work, a new Point-of-care test (POC-test) using a microfluidic paper-based analytical device (μPAD) for calcium quantification in saliva samples is described. The developed μPAD was based on the colorimetric reaction between calcium and cresolphthalein complexone (CPC) which forms an intense purple colour product. The developed device enabled calcium quantification in the range of 0.27–4.50 mmol/L (11.0–180 mg/L) with a detection limit of 80 μmol/L (3.2 mg/L). The accuracy of the developed μPAD was confirmed by analysing saliva samples (#10) and comparing the results obtained with the atomic absorption spectrometry reference procedure; the relative deviation between the two sets of results was below 10 %. A correlation between salivary calcium content and calcium content in blood was established and it was possible to conclude that salivary calcium concentrations above 1.55 mmol/L is an indicator of hypercalcemia. The developed device was stable for 2 weeks when stored at room temperature in vacuum conditions.

Introduction

The clinical interest in salivary metabolites has been growing because it is a biological fluid that contains many of the same biomarkers as blood and serum samples. Therefore, saliva can be used for the potential detection of diseases and follow up the patient's health status [1–3]. Saliva offers many advantages compared to other biological samples namely being non-invasively, painless collection with cheap consumables. The collection procedure is simple, and stress-free, and it can be self-collected suitable for clinically difficult situations, such as patients who suffer from anxiety from blood collection, and with children [4,5].

Calcium is one of the inorganic electrolyte components in saliva, found in its ionic form, which plays an important role in numerous body functions [6]. It is important to establish a correlation between salivary content and serum content due to its physiological meaning. Calcium level in the blood is regulated by two hormones: PTH – parathyroid hormone, produced by the parathyroid glands, and calcitonin, produced by thyroid glands. Thus, calcium monitoring is essential for the diagnosis of hyperparathyroidism, which represents one of the most common endocrine disorders worldwide [7]. The clinical consequences are typically hypercalcaemia (high calcium concentrations) and excessive secretion of PTH [8]. Hypercalcaemia is also related to other health issues, such as high blood pressure, kidney stones, chronic fatigue, and osteoporosis [9,10]. The most common cause of hypocalcaemia (low calcium concentrations) is Vitamin D deficiency [6].

Most of the commonly used methods for the determination of salivary metabolites include high-performance liquid chromatography with mass spectrometry (HPLC-MS), atomic absorption spectrometry (AAS), and two-dimensional gas chromatography (GC). Although these methods offer high analytical performance, they also involve a significant cost of analysis, because are technologies that require sophisticated instrumentation and highly trained technicians [11]. Aiming to make saliva analysis more affordable and accessible, new diagnosis approaches based upon microfluidics have been developed following the World Health Organization (WHO) guidelines for affordable tools [12–14]. Microfluidic paper-based analytical devices (μPADs) are low-cost, portable, and disposable and have emerged as a promising alternative diagnostic tool to conventional methods [15–21]. Furthermore, μPADs are particularly useful in resource-limited settings where

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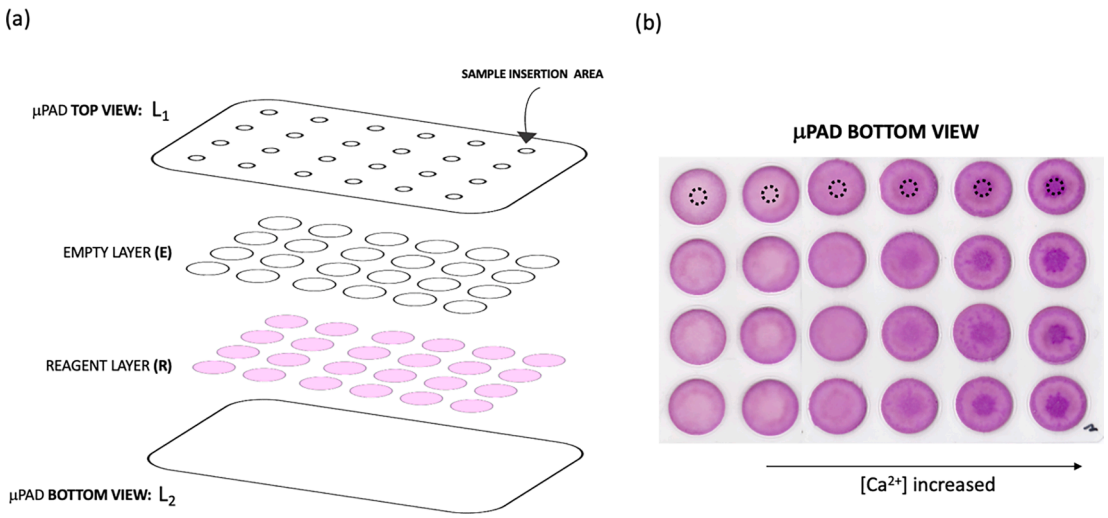

Fig. 1. Schematic representation of the μPAD assembly for calcium determination; (a) paper discs alignment and the respective layers of the μPAD; L_1 and L_2, laminating pouches sheets and the top and bottom view of the μPAD, respectively; E, empty layer; R, reagent layer; (b) image of the μPAD detection (bottom view) after loading the calcium standard solutions; the dashed circles represent the measurement colour areas.

access to laboratory equipment and trained technicians may be limited. They are also ideal for point-of-care (POC) testing, as they are hand-sized (small and portable) and can be used in remote areas.

Until now, only one microfluidic paper-based device was designed for the assessment of calcium levels in biological saliva samples [22]. However, this device involves wax printing, which entails the utilization of costly wax materials and an extra step of heating, necessitating higher temperatures (approximately 120 °C). In this context, the aim of this work was to develop a user-friendly μPAD for calcium quantification in human saliva samples, that doesn't require specialized technicians or complex equipment (like wax printers). The colorimetric reaction was based upon the reaction between calcium and cresolphthalein complexone, forming a strongly purple coloured complex. The paper device was studied to be used as a simpler, faster, and POC-test alternative in the context of clinical diagnosis for diseases associated with calcium levels and regarding a patient's health status.

Materials and methods

Reagents and solutions

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

A calcium standard stock solution of 13.6 mM (545 mg/L) was prepared by dissolving 100 mg of calcium chloride dihydrate (Merck, Germany) in 50 mL of water. From this solution, working standards within the range of 0.27–4.50 mM (11.0–180 mg/L) were prepared in synthetic saliva with BSA protein. According to Eisenburger et al. [23], synthetic saliva solution was prepared by dissolving 2250 mg of KCl (Merck, Germany), 544 mg of KH_2PO_4 (Merck, Germany), 4775 mg of HEPES (Sigma, Germany) and 2700 mg of BSA - Bovine Serum Albumin (Sigma, Germany) in 1 L of water. This composition mimics the ionic content and the viscosity of saliva samples.

The cresolphthalein complexone (CPC) solution was prepared weekly by dissolving 20 mg of the solid (Sigma, Germany) in 10 mL of 14 g/L of AMP solution to a final concentration of 2.0 g/L CPC. The 14 g/L 2-amino-2-methyl-1-propanol (AMP) buffer solution (Sigma, Germany) was prepared by dissolving 1.4 g of solid (Merck, Germany) in 100 mL of water [24].

μPAD assembly

The assembly of the μPAD consisted of a hand-sized card composed of 24 reading units (hydrophilic areas), in an array of 4 rows × 6 columns (Fig. 1a), under the 4 mm holes previously perforated in a laminating pouch (Q-connect, 75 × 110 mm, glossy, 125 microns: 75 microns polyester, 25 microns low-density polyethylene, 25 microns ethylene vinyl acetate glue). Each reading unit comprised two layers: the top layer, E, consisted of an empty Whatman Grade 4 (W4) filter paper disc with 1.27 cm diameter; and the bottom layer, R, consisted of a Whatman Grade 50 (W50) filter paper disc with 1.27 cm diameter loaded with reagent solution. The reagent discs, layer R, were prepared by loading 30 μL of CPC coloured reagent in the W50 paper discs and drying them in the oven for 20 min at 50 °C for. The reading unit was assembled by stacking the two paper discs of each layer to ensure layer overlapping and a vertical flow approach.

All the paper units were aligned with the 4 mm sample insertion holes of the laminating pouch and the μPAD assembly was completed with the lamination process (United Office – ULG 300 B1) creating a strong physical separation between the hydrophilic area (paper units) and the hydrophobic zone (laminating pouches). Having the empty paper disc on top of the reagent disc when aligning with the sample insertion holes minimizes the reagent-loaded disc contact with air.

Calcium measurement procedure

After μPAD assembly, 30 μL of standard/sample was loaded through the sampling hole, which took approximately 2 min to ensure the total absorption. Then, the sampling holes were covered with adhesive tape, to guarantee minimal contact of the operator with the biological samples. The standard/sample flows through the unit in a vertical flow approach (Fig. 1a) and the reaction between CPC and calcium forms an intense purple complex in the bottom/reagent layer (R).

This layer became the μPAD detection zone and, ten minutes after loading the standard/sample volume was scanned using a flatbed scanner (Canon LIDE 120) for imaging acquisition. The images were processed using ImageJ software (ImageJ, National Institutes of Health, USA) to measure the colour intensity. To obtain the highest intensity, a colour filter from RGB format with the complementary colour (green filter) of the reaction product, was used. For each paper unit, a measurement area in the reagent layer was set as corresponding to the μPAD sample hole (4 mm = 100 × 100 pixels) (ESM Fig. 1).

The intensity values were then converted into absorbance values

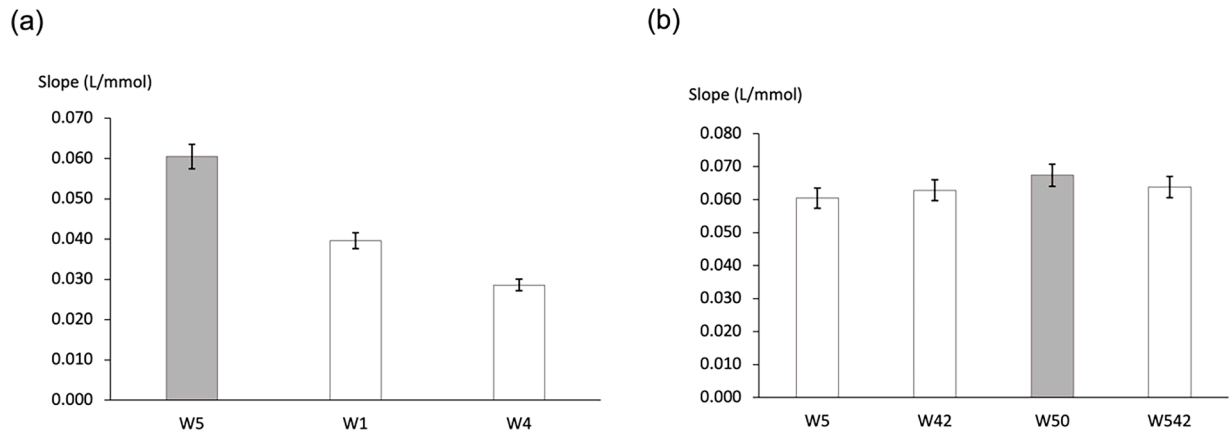

Fig. 2. Study of the influence of different filter papers in the calibration curve slope: (a) different paper porosities and (b) different types of filter paper; the grey bars represent the chosen option; the error bars represent 10 % deviation of the measurements.

using the Beer-Lambert law equation: $A = \log_{10}(I_0 / I_S)$, where A is the absorbance value, I_0 is the average measured intensity of the blank signal and I_S is the average measured intensity of the standard/sample signal. The purple colour product became more intense with increasing calcium standards concentration (Fig. 1b) so, with the calculated absorbance values (corresponding to an average of 4 readings), a calibration curve was established.

Sample collection

The saliva collection procedure consisted of placing a 5 × 5 cm sterilized gauze in the oral cavity for approximately 2 min. After that, the gauze was filtered through a 0.2 μm syringe filter to Eppendorf tube. The samples were loaded directly into the μPAD without any dilution or sample pre-treatment. In cases where the samples were not used on the same day as collection, they 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, all rights were respected.

Ethics declarations

The saliva samples used in this work were blind samples, with no identification or any information required or registered, and were obtained from volunteer participants with informed consent. There was no association with a clinical trial.

Reference procedure – accuracy assessment

To assess the accuracy of the developed device for calcium determination, 10 saliva samples were analysed with the developed μPAD method and by atomic absorption spectroscopy (AAS) reference procedure for water analysis [25], since there is no reference method for saliva samples analysis.

Results and discussion

The studies performed to optimize the developed μPAD were made by comparing the slope (sensitivity) of the calibration curves obtained using calcium standard solutions within the concentration range of 0.30–5.0 mM (12.0–200 mg/L). The chosen option consisted of the highest slope as an indication of higher sensitivity.

Preliminary studies

The first study consisted in comparing different colour reagents for calcium determination namely 4-(2-pyridylazo) resorcinol (PAR), Zincon, 1-(2-pyridylazo)−2-naphthol (PAN) and σ-cresolphthalein complexone (CPC). Calibration curves were established, in a batchwise procedure, and the results obtained showed a significantly higher sensitivity for the reaction with CPC (ESM Fig. 2), so it was the reagent chosen for further studies.

Then, the colorimetric reaction was performed on paper and a μPAD

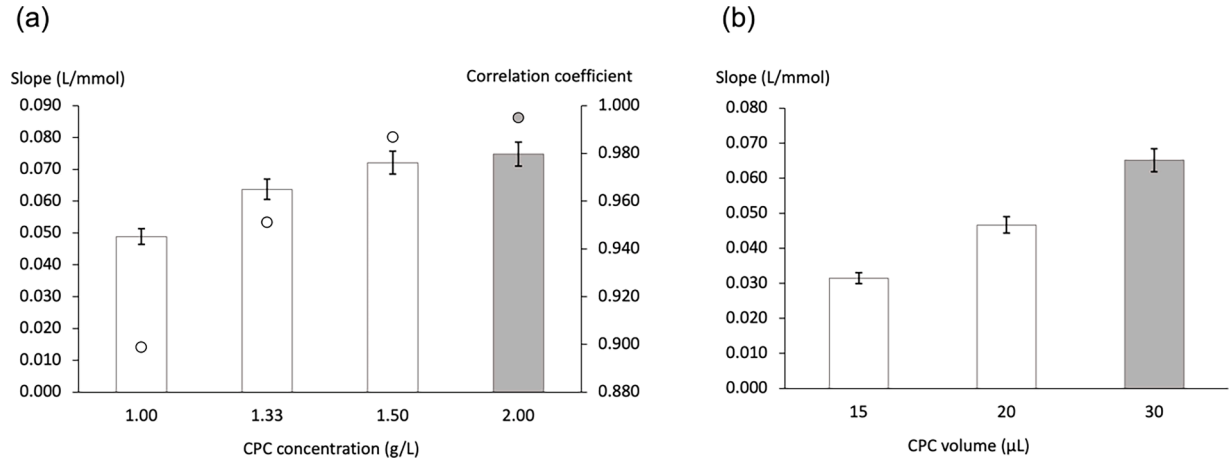

Fig. 3. Study of the influence in the calibration curve of (a) different CPC concentrations, and (b) different CPC solution volumes; bars represent the calibration curve slope; the circles represent the correlation coefficient; the grey filling represents the chosen options; the error bars represent 10 % deviation of the measurements.

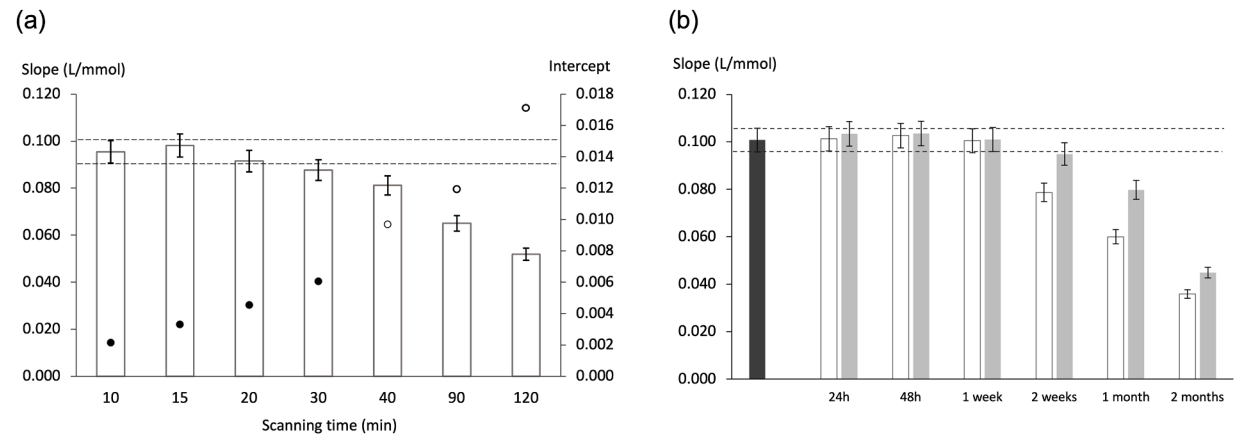

Fig. 4. Stability assessment of the developed μPAD for calcium determination; (a) the colour product stability, comparing the calibration curve slope (bars) and intercept (circles) with the interval corresponding to the 10 % deviation of the 10 min calibration curve (horizontal dashed lines); (b) the μPAD storage stability, in contact with air (white bars) and in vacuum (light grey bars) comparing to freshly prepared (dark grey bar) μPAD 10 % deviation (horizontal dashed lines).

with two paper layers was assembled, to ensure a vertical flow approach. For this first paper approach, both layers consisted of 12.7 mm Whatman 1 filter paper discs with an empty paper disc placed on top of a paper disc loaded with the reagent. A calibration curve was established by loading 15 μL of standard and it was observed that it took about 2 min to be absorbed.

μPAD assembly

Study of the filter paper type

The type of filter paper used to load the reagent, consisting of the reagent layer, was tested, evaluating the influence of different papers on the calibration curve. First, Whatman qualitative filter papers with different pore sizes, 2.5 (W5), 11 μm (W1) and 20–25 μm (W4), were compared and the highest sensitivity resulted from the paper with the smallest pore size, W5, so it was the one chosen (Fig. 2a). Then, with the chosen pore size of 2.5 μm, Whatman filter paper with different treatments, were also tested: qualitative (W5), ashless (W42), hardened low ash (W50) and hardened-ashless (W542). The results obtained showed (Fig. 2b) that the calibration curve slopes were similar (slope deviation < 10 %), being slightly higher with W50 (slope deviation $= 12$ %), so, it was the paper chosen.

For the top layer of the μPAD, being an empty layer, the influence of the type of paper on the calibration curve was not evaluated and qualitative paper was set. However, considering the potential influence of paper porosity in the vertical flow, the pore size influence in the calibration curve was assessed. The papers porosity tested was the same as previously used for the reagent layer paper discs namely 2.5, 11 and 20–25 μm (W5, W1 and W4, respectively). Comparing the calibration curves slope (ESM Fig. 3), the paper with the highest porosity (W4) was chosen because displayed the highest sensitivity.

CPC concentration and volume

The influence in the calibration curve slope of both the concentration and volume of the CPC reagent solution was evaluated. The CPC concentration was varied within the range 1.0–2.0 g/L and the sensitivity (calibration curve slope) increased with the increase of concentration (Fig. 3a). However, the results showed that the increase was not statistically significant (slope deviation < 10 %) when comparing the calibration curve slope of 1.5 and 2.0 g/L. Still, 2.0 g/L was the concentration chosen because it presented the highest correlation coefficient ($R^2 > 0.99$) indicating better linearity.

Then, different CPC solution volumes were tested within the range 15–30 μL (Fig. 3b), and 30 μL was the chosen volume because it presented the highest sensitivity. Higher volumes were not tested because the 30 μL corresponded to the maximum volume that could be loaded into the paper disc.

Image processing

After setting the μPAD assembly, some studies related to the image treatment were performed. The image of the μPAD detection zone was obtained by scanning the bottom layer, then the colour intensity was calculated by converting that image to RGB staking in the imaging processing software (ImageJ). As both the reagent and the reaction product presented colour, two potential complementary colour filters were tested: one targeting the purple-formed product and one targeting the dark pink reagent, green and red, respectively. The green filter, targeting the product formed, resulted in calibration curves with a higher sensitivity (ESM Fig. 4), so it was the filter chosen.

As mentioned, the reaction between CPC and calcium formed an intense, purple-coloured product, as shown in Fig. 1b, which proved to be more intense in the centre of the paper discs (Fig. 1b). So, the size of the selected area at the image processing software for measuring colour intensity was studied. Circles with different diameters, ranging from 100 to 270 pixels, were tested corresponding to the 4 mm diameter of the μPAD sample hole (100 pixels) and the 12.7 mm diameter of the paper disc (270 pixels). The calibration curves slope obtained for each tested diameter showed that increasing the selection area resulted in decreasing the sensitivity (ESM Fig. 5). So, the selection area with 100 pixels, corresponding to the 4 mm sampling hole, was chosen because presented a higher sensitivity.

Sample volume and saliva sample matrix

Sample volume

With the aim of further increasing the sensitivity of the calcium determination and obtaining the lowest limit of detection possible, sample volumes in the range of 20–40 μL were tested. By increasing the sample volume, the sample absorption time also increased from 2 to 30 min, respectively. The calibration curves slopes were compared (ESM Fig. 6) and 30 μL was the chosen volume because higher volumes did not result in a significant sensitivity increase (slope increase < 10 %) and simultaneously ensured a faster analysis time, sample absorption time of about 5 min.

The scanning was performed 10 min after sample/standard loading to ensure not only complete absorption but also to display enough time for the formation of the colour product. The covering of the sample hole with tape, for operator safety towards the biological sample, was made just before scanning the μPAD.

Table 1
Summary of the analytical features of the developed μPAD for calcium determination in saliva samples; LOD, limit od detection; LOQ, limit of quantification; RSD, relative standard deviation.

Dynamic range (mmol/L)	Calibration curve [a] $A = S (\pm SD) \times [Ca^{2+}] + b (\pm SD)$	LOD	LOQ	RSD (%)	
		(mmol/L)		Intraday	Interday
0.270 – 4.50	$A = 0.0950 (\pm 0.0027) \times [Ca^{2+}] + 0.000 (\pm 0.002)$ $R^2 = 0.9993 \pm 0.0003$	0.078	0.260	1 %	3 %

[a] $n = 4$.

Table 2
Recovery percentages (R) calculated by using the concentrations obtained by the μPAD method, before ($[Ca^{2+}]_{initial}$), and after addition ($[Ca^{2+}]_{found}$); values represent the average of four replicas; SD, standard deviation, E, obtained by error propagation calculation.

Saliva samples (S#)	$[Ca^{2+}]_{initial} \pm$ SD (mmol/L)	$[Ca^{2+}]_{added}$ (mmol/L)	$[Ca^{2+}]_{found} \pm$ SD (mmol/L)	$R \pm E$ (%)
S1	1.13 ± 0.11	0.65	1.75 ± 0.16	95.4 ± 0.3
		2.27	3.23 ± 0.26	92.5 ± 0.1
S2	1.42 ± 0.14	0.65	2.04 ± 0.19	94.9 ± 0.4
		2.27	3.66 ± 0.31	98.7 ± 0.1
S3	1.43 ± 0.13	0.65	2.11 ± 0.21	104 ± 1
		2.27	3.59 ± 0.30	95.1 ± 0.1
S4	1.50 ± 0.15	0.65	2.15 ± 0.19	100 ± 1
		2.27	3.64 ± 0.29	94.3 ± 0.1
S5	1.84 ± 0.19	0.65	2.44 ± 0.23	92.3 ± 0.5
		2.27	3.98 ± 0.32	94.3 ± 0.2
S6	1.73 ± 0.17	0.65	2.38 ± 0.26	100 ± 1
		2.27	4.11 ± 0.34	105 ± 1
S7	1.19 ± 0.16	0.65	1.83 ± 0.23	98.5 ± 0.4
		2.27	3.61 ± 0.39	107 ± 1

Sample matrix

Aiming for the application of the developed μPAD to saliva samples, the potential interference from the salivary matrix was assessed. Two sets of calcium standards were prepared, one in water and one in synthetic saliva, and calibration curves were established. Comparing the calibration curve slopes, the calibration curve obtained with the standards prepared in synthetic saliva was higher indicating some positive interference from one (or more) synthetic saliva constituents. Suspecting this positive interference could be due to the presence of magnesium or protein in the synthetic saliva, calcium standards with different compositions of synthetic saliva were compared: synthetic saliva (SS); synthetic saliva without magnesium and without BSA protein (InSS); synthetic saliva without BSA protein (InSS_Mg,). The calibration curve slopes obtained were similar (slope deviation < 5 %) except for the calibration curve of the standards prepared in the complete synthetic saliva, which showed a slope 22 % higher (ESM Fig. 7). This indicates that there was no individual interference, but the overall matrix affected the determination so, to minimize the matrix interference for the sample application, the calcium standards were prepared in synthetic saliva to mimic the sample matrix.

Stability studies

One of the main objectives in developing this μPAD, as most μPADs in general, was its portability and potential "outside the lab" use for *in-situ* analysis. In this context, the μPAD stability was a key parameter to assess and so stability studies were performed. These studies include not only the storage stability of the developed μPAD before application but also the stability of the coloured product formed after the sample loading.

Coloured product stability

The coloured product stability was assessed by scanning the μPAD at different time intervals, from 10 min to 2 h, and the established calibration curve slopes were compared (Fig. 4a).

No significant differences were observed up to 30 min (slope deviation < 10 %) thus indicating that the calcium determination could be carried out within the time period without affecting the results.

μPAD stability

To assess the stability of the developed μPAD, several devices were prepared and stored at room temperature (≈21 °C) and protected from light (covered with aluminium foil). Two different atmospheric conditions were used: in contact with air and in a vacuum, using a plastic bag and a vacuum packaging machine. All the devices were stored for time periods from 24 h to 2 months.

For each period and storage condition, the stored μPAD was used to perform a calibration curve and was compared to the calibration curve of a freshly assembled device. The results obtained indicate that the μPAD could be stored for 2 weeks in a vacuum because similar sensitivity was observed up to 2 weeks (slope deviation < 10 %) at those stored conditions (Fig. 4b). However, when the storage was not in a vacuum, the μPADs can only be kept for one week, similar sensitivity (slope deviation < 10 %).

Features of the developed μPAD for calcium determination

The analytical features of the developed μPAD for calcium determination in saliva samples under optimized conditions are summarized in Table 1.

Following the recommendations of the International Union of Pure and Applied Chemistry (IUPAC) [26], the minimum detectable concentration or limit of detection (LOD) and the limit of quantification (LOQ) were calculated as three and ten times the standard deviation of the intercept ($n = 4$) divided by the calibration curve slope average ($n = 4$).

The μPAD repeatability was assessed by calculating the relative standard deviation of four calibration curves in the same day (intraday RSD) and four calibration curves on consecutive days (interday RSD). The reagent consumption was calculated based upon the total volume of all reagent solutions in the 24 paper discs of one μPAD: 1.44 mg of CPC and 10.1 mg of AMP. The sample consumption was calculated per determination as 120 μL, considering four paper units discs per determination and 30 μL of the sample per paper unit.

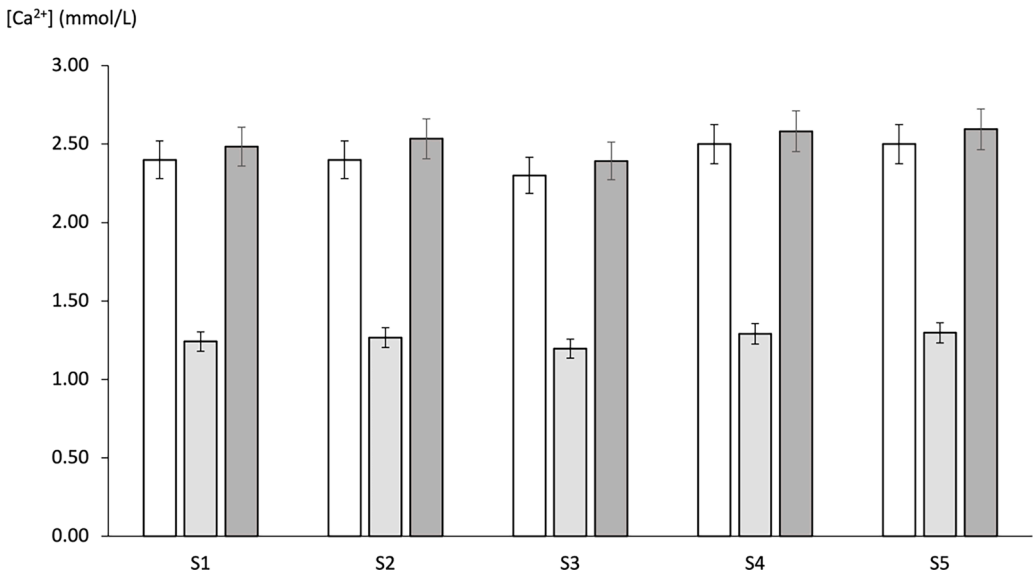

Fig. 5. Evaluation of the correlation between blood and saliva calcium content where the white bars represent the calcium content in the blood, the light grey bars represent the salivary calcium content obtained with the developed μPAD; the dark grey bars represent a simulation of the expected blood content considering that salivary calcium corresponded to 50 % of the calcium in serum; the error bars represent 10 % deviation of the measurements.

Application to saliva samples

Accuracy assessment

For the accuracy assessment, saliva samples (#10) were analysed with the developed μPAD and with atomic absorption spectrometry (AAS). The obtained results were compared and a linear relationship between the two sets of calcium concentration was established (ESM Fig. 8). The equation for the correlation (t-student analysis) between the concentrations obtained with the proposed μPAD ($[Ca^{2+}]_{\mu PAD}$) and the concentrations obtained with AAS ($[Ca^{2+}]_{AAS}$) was: $[Ca^{2+}]_{\mu PAD} = 1.06$ ($(\pm 0.04) \times [Ca^{2+}]_{AAS} - 0.156$ (± 0.061), where the values in brackets represent the 95 % confidence interval. No statistical differences between the two methods were observed, as the slope and the intercept were not statistically different from 1 to 0, respectively. So, the proposed paper-based device can be considered accurate.

Recovery studies

Since the calcium concentration values of the analysed saliva samples were all below 2.00 mmol/L, recovery percentages assessment was performed to analyse μPAD accuracy for higher calcium concentrations. For this study, saliva samples (#7) were spiked with a concentrated calcium stock solution (13.6 mmol/L) to final added concentrations of 0.65 and 2.27 mmol/L and the recovery percentages calculated, according to the IUPAC recommendations [27] (Table 2).

The average was 98.1 %, with a relative deviation of 4.7 %, and a statistical test (*t*-test) proved that it was not significantly different from 100 % at a 95 % significant level, where the calculated t-value was −1.592, with a correspondent critical value of 2.533. Therefore, the statistical *t*-test indicated the absence of multiplicative matrix interferences, and the applicability of the developed device to different saliva samples was proved.

Calcium levels correlation between blood and saliva samples

Saliva samples (#5) were analysed using the developed device and the calcium concentration values obtained were compared to the blood calcium content. According to the results obtained (Fig. 5), the salivary calcium concentration (light grey bars) would correspond to 50 % of the calcium concentration in serum (white bars). To test this hypothesis, a comparison was made between the reported serum calcium content, and expected calculated values considering 50 % of the salivary calcium (dark grey bars). No statically significant differences were observed between the two sets of values as the relative deviation was < 10 % (Fig. 5).

These results indicate that, based upon the salivary calcium assessed with the developed μPAD, it could be possible to infer the calcium content in the blood, an important biomarker. Thus, by quantifying calcium content in saliva it would be possible to monitor patients with hypercalcaemia without blood sampling.

Conclusions

This work reports the development of a simple microfluidic paper-based analytical device (μPAD) for calcium quantification in saliva samples. This μPAD not only proved to be an accurate method, but it also provided fast, in-situ determination enabling point-of-care analysis. Furthermore, it could be an excellent alternative for clinical diagnosis of health conditions related to calcium content in the body. The described device was successfully applied to saliva samples in a range of 11.0–180 mg/L (0.300–4.50 mmol/L) with a minimum detectable calcium concentration of 3.2 mg/L. One of the main advantages of this μPAD is that only uses 120 μL of sample, which represents a very positive aspect when biological samples were used.

If compared to the most used methods for salivary metabolites determination, for example AAS, the developed device does not require expensive equipment or specialized technicians, providing low-cost analysis. Furthermore, this μPAD proved to be ideal for frequent self-monitoring of health conditions.

A correlation between salivary calcium content and serum calcium content was made and it was possible to conclude that salivary calcium represents approximately 50 % of the calcium content in serum. The normal calcium concentration in the blood range within 2.10–2.55 mmol/L [9], which means that in saliva the normal range is 1.05–1.28 mmol/L. Hypercalcaemia is usually not more than 0.25 mmol/L above the upper limit of the normal range [6]. Thus, it was possible to conclude that salivary calcium above 1.55 mmol/L indicates a patient with hypercalcaemia.

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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.talo.2023.100254.

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