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A Sensitive Paper-Based Colorimetric Assay for Creatinine Detection in Biological Fluids

Oinam Robita Chanu [a], Subramaniam Pushpavanam [b], Ashish Kapoor [c], Varshini Karthik [a, *]

[a] Department of Biomedical Engineering, SRM Institute of Science and Technology, Kattankulathur-603203, Tamil Nadu, India
[b] Department of Chemical Engineering, Indian Institute of Technology Madras, Chennai-600036, Tamil Nadu, India
[c] Department of Chemical Engineering, Harcourt Butler Technical University, Kanpur-208002, Uttar Pradesh, India
* Corresponding Author Email: varshink@srmist.edu.in

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Abstract: Chronic kidney disease (CKD) is associated with elevated creatinine levels due to impaired kidney function. Conventional creatinine determination is generally performed using blood serum or plasma, requiring invasive sample collection and laboratory-based instrumentation. Although microfluidic paper-based analytical devices (μPADs) have been widely investigated for point-of-care analysis, many reported designs require flow channels, multilayer structures, or complex fabrication procedures. In this study, a spot-based laser-printed microfluidic paper-based analytical device (LP-μPAD) was fabricated on Whatman Grade 4 filter paper for colorimetric creatinine detection. Hydrophobic barriers and hydrophilic detection zones were produced by laser printing followed by thermal treatment. Creatinine was detected using the Jaffe reaction, and the colour formation resulted from the Janovsky complex, which was further analyzed from smartphone images using ImageJ software. The LP-μPAD exhibited a linear response over the concentration range of 0-20 mg/dL, with a limit of quantification of 0.49 mg/dL and limit of detection of 0.15 mg/dL. The assay showed good precision with an RSD of less than 10%, satisfactory selectivity against representative interfering species, and acceptable storage stability under refrigerated conditions. Bland-Altman analysis showed good agreement between the LP-μPAD and the reference UV-Vis method. Recovery values of 99.0-107.5% for artificial saliva and 99.13-112.0% for artificial urine demonstrated satisfactory analytical performance. The proposed spot-based LP-μPAD provides a simple approach for creatinine determination in simulated biological samples.

Keywords: Paper-Based Analytical Device, Creatinine, Janovsky Complex, Colorimetric Sensing, Non-Invasive Diagnostics.

1. Introduction

Chronic kidney disease (CKD) involves a gradual decline of renal function and is increasingly prevalent worldwide, primarily due to diabetes and hypertension [1, 2]. Regular biochemical analysis is essential for diagnosis and monitoring. Creatinine, a metabolic byproduct excreted by the kidneys, is widely used as an indicator of renal function [3, 4].

Conventional gold-standard techniques for creatinine determination are based on blood serum analysis. In healthy individuals, serum creatinine values range from 0.6 to 1.5 mg/dL, and values beyond this range indicate impaired kidney function [5, 6]. Although serum analysis is widely used, it involves invasive procedures that may cause discomfort and anxiety. In CKD patients, repeated blood sampling during dialysis can lead to cumulative blood loss (approximately 4-20 mL per session), along with additional loss due to frequent testing. Furthermore, there are high chances of blood-borne infections such as hepatitis C and B, posing risks to both patients and healthcare professionals [5-7].

Alternative biological samples for creatinine determination include urine and saliva. Urine has been widely used in clinical diagnostics, typically through spot (random) urine tests or 24-hour urine collection methods [8]. In healthy individuals, creatinine levels in spot urine samples range from twenty to 20 to 320 mg/dL for men and 20 to 280 mg/dL for women, while 24-hour excretion ranges from 500 to 2000 mg/day [9-12]. Despite being non-invasive, urine-based methods have limitations, including sensitivity to hydration levels, variability in voiding time, and challenges associated with complete and accurate sample collection, which may lead to under- or overestimation.

Recent advances in bioinformatics, metabolomics, genomics, and proteomics have

significantly accelerated salivary research. Saliva is increasingly being explored as a useful diagnostic sample. It has been studied for detecting a range of conditions, including oral, head and neck cancers, as well as cancers of the lung, pancreas, breast, and ovary. It has also shown potential for monitoring systemic conditions such as type 2 diabetes [13-16]. In recent years, studies have highlighted the potential of saliva as an alternative to serum or plasma for creatinine-based diagnosis of CKD, particularly in end-stage renal disease (ESRD) [17-20].

Various analytical methods are used to measure creatinine, including capillary electrophoresis (CE), UV-Vis spectroscopy, high-performance liquid chromatography (HPLC), tandem mass spectrometry, flow injection analysis (FIA), and surface-enhanced Raman spectroscopy [7, 21]. However, these techniques typically require skilled personnel, expensive equipment, and longer analysis times, limiting their suitability for on-site applications.

µPADs have emerged as a simple platform for chemical and biochemical analysis because they require only small amounts of sample and reagent and can be fabricated at low cost [13-15]. Different fabrication methods, including photolithography, wax printing, inkjet printing, laser printing, and flexographic printing, have been used to generate hydrophobic barriers and hydrophilic reaction zones on paper substrates [16-19]. When combined with colorimetric detection, the analytical response can be recorded using readily available devices such as smartphone cameras, enabling quantitative analysis without sophisticated laboratory instrumentation [20-25].

A variety of paper-based methods have been reported for creatinine determination. Melo et al. developed a disposable microfluidic paper-based device for the on-site quantification of urinary creatinine using the Jaffe reaction [26]. Gautam *et al.* reported a paper-based device coupled with an RGB sensor for creatinine quantification in whole blood [27], whereas Ko *et al.* developed a handheld colorimetric microfluidic device for the concurrent determination of blood urea nitrogen and creatinine [28]. Electrochemical and potentiometric paper-based creatinine sensors have also been described by Manikandan *et al.* and Kamel *et al.,* respectively [29, 30]. In addition, paper–polymer hybrid and other microfluidic configurations have been investigated for creatinine analysis [31-35]. Although these approaches have demonstrated satisfactory analytical performance, several require patterned flow channels, multilayer assembly, polymeric components, or multiple fabrication steps, thereby increasing manufacturing complexity. Previous paper-based creatinine assays have commonly employed cellulose filter paper or chromatography-paper substrates [26, 33, 35, 36, 37-39].

In the present study, a spot-based laser-printed microfluidic paper-based analytical device (LP-µPAD) was fabricated on Whatman Grade 4 filter paper for creatinine determination. The device consists of a single paper layer, where the reaction is confined to a defined spot without the use of flow channels or stacked paper layers. Hydrophobic barriers were formed by laser printing followed by thermal treatment, resulting in a straightforward fabrication process. Creatinine was determined by the Jaffe reaction, and the colour intensity was quantified from smartphone images captured inside a custom-designed 3D-printed light box using ImageJ software. The analytical performance of the LP-µPAD was evaluated in terms of linearity, sensitivity, precision, selectivity, storage stability, agreement with a UV-Vis reference method, and recovery using artificial saliva and urine samples.

2. Experimental

2.1 Materials and Chemicals

Chemicals of analytical grade were used in this study. Creatinine and picric acid were obtained from Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, while sodium hydroxide was sourced from Merck Life Science Pvt. Ltd., Mumbai. Potassium chloride (≥99.5%), sodium carboxymethyl cellulose (≥99%), calcium chloride dehydrate (≥99.5%), magnesium chloride hexahydrate (≥99.5%), dipotassium hydrogen phosphate (≥99.5%), potassium dihydrogen phosphate (≥99%), urea (≥99.5%), sodium chloride (≥99%), sodium phosphate dibasic (≥99%), methyl p-hydroxybenzoate (≥99% purity), and albumin (≥99%) were sourced from SRL and Merck Life Science Pvt. Ltd. For the µPADs, Whatman No. 4 filter paper (size: 460 × 570 mm) was purchased from Cytiva Life Sciences, USA.

The glassware used in the experiments was obtained from BOROSIL®. Before use, it was thoroughly cleaned, rinsed with 0.1% HNO_3 followed by deionized (DI) water, and then dried at 80 °C for 12 hours.

2.2 Instruments

An HP LaserJet Pro P1606dn monochrome laser printer was used to design and fabricate the µPADs. Hydrophobic barriers were created on the paper substrates using the toner cartridge (CE 278A), which consists of the styrene acrylate resin (<55%), ferrite (<45%), and wax (<10%). A CWF1200 Carbolite Gero furnace was also used for sample drying.

To capture assay images without interference from external light and to maintain consistent illumination with an improved signal-to-noise ratio, a custom 3D light box was developed. The box was 3D-printed using black acrylonitrile butadiene styrene (ABS) to minimize light leakage. Its dimensions were 12 cm × 12 cm × 12 cm. A 5 cm diameter opening on the top panel was provided for image acquisition. The illumination system consisted

of a white LED strip (30 LEDs, 6000 K) installed along the inner walls of the box at a height of 6 cm from the base and powered by a 12 V DC supply to provide uniform lighting. During image acquisition, the paper device was positioned at a fixed location inside the imaging box, and all measurements were performed under identical imaging conditions. The acquired images were processed using ImageJ software, and the normalized green intensity (NGI) was used for quantitative analysis. Further details are available in our previously published work [40]. A comparison of calibration performance using a second smartphone model is provided in Figure S2.

2.3 Preparation of Sample

2.3.1 Preparation of Artificial Saliva and Urine Samples

The artificial saliva (AS) formulation was prepared following previously reported methods [13, 40]. To prepare artificial saliva, 2 g of methyl p-hydroxybenzoate was dissolved in 800 mL of purified water. From this solution, 20 mL was set aside for later use, and the remaining portion was stored under refrigeration. In a separate step, 10 g of sodium carboxymethyl cellulose was slowly added to 200 mL of boiled distilled water with continuous stirring until a uniform solution formed. After cooling, the methyl p-hydroxybenzoate solution was added to this mixture, resulting in gel formation.

Next, in the reserved 20 mL methyl p-hydroxybenzoate solution, 0.166 g of calcium chloride dihydrate, 0.804 g of dibasic potassium phosphate, 0.059 g of magnesium chloride hexahydrate, 0.625 g of potassium chloride, and 0.326 g of monobasic potassium phosphate were dissolved. This salt solution was then added gradually to the gel with continuous mixing to ensure uniformity. Finally, the pH was balanced to 6.75 using potassium hydroxide and this artificial saliva was stored at ambient temperature.

Artificial urine was synthesized with an earlier reported protocol [31, 38, 41]. Briefly, 1.82 g of urea, 0.48 g of dibasic sodium phosphate, 0.75 g of sodium chloride, 0.45 g of potassium chloride, and 5 mg of albumin were prepared in 100 mL of DI water. The solution was stirred with a magnetic stirrer until complete dissolution was achieved.

2.3.2 Preparation of Colorimetric Reagent

Jaffe's reagent, a colorimetric reagent, was prepared using 2 M NaOH and 25 mM picric acid. The two solutions were prepared separately to a final volume of 50 mL each, with the NaOH prepared in deionized (DI) water.

The picric acid solution (25 mM) was prepared in a 50 mL mixture of methanol and DI water (60:40, v/v) to improve stability without affecting the Jaffe reaction. The picric acid solution was protected from light by covering the container with aluminum foil due to its photosensitive nature.

2.3.3 Preparation of Creatinine Solution

The primary creatinine stock solution had a concentration of 200 mg/dL, from which working standard solutions were prepared. Calibration standards ranging from 0 to 20 mg/dL were subsequently obtained by serial dilution of an intermediate 50 mg/dL solution derived from the primary stock.

2.4 Design and Fabrication of LP-µPADs

LP-µPADs were fabricated following the protocol previously reported by our research group [42]. Different patterns were designed using Microsoft PowerPoint. The paper substrate used for the LP-µPADs was Whatman filter paper of Grade 4 with a thickness of 205 µm and a pore size of 20-25 µm. Hydrophobic barriers were printed onto the paper using a laser printer at maximum resolution and print quality. The circular spot design was then subjected to thermal treatment at 165 °C for 10-15 min in a muffle furnace to allow the toner to penetrate the paper matrix. After complete penetration of the toner through the paper substrates, the LP-µPADs were available for use.

Each device consisted of circular spots with a diameter of approximately 5 mm and a spacing of 10.16 mm (0.4 inch) between adjacent spots to minimize cross-contamination. The printed toner thickness was approximately 1 pt (~0.35 mm), forming the hydrophobic boundary around each spot. The overall layout consisted of a 25 × 25 array of spots, with a total of 625 spots on an A4 sheet.

To evaluate fabrication reproducibility, µPADs were prepared on four separate A4 sheets under identical conditions. Representative spots from each sheet were tested at a fixed concentration. The analytical response showed minimal variation, with a relative standard deviation (RSD) below 5%, indicating consistent fabrication.

2.5 Determination of Creatinine

The steps involved in the proposed creatinine-detection procedure are illustrated in Figure 1.

On the fabricated LP-µPADs, 5 µL each of NaOH and picric acid (Jaffe's reagent) were sequentially loaded onto a detection zone and allowed to dry for approximately 7 min. The dried assay exhibited a yellowish color, after which 5 µL of the creatinine samples at varying concentrations were introduced. A Janovsky complex, producing a yellow-to-orange-red colour, formed when creatinine reacted with picric acid under alkaline conditions as shown in Figure 2.

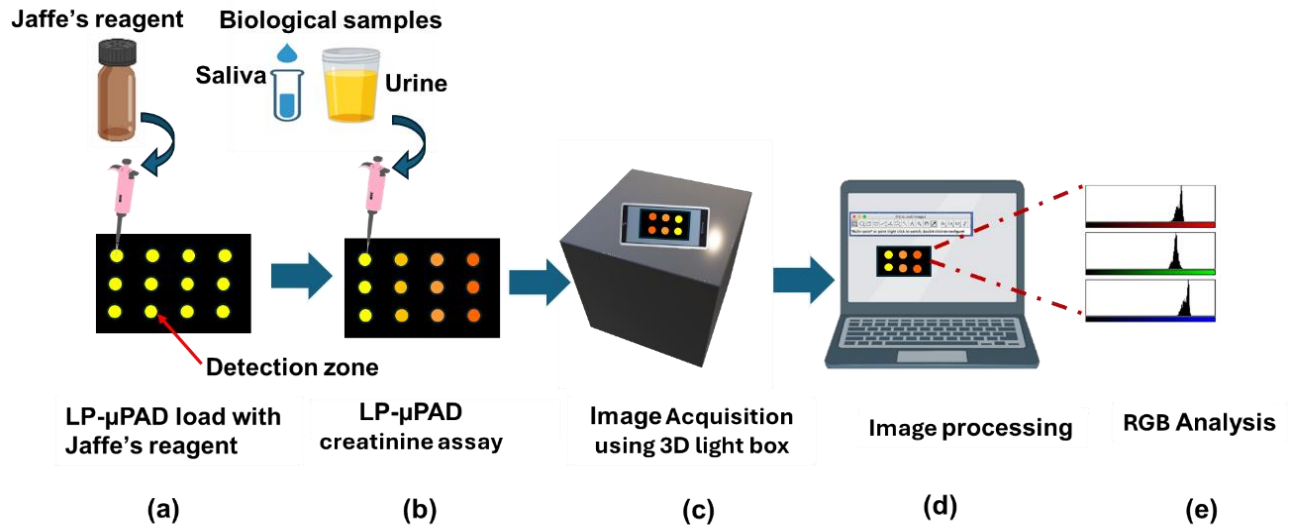

Figure 1. Schematic illustration of the creatinine detection process: **(a)** loading Jaffe's reagent onto the detection zone of the LP-µPAD, **(b)** addition of creatinine from the biological sample, **(c)** image acquisition using a 3D light box, **(d)** image processing using ImageJ software, and **(e)** conversion of images into RGB data for quantitative analysis.

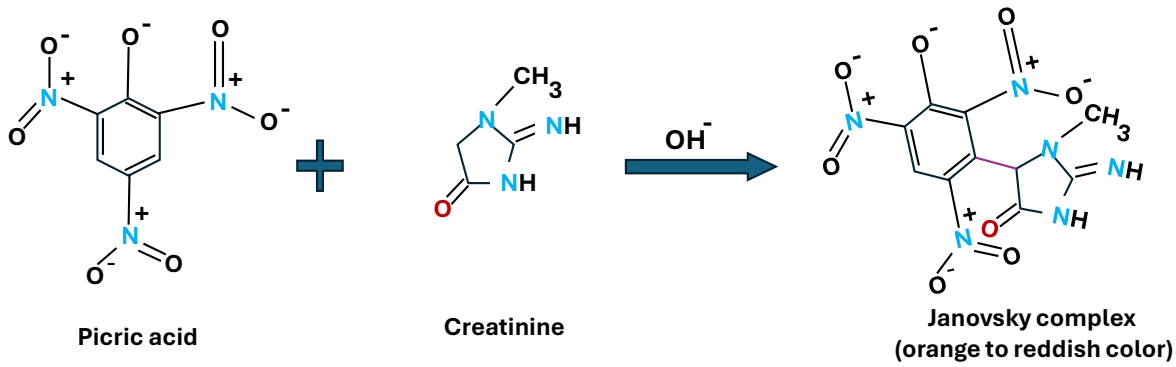

Figure 2. Mechanism of Jaffe reaction forming the Janovsky complex.

The colour intensity increases with creatinine concentration. All experiments were carried out at ambient temperature (~30 °C).

Images were captured with Samsung Galaxy M51 smartphone in default mode, and the camera settings (f/1.8 aperture, 1/100 s shutter speed, 5. 2 mm focal length, ISO 80, with flash off) inside a 3D light box for quantitative analysis. This procedure was followed for all experiments.

The captured images were analyzed with ImageJ software to extract RGB intensity values and and the normalized grayscale intensity was then calculated (NGI) using Eq. (2.1) [42, 40].

$$NGI = 255 - (0.2126 * R + 0.7152 * G + 0.0722 * B) \quad (1)$$

Where

R, G, and B correspond to red, green, and blue intensity, respectively.

The NGI values were directly proportional to the creatinine concentration. All experiments were performed with five to six replicates. For calibration, regression analysis was conducted by fitting average values of NGI versus creatinine concentration (0 to 20 mg/dL).

2.6 Analytical Procedure

Calibration curves were established by plotting NGI against creatinine concentrations ranging from 0 to 20 mg/dL. The limit of detection (LOD) and limit of quantification (LOQ) were computed using Eq. (2.2) and (2.3) [43].

$$LOD = \frac{(3*\sigma)}{S} \quad (2)$$

$$LOQ = \frac{(10*\sigma)}{S} \quad (3)$$

Here S is the slope of the calibration curve and σ represents the standard deviation of the control sample.

2.6.1 Evaluation of Jaffe's Reagent

To improve the sensitivity of the NGI response, the concentrations of picric acid (PA) and sodium hydroxide (NaOH) in Jaffe's reagent were optimized. The concentration of NaOH was varied (1 to 4M) while keeping the concentration of PA constant. In a similar manner, the concentration of PA varied from 10 to 100 mM while maintaining a fixed NaOH concentration. The optimal concentrations were selected based on the observed NGI response.

2.6.2 Reaction-Time Optimization

The development of colour from yellow to orange-red in the colorimetric Jaffé reaction must be monitored over time. This monitoring identifies the time at which the signal reaches its maximum intensity and determines when it becomes saturated. Experimentation was performed for two different creatinine concentrations that were loaded onto the reagent preloaded LP-μPAD. In the next step images were captured at different time points over 60 min. At each time point, NGI values were recorded to observe changes in color intensity signal. Based on these observations, the appropriate time for image capture and signal measurement was selected between the point of maximum intensity and the stage where the signal became stable. This enables consistent estimation of creatinine across samples.

2.6.3 Analytical Performance Analysis

Stability and reproducibility studies of the reagent were conducted to evaluate its shelf life and assess the suitability of the device for real-time, on-site applications.

To evaluate the practical applicability of the spot-based LP-μPAD for creatinine detection in biological samples such as saliva and urine, experiments were performed using artificial saliva and urine matrices. The device performance was further assessed by determining the percentage recovery of spiked samples. Predefined concentrations of creatinine were added to the artificial samples, and the corresponding concentrations were estimated from the calibration curve. The recovery percentage was calculated using the following Eq. (2.4) [44]:

$$Recovery\ \% = \frac{C_f}{C_s} \times 100 \qquad (4)$$

Here C_s is the spiked concentration and C_f is the derived value from the calibration slope.

The selectivity and interference of the μPAD-based creatinine assay were evaluated using common species present in biological fluids, including sodium chloride (NaCl), glucose, potassium chloride (KCl), ascorbic acid (AA), urea, uric acid (UA), calcium chloride (CaCl2), and sodium nitrite ($NaNO_2$).

For selectivity assessment, the concentration ratio of creatinine to interferents was maintained at 1:5 (5 mg/dL and 25 mg/dL). Individual solutions of each interfering species (25 mg/dL) were separately tested in the absence of creatinine to evaluate their response under the assay conditions. For interference evaluation, creatinine (5 mg/dL) was analyzed in the presence of each species (25 mg/dL) to examine any effect on the analytical signal.

All measurements were carried out following the creatinine detection procedure described earlier. A blank sample (DI water) was used to establish the baseline response. The signal was expressed as mean normalized gray intensity (MNGI), reported as the mean ± standard deviation (n = 5).

3. Results and Discussion

3.1 Jaffe's Reagent Optimization

An investigation of Jaffe's reagent components, picric acid and sodium hydroxide (NaOH), was conducted according to the protocol described in Section 2.6.2. In this study, picric acid concentrations ranging from 10 to 100 mM were applied to the detection zone of spot LP-μPADs containing 2 M NaOH. for each assay, a creatinine sample (10 mg/dL) was added. Color variation from yellow to orange was observed after the addition of creatinine. The images were captured and analyzed to obtain NGI values, as shown in Figure 3(a). The maximum NGI was observed at a picric acid concentration of 25 mM.

The picric acid concentration was then fixed at 25 mM, and the same procedure was followed to optimize the NaOH concentration. NaOH concentrations ranging from 0.5 to 4 M were tested. After the addition of picric acid and creatinine, the NGI values were obtained and plotted, as shown in Figure 3 (b). The highest NGI was observed at 2 M NaOH.

The results are consistent with the findings reported by Sununta *et al.,* who reported that the same NaOH and picric acid concentrations produced a higher colour intensity [45]. Based on these results, 2 M NaOH and 25 mM picric acid were selected as the optimized conditions for subsequent experiments.

3.2 Optimization of Reaction Time

The effect of reaction time on the colorimetric response following creatinine addition was studied. In this experiment, creatinine solutions at concentrations of 2.5 and 10 mg/dL were added to preloaded spot-based LP-μPADs. After the addition to the detection zone, images were captured over a period of 60 min. The NGI values were obtained and plotted, as shown in Figure 4.

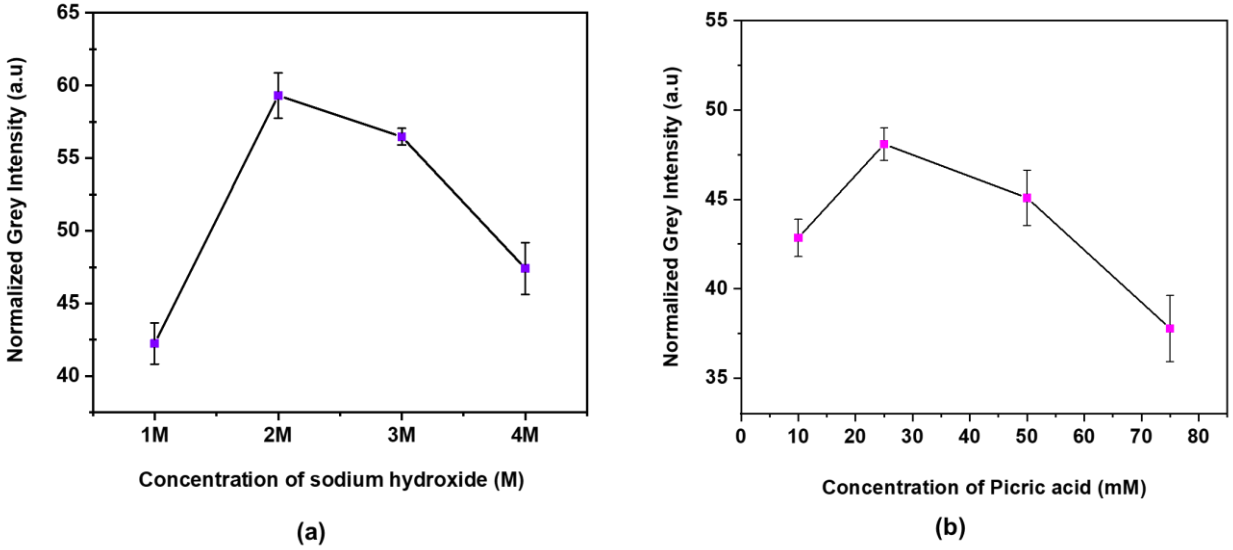

Figure 3. Reagent optimization **(a)** picric acid concentration and **(b)** sodium hydroxide (n=3).

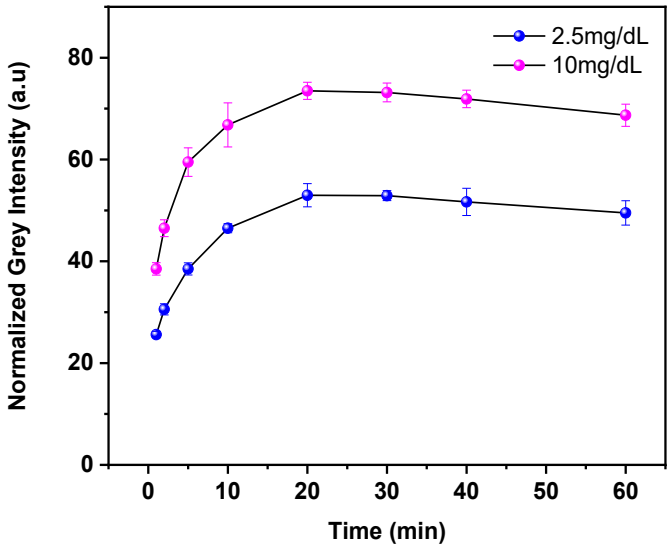

Figure 4. Impact of reaction time on creatinine determination (n=3).

The time-versus-NGI plot showed that the signal intensity reached a maximum at 20 min and remained relatively stable between 30 and 40 min, followed by a gradual decrease at approximately 60 min. To justify the selection of the analytical readout time, assay precision, between-spot variation, and signal drift were evaluated at 20, 30, and 40 min using three independent paper spots (n = 3) at creatinine concentrations of 2.5 and 10 mg/dL. The coefficient of variation (CV) ranged from 1.85% to 5.18% for 2.5 mg/dL and from 2.28% to 2.52% for 10 mg/dL, indicating good reproducibility. Relative to the 20 min measurement, the signal drift was 0.14% and 0.45% at 30 min for the 2.5 and 10 mg/dL concentrations, respectively, and remained below 2.5% at 40 min for both concentrations. Although the signal remained relatively stable after 20 min, a slight reduction in signal intensity was observed at longer reaction times without any improvement in assay precision. Therefore, 20 min was selected as the optimum time for image acquisition and creatinine quantification because it provided the highest signal response with good reproducibility and minimal signal drift. These results are consistent with previous reports describing rapid color development in the Jaffe reaction followed by a gradual decrease in signal with prolonged reaction time [45].

3.3 Creatinine Determination

Under the optimized conditions, creatinine detection was performed. A visible color change was observed with varying concentrations of creatinine (0 to 20 mg/dL), as shown in Figure 5(a), and the corresponding calibration curve is presented in Figure 5(b). From the calibration plot, linear regression over the concentration range of 0–20 mg/dL yielded the equation ($y = 34.0381 + 2.7577x$) with an R^2 value of 0.9594.

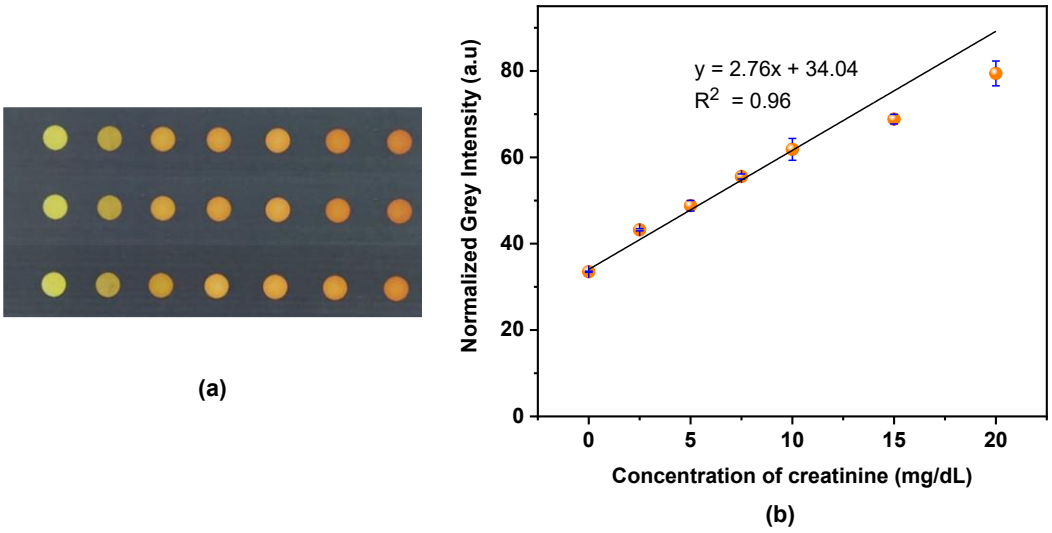

Figure 5. (a). Different concentrations of creatinine loaded onto spot-based LP-μPADs preloaded with Jaffe's reagent, **(b)** linear calibration plot of creatinine concentration versus normalized grayscale intensity obtained from front-side images (n=6).

Table 1. Comparative performance of LP-μPAD with existing creatinine detection

Fabrication process of μPAD	Fabrication complexity	Dynamic range (mg/dL)	Image acquisition system	LOD (mg/dL)	LOQ (mg/dL)	Sample	Reference	Year
Xerox Solid Ink Black	Single filter paper	5 - 60	Desktop scanner	1.57	5.24	Artificial urine	Rossini *et al.* [38]	2018
Solid ink printer with lamination method	Single filter paper	11.88-226.24	Smartphone camera	11.88	NR	Artificial urine	Lewińska *et al.* [2]	2021
Mold immersed in polystyrene toluene	Single filter paper	3.39–56.6	Smartphone camera	2.48	NR	Serum and Urine	Shariati and Khayatian [35]	2022
Lamination method	Multilayer stacking paper	2.2-35	Desktop scanner	0.66	2.2	Synthetic urine	Melo *et al.* [26]	2023
PMMA hybrid model	Single paper	11.31 - 113.12	Camera	0.45	NR	Artificial urine	Sheeraz *et al.* [31]	2024
DMF integrated model	Single filter paper	2 - 32	Desktop scanner	1.4	2	Artificial urine	Velasco *et al.* [33]	2025
LaserJet printer	Single filter paper	0 -20	Smartphone camera	0.15	0.49	Artificial saliva and urine	Current work	2026

*NR- Not reported

The standard errors of the intercept and slope were 0.6693 and 0.2536, respectively, with corresponding 95% confidence intervals of 32.317–35.759 for the intercept and 2.106-3.410 for the slope. The residual plot showed no systematic deviation from linearity over the investigated concentration range. The standard deviation of the blank response used for the calculation of the limit of detection (LOD) and limit of quantification (LOQ) was 0.1344. Based on Eq. (2.2) and (2.3), the LOD and LOQ were determined to be 0.15 mg/dL and 0.49 mg/dL, respectively.

The analytical performance of the proposed LP-μPAD was compared with previously reported μPAD-based creatinine sensors, as summarized in Table 1.

The proposed assay exhibited a lower LOD than several previously reported paper-based creatinine detection methods. This performance may be attributed to the spot-based configuration on a single layer of Whatman Grade 4 filter paper, which eliminates the need for a flow channel and confines the reaction within a defined detection area. The localized colour development produced by this design provides a consistent region for image analysis. The linear working range obtained in this study is expected to be suitable for creatinine determination in saliva and urine, subject to further validation using real biological samples for chronic kidney disease.

3.4 Stability and Reproducibility Assessment

Stability assessment was carried out using preloaded Jaffe's reagent spot-based assays under two drying conditions: air drying at room temperature and vacuum drying. After drying, all assays were encased in aluminum foil and stored in airtight zip-lock covers at 4 °C to prevent light exposure, as picric acid is light-sensitive.

On the 7th day, assays from both conditions were tested by adding creatinine solutions of varying concentrations 0 to 20 mg/dL. The images were captured and analyzed to obtain slope values, which are presented in the bar graph in Figure 6. The results showed that vacuum-dried assays had higher stability than air-dried assays, possibly because exposure to air during drying led to some oxidation of the reagent. Hence, vacuum-dried assays were utilized for the reagent shelf-life study.

The shelf life of preloaded, vacuum-dried LP-µPAD assays was studied for creatinine detection over a 30-day period (Figure 6). At each time point, calibration curves (Figure S1) were generated, and sensitivity was calculated using linear regression analysis. On day 0, the calibration curve was y = 2.7271x + 43.418. A similar response was seen on day 7 (y = 2.6715x + 26.98). After this point, sensitivity gradually decreased, as shown by the results on day 10 (y = 2.0767x + 34.244), day 20 (y = 2.0689x + 28.82), and day 30 (y = 2.0696x + 32.122).

Overall, the first week (day 0 and day 7) showed higher sensitivity than at later time points, with no clear difference between them. After day 7, the response dropped slightly and then stayed steady through the rest of the storage period, with only small variations between days 10, 20, and 30. Overall, the assay showed a slight drop in performance after one week, followed by stable behavior up to 30 days. This indicates minimal self-oxidation of Jaffe's reagent and good storage stability of the LP-µPADs at 4 °C. The relatively small change in calibration slope during the first week indicates that the analytical performance was maintained over this period. Therefore, storage for up to one week is recommended to ensure consistent analytical performance under the storage conditions investigated, whereas recalibration is recommended if the devices are used after longer storage periods.

To evaluate the precision of the LP-µPADs, reproducibility of creatinine detection was examined over seven consecutive days. Creatinine concentrations ranging 0 to 20 mg/dL from were added to preloaded, vacuum-dried LP-µPADs. After the reaction, images were captured and used to obtain NGI values for each concentration, as shown in Figure 7.

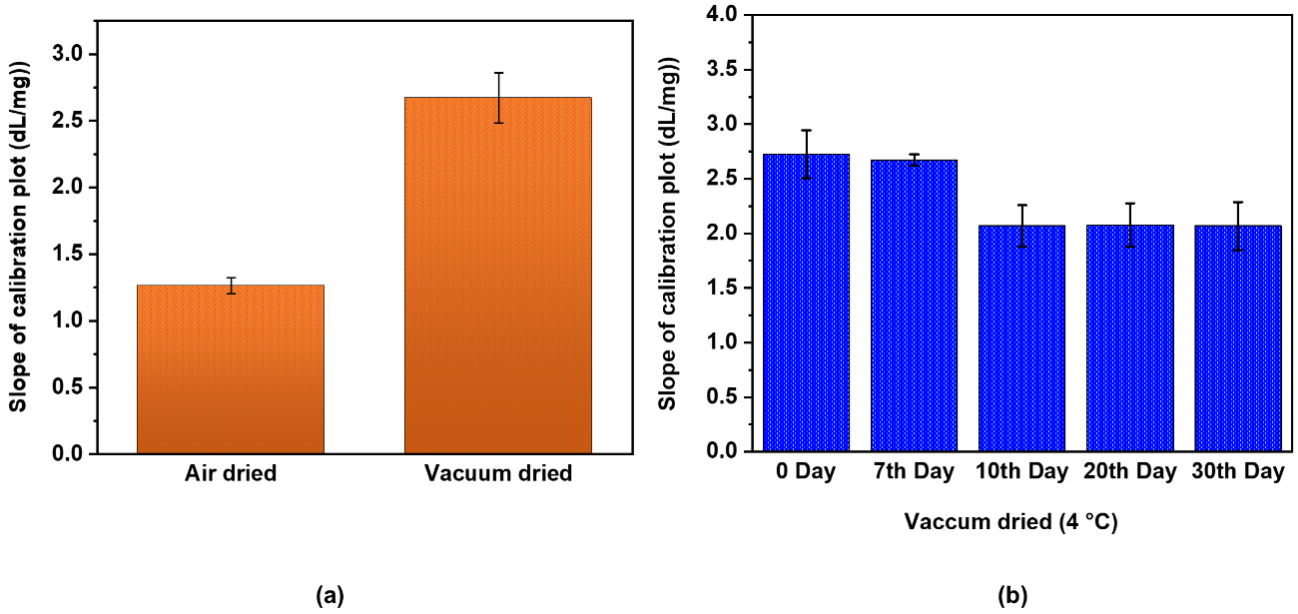

Figure 6. Stability of reagents on LP-µPADs **(a)** effect of drying conditions **(b)** performance of vacuum-dried reagents over different days (n=3).

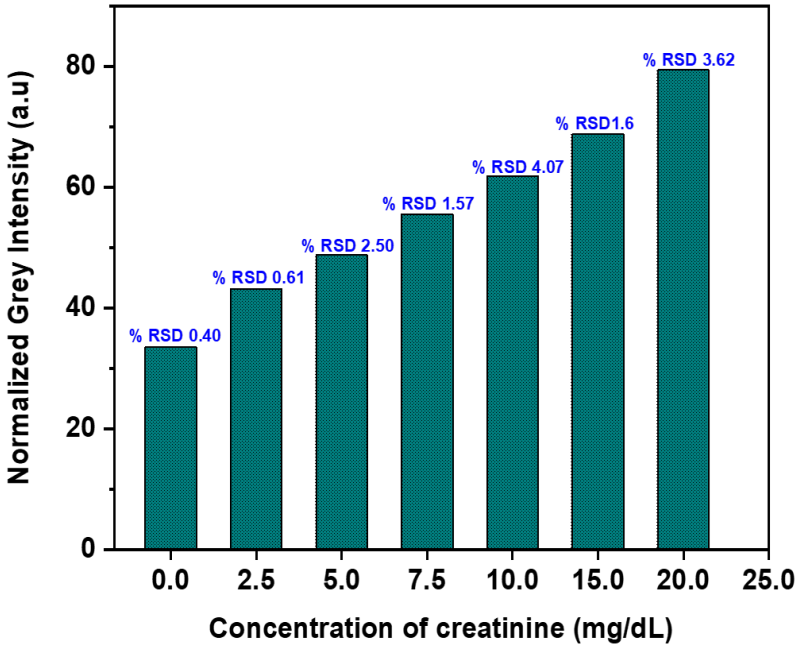

Figure 7. Reproducibility of creatinine determination under inter-day conditions (n=3).

The relative standard deviation (RSD) was evaluated as RSD (%) = (s_i / $\bar{x}$) × 100, where s_i is the standard deviation and $\bar{x}$ is the mean value [46]. The %RSD values ranged from 0.4 to 3.62% for all concentrations. According to AOAC guidelines, inter-day reproducibility should be ≤ 10% RSD [46, 47]. The values obtained were within this limit, indicating good precision of the device. The measurements were carried out in triplicate.

3.5 Selectivity and Interference Study

The selectivity of the assay was determined by measuring the response of individual interfering species, as described in Section 2.6.3, in the absence of creatinine. The results are shown in Figure 8(a).

The tested interferents produced MNGI values close to the baseline, indicating negligible color development under the assay conditions. This suggests that these species do not generate measurable signals that could interfere with creatinine detection. The effect of interfering species on creatinine detection was examined by measuring the response of creatinine (5 mg/dL) in their presence shown in Figure 8(b). The MNGI value for creatinine alone (58.65 ± 1.28) showed only slight variation when interferents were added, with values ranging from about 56.4 to 58.4. The overall change in signal was small (≤ ~2 NGI units), suggesting that these species have little impact on the measurement. These observations indicate that the assay maintains a consistent response for creatinine even in the presence of common coexisting compounds. These observations demonstrate that the proposed LP-μPAD provides a consistent response for creatinine in the presence of the selected interfering compounds. The selectivity study was performed within the physiological pH ranges of urine (4.5-8.0) and saliva (6.2-7.6) [8]. Although the results indicate good selectivity under the investigated conditions, biological fluids contain additional endogenous constituents that may contribute to matrix effects. Therefore, further studies involving a broader range of interfering species at physiologically relevant concentrations, together with validation using real saliva and urine samples, are required to further establish the selectivity of the proposed assay.

3.6 Accuracy Assessment

The performance accuracy of the LP-μPAD was evaluated by comparing the creatinine values obtained using the developed device with those measured by a UV-Vis spectrometer. Eight concentrations within the range of 0-20 mg/dL were selected for the comparison study. Bland-Altman analysis was carried out to examine the agreement between the two methods, and the corresponding plot is shown in the Supporting Information (Figure S3). The analysis yielded a mean bias of 0.07, with 95% limits of agreement ranging from −0.411 to 0.551, indicating good agreement between the LP-μPAD and the reference method. The differences were distributed on both sides of zero across the investigated concentration range, indicating that the LP-μPAD did not show a systematic positive or negative bias relative to the UV-Vis method. Although slightly larger deviations were observed at higher concentrations, all measurements remained within the calculated limits of agreement. These results indicate that the proposed LP-μPAD provides analytical performance comparable to that of the UV-Vis method for prepared creatinine solutions in artificial saliva and artificial urine under the conditions investigated in this study.

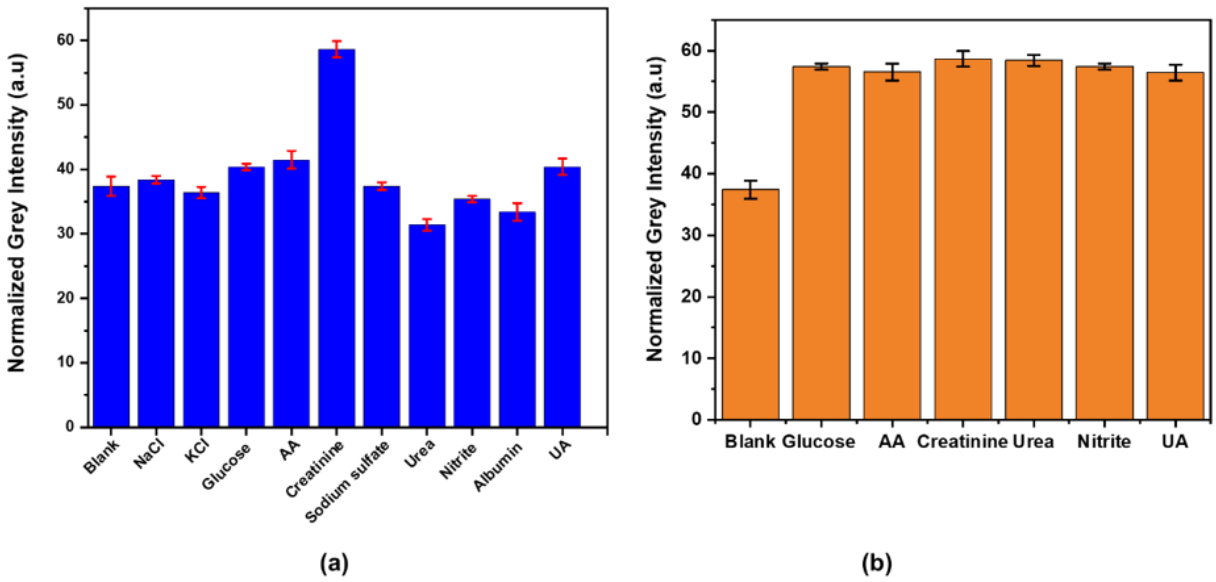

Figure 8. (a) Selectivity study of the Jaffe reagent toward creatinine and common interfering species and **(b)** Interference study of creatinine detection in the presence of interfering species (n = 3).

Table 2. Recovery analysis of creatinine in artificial saliva and urine samples (n=6)

Sl. No.	Simulated Samples	Spike (mg/dL)	Found (mg/dL)	Recovery (%)
1	Artificial saliva	0.2	0.198	99
		0.4	0.43	107.5
		0.6	0.64	106.7
2	Artificial urine	5	5.6	112.0
		10	10.72	107.50
		15	14.87	99.13

Further validation using independent biological samples and real saliva and urine specimens will be carried out in future work to further evaluate the applicability of the proposed assay.

To assess the applicability of the spot-based LP-μPADs, creatinine determination was performed in artificial saliva and urine, which have compositions similar to those of human biological samples. The performance of the proposed device was assessed using a spiking method, in which creatinine standard solutions were added at concentrations of 0.2 to 0.6 mg/dL for artificial saliva and 5 to 15 mg/dL for artificial urine, as summarized in Table 2.

The percentage recovery of creatinine was calculated using Eq. (2.4), and the results are presented, as summarized in Table 2. The recovery values ranged from 99 to 107.5 % for artificial saliva and 99.13 to 112.0 % for artificial urine samples, demonstrating the accuracy and reliability of the device for analysis of biological samples.

4. Conclusion

A spot-based LP-μPAD was fabricated on Whatman Grade 4 filter paper using a laser-printing method for colorimetric creatinine detection. The single-layer spot-based design eliminated the need for flow channels and multilayer assembly, providing a simple fabrication process. Creatinine detection was based on the Jaffe reaction, and the colour response was quantified from smartphone images using ImageJ software. The developed assay exhibited a linear response over the concentration range of 0-20 mg/dL, with a limit of detection of 0.15 mg/dL, a limit of quantification of 0.49 mg/dL, and %RSD values below 10%. The assay showed satisfactory selectivity toward representative interfering species and good agreement with the reference UV-Vis method, as confirmed by Bland–Altman analysis. Recovery values of 99.0-107.5% for artificial saliva and 99.13-112.0% for artificial urine demonstrated satisfactory analytical performance in simulated biological samples. The assay maintained analytical performance during refrigerated storage,

although the calibration sensitivity decreased gradually with storage time; therefore, a storage period of up to one week is recommended to ensure consistent analytical performance under the investigated conditions. Future work will focus on evaluating the assay using real saliva and urine samples, together with broader validation of selectivity and smartphone-based image acquisition, to further establish its applicability for practical creatinine analysis.

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Authors Contribution Statement

Oinam Robita Chanu: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – Original Draft. Subramaniam Pushpavanam: Resources, Methodology, Supervision, Writing – review & Editing. Ashish Kapoo: Formal analysis, Methodology, Writing – Review & Editing. Varshini Karthik: Supervision, Validation, Writing – Review & Editing. All the authors have read and agreed to the published version of the manuscript.

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Data Availability

The data supporting the findings of this study can be obtained from the corresponding author upon reasonable request.

Has this article screened for similarity?

Yes