CHAPTER 3: SMARTPHONE-BASED AUTOMATED ESTIMATION OF PLASMA CREATININE FROM FINGER-PRICKED BLOOD ON A PAPER STRIP VIA SINGLE-USER STEP SAMPLE-TO-RESULT INTEGRATION

3.1 Chapter overview

This chapter describes the development of a low-cost and simply-fabricated microfluidic paper-based analytical device (μPAD) to estimate plasma creatinine from the finger-pricked whole human blood. A simple paper strip is proposed to accurately measure creatinine concentration using just 10 μL of figure-pricked blood. The proposed device mainly involves three steps. First step is separation of plasma from the whole blood, second step is transportation of plasma to the detection zone by exploiting the capillary properties of the paper matrix, and third step is the quantification of the creatinine concentration using an in-house developed app. The creatinine concentration of same samples are then estimated by biochemical auto-analyzer and compared with the values obtained from our proposed device. The results obtained by our device show a reasonable accuracy in comparison to the gold standard method.

3.1.1 Introduction

Creatinine is a breakdown product molecule of creatine and phosphocreatine produced in the human body as a result of muscle metabolism. Spontaneous conversion of creatine to creatinine occurs during movements of muscle contraction and is subsequently transported via the bloodstream to the kidneys and finally excreted through urine. Further, in addition to being one of the common markers of renal disease progression, blood creatinine levels may also be implicitly correlated with heart failure (Ko et al. 2007; ter Maaten et al. 2014),

cancer (Kent et al. 2017; Williams et al. 2013), and muscular dystrophy (Debus et al. 2015; Randviir and Banks 2013). Therefore, estimating creatinine levels in the body fluids may be considered as a first level of intervention to decide on the suggestive recommendations of further specific diagnostic assessments and clinical decision making. Considering such decisive implications in primary healthcare and remedial policies for combating non-communicable lifestyle diseases, point-of-care (POC) testing for creatinine concentrations is turning out to be progressively more imperative than ever before.

In POC-based applications, non-invasive testing holds obvious practical benefits as compared to blood-extraction based procedures. From such perspectives, measurements of creatinine from urine samples hold key advantages due to the non-invasive nature of the sample collection procedure (Jalal, Jin, and Shim 2017). However, the accuracy of the resulting prediction is often masked by diet, water intake, hydration level, etc. Therefore, a single measurement from urine does not provide a decisive picture. Rather, for accurate urinalysis, the periodic collection of urine samples over an entire 24 h period often renders to be essential to arrive at clinically-relevant inferences (Nabavizadeh, Ghadermarzi, and Fakhri 2014). As an alternative proposition to this, non-invasive blood-based creatinine level determination has also been attempted using the principle of near-infrared (NIR) spectrometry (Burbea 2019). However, this method has been reported to lack the envisaged specificity as compared to the established laboratory-based gold standards.

The gold standard method of creatinine level detection from blood plasma, despite being robustly founded from the first principles, suffers from several implementation constraints for POC applications, such as specialized infrastructure and human-resource-intensive dissemination framework that cannot be readily translated to under-resourced settings without compromising the cost and operational simplicity. To circumvent these limitations, minimally invasive blood sample collection procedures (such as finger-

pricking) have emerged over the recent past, in an effort to perform essential screening tests for detecting potentially vulnerable cases falling outside the limits of the normal ranges of plasma creatinine concentration in the human blood, i.e., 0.5 to 1.0 mg/dl for women and 0.7 to 1.2 mg/dl for men (Songjaroen et al. 2009). However, precise quantifications in this regard are clinically more preferred, to enable decisive staging of progressive renal malfunctioning (see Table 3.1), as opposed to mere qualitative screening. Towards that, it renders imperative to innovate a POC-based testing procedure that is aligned with the established biochemical gold-standards, ensuring high analytical efficacy, and at the same time, conveniently miniaturized and adapted for use in resource-constrained settings.

Table 3.1 The various phases of kidney disease with representative blood plasma creatinine concentration values

Concentration of Plasma Creatinine (mg/dl)	**Stages of Kidney Functions**
0.5-1.2	Normal ranges
1.6	50% of kidney function is already lost
5.0-7.0	Severe kidney damage
9.0	Late-stage of chronic kidney disease (CKD)
10.0	90% of kidney function has already been lost
>10.0	Need dialysis until getting a renal transplant

Jaffé reaction has been established as a classical means of measuring the creatinine concentration in human blood and urine samples over almost a century, due to its simplicity

(Blass, Thibert, and Lam 1974), and appears to be ideal for direct biochemical sensing as against other alternative indirect modes of measurement. The contextual efficacy of this reaction is centered around the fact that creatinine reacts proportionately with picric acid to produce a colored complex in the presence of an alkaline solution. Similar principles of detection have subsequently been advanced using other alternative reagents such as 3,5-dinitrobenzoic acid (Langley and Evans 1936), 3,5-dinitrobenzoyl chloride (Parekh et al. 1976), methyl-3,5-dinitrobenzoate in a mixture of dimethyl sulfoxide, methanol, and tetramethylammonium hydroxide (Sims and Parekh 1977). With further advancements in POC-based technologies, the classical Jaff´e kinetic assays (Blel et al. 2017; Chen et al. 2016; Wang et al. 2016) have been substituted by other alternative measurement techniques as well, such as, capillary electrophoresis (Vitali et al. 2017), chromatography (Langsi et al. 2017), chemiluminescence (Hanif et al. 2016), spectrophotometry (Krishnegowda et al. 2017; Saidi et al. 2018), colorimetry (Alula et al. 2018; Hall et al. 2017; Sutariya et al. 2016), potentiometry (Guinovart et al. 2017), amperometry (Kumar, Jaiwal, and Pundir 2017; Nieh et al. 2013), electrochemical sensors (Hooshmand and Es'haghi 2017; Raveendran et al. 2017), and pH sensors (Pal et al. 2016). However, the act of striking a balance between adhering to the fundamental scientific gold-standards and exploiting the emerging sensor technologies has continued to remain challenging from the purview of providing affordable albeit highly accurate diagnostic solutions.

Microfluidic paper-based analytical devices (μPADs) have off-late ushered great promises to translate gold standard testing methods to extreme POC-settings. Various fabrication technologies have been used for the said purpose for developing the test strips, such as wax printing (Lu et al. 2010), inkjet printing (Abe, Suzuki, and Citterio 2008), photolithography (Carrilho, Phillips, et al. 2009), and screen-printing (Renault et al. 2013). These methods have their own merits and limitations including resource-intensiveness and

complexity, but have been found to be readily adaptable for developing test kits for assessing the critical parameters of renal function (Huang et al. 2011; Lin et al. 2013; Tambaru et al. 2017). Sittiwong and Unob (Sittiwong and Unob 2016) presented a paper-based platform for colorimetric detection of creatinine levels over the range of 1-6 mg/dl, albeit being mediated by a complex surface modification process. An integrated Self-powered Imbibing Microfluidic Pump by Liquid Encapsulation (SIMPLE) based-biosensor (Creasensor) (Dal Dosso et al. 2018) has been proven to act as a point-of-care (POC) device effective over the creatinine range of 0.67-20 mg/dl, despite being constrained by over-duly complicated manufacturing procedure as well as device architecture. Talalak et al. (Talalak et al. 2015) developed a highly-specific low-cost enzymatic paper-based analytical device (enz-PAD) for creatinine assay by colorimetric detection, albeit suffering from limited operational stability. Sununta et al. (Sununta et al. 2018) proposed a μPAD for determining the creatinine concentration in urine samples utilizing the Jaffé reaction process. Tseng et. al. (Tseng et al. 2018) extended the same reaction principle to an integrated 3-D μPAD platform for the detection of whole blood creatinine levels. In line with this, a paper-based chip was developed for the same purpose with a CMOS camera and USB connection, selectively effective over the creatinine range of 0.19–8.0 mg/dl (Fu et al. 2018). Chaiyo et al. (Chaiyo et al. 2018a) developed a novel paper based device for determining albumin to creatinine ratio using colorimetry of urine samples. Similar sensors have also been reported with more favourable user-friendly features such as smartphone integration (Lewińska et al. 2021), extendible to the estimation of other renal function biomarkers as well, from urine samples (Rossini et al. 2018).

While intuitively obvious, it has not been by any means trivial to extend the above urine-based sensors for blood-based analytics, as attributed to certain additional compelling issues, including but not limited to plasma separation, colour-interference

factors etc. An inventive approach to circumvent this challenge has later been introduced by using an aerosolized polymeric solution and substrates masked with painter's tape (Heist et al. 2018). However, the aspects of complicated fabrication and use of specialized reagents such as chitosan could not be obviated in the procedure (Hou et al. 2011; Kim, Kim, and Kim 2020; Yung et al. 2009). Nilghaz and Shen (Nilghaz and Shen 2015) offered a potentially alternative approach of separation of plasma from whole human blood on μPADs using saline (NaCl) and magnesium chloride ($MgCl_2$) solutions, for functionalizing the patterned paper. However, the resulting assay suffered from poor quantitative accuracy.

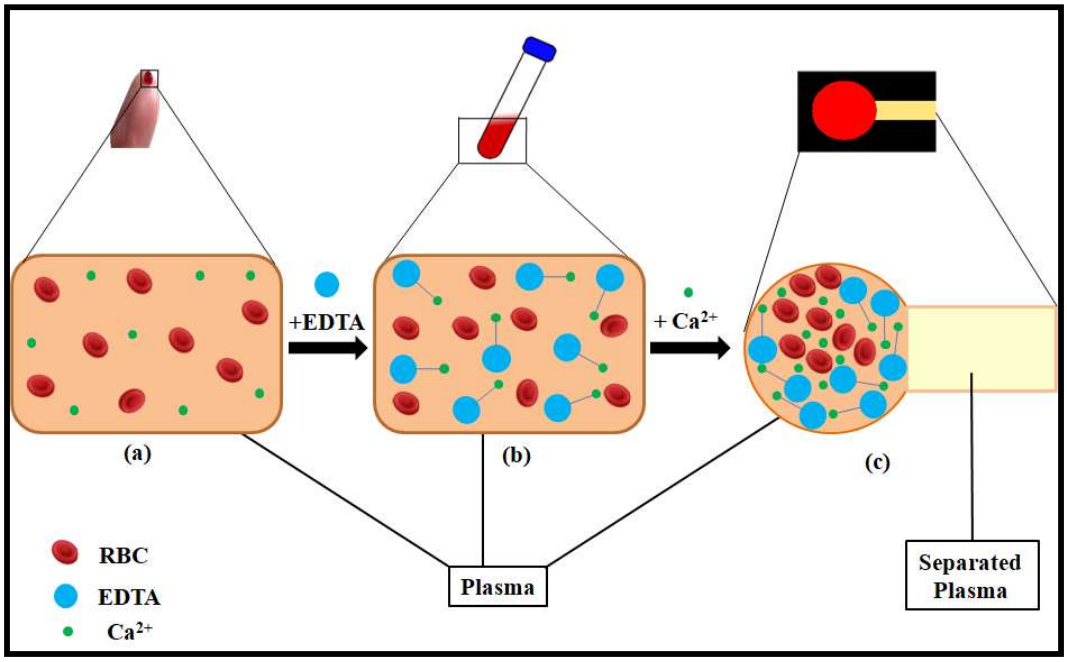

Fig. 3.1 Illustration of the aggregation of RBCs: (a) Whole blood containing RBC, WBC (not shown in the figure), Ca^{2+} etc. dispersed in plasma in vivo, (b) Addition of anti-coagulant (EDTA) that binds to Ca^{2+} to prevent RBCs aggregation in vitro, (c) Addition of excess Ca^{2+} initiates aggregation of RBCs and plasma separation in vitro.

Here, we introduce a simply-fabricated paper microfluidic strip for evaluating plasma creatinine levels in the blood, facilitated by quantitative colorimetric image analysis, in a unified single-step procedure achieving sample-to-result integration inclusive of a spontaneous separation of blood plasma via mere salt-functionalization of the paper-strip kit (as schematically delineated in Fig. 3.1) with no additional complex intervention. The simple plasma separation approach reported herein harnesses the coagulation ability of Ca^{2+} ions (Li et al. 2014), which can reduce the charge repulsion between the red blood cells (RBCs), exhibiting aggregation of RBCs at high salt concentration (Ataullakhanov et al. 1994). On the other hand, the addition of ethylenediaminetetraacetic acid (EDTA) to fresh human whole blood outside the vascular system (*in vitro*) chelates Ca^{2+} and, hence, prevents aggregation of RBCs. Here, anti-coagulating EDTA-treated whole human blood is accordingly used as test samples. Our studies also substantiate the supreme stability of the detection kit, rendering usability for a prolonged time and preservability at room temperature. As a further decisive advantage in sharp contrast with other reported POC devices for blood creatinine estimation via Jaffé reaction, the present approach does not require any mandatory heating step and hence can be executed without any special environmental control in resource-limited settings.

3.2 Experimental details

3.2.1 Chemicals and materials

Creatinine (anhydrous, ≥98%) and picric acid (moistened with water, ≥98%) were purchased from Sigma (India). Sodium hydroxide (NaOH) and calcium chloride ($CaCl_2$) were of analytical grade and are obtained from Merck (India). Deionized (DI) water was used for the preparation of 10% (w/v) aqueous NaOH solution, which was mixed with picric acid in a ratio 1:5 to prepare an alkaline picrate. DI water was also used to prepare

different creatinine concentrations for the calibration curve. 100 mM aqueous $CaCl_2$ solution was prepared and was serially diluted to different concentrations (10, 20, 40, 50, and 60 mM) for functionalizing the sample inlet zone of the μPAD. In this work, $CaCl_2$ is used for the salt solution because it is easily available as well as inexpensive.

3.2.2 Device design and fabrication

We used Whatman cellulose paper (Grade 4, GE Healthcare, UK) for the kit preparation. Dry paper gets wetted by the blood sample via capillary action, which can be used as chromatographic support (Metcalf, Morgan, and Dean 1982). This principle is inherent to several paper-based applications, including lateral flow assays (Chin, Linder, and Sia 2007; Liu, Mazumdar, and Lu 2006; Wong et al. 2006). The paper channels were designed in Inkscape software and fabricated on the cellulose paper (mean pore diameter, $2r_m = 20 – 25$ μm; where r_m stands for the mean pore radius) by an office-executable laser printing procedure (Dey et al. 2015). The fabrication technique directly employs the toner cartridge for fabricating the hydrophobic barriers surrounding the desired geometry of the paper channels. In sharp contrast to the wax printing (Carrilho, Martinez, and Whitesides 2009), this method is simpler without compromised scalability for mass-scale manufacturing. In our in-house facility, the design of the microchannel was printed on both sides of the paper by a laser printer (HP Colour LaserJet 2600n). Subsequently, the pattern-printed paper is heated on a hot plate at 180 °C for 4-5 min. During the heating process, the printer's toner particles melt and the molten ink penetrates through the porous paper matrix, thereby generating a well-defined hydrophobic barrier spanning across the paper thickness. The paper cartridge, fabricated following the above-mentioned procedure, forms a stand-alone strip for the detection purpose, as illustrated in Fig. 3.2.

3.2.3 Collection of blood samples and processing

Blood samples were collected from the patients (aged between 10-60 years) through finger-prick, maintaining a standard protocol guideline, at the in-house hospital of the parent institute of the corresponding author. The hematocrit measurements of those patients all lie in the normal range (36-51%) as found by the Automated Hematology Analyzer. An approval of ethical clearance was obtained according to the study protocol evaluated by the Institutional Ethical Committee of the Indian Institute of Technology Kharagpur. The fingertip skin was punctured using a safety lancet, and the first drop of blood was wiped using cotton to minimize excess tissue fluids oozing out. Subsequent blood drops were then collected into anti-coagulant dipotassium ethylenediaminetetraacetate (K_2-EDTA)-coated tubes giving a minimum volume (0.1 mL) of blood.

After collection, the samples were processed immediately, for testing and analytics. The sample was gently shaken to make a homogeneous suspension before any experimental run. The creatinine content was also measured using an Erba biochemical auto-analyzer (EM360) which is generally considered as a standard pathological laboratory instrument.

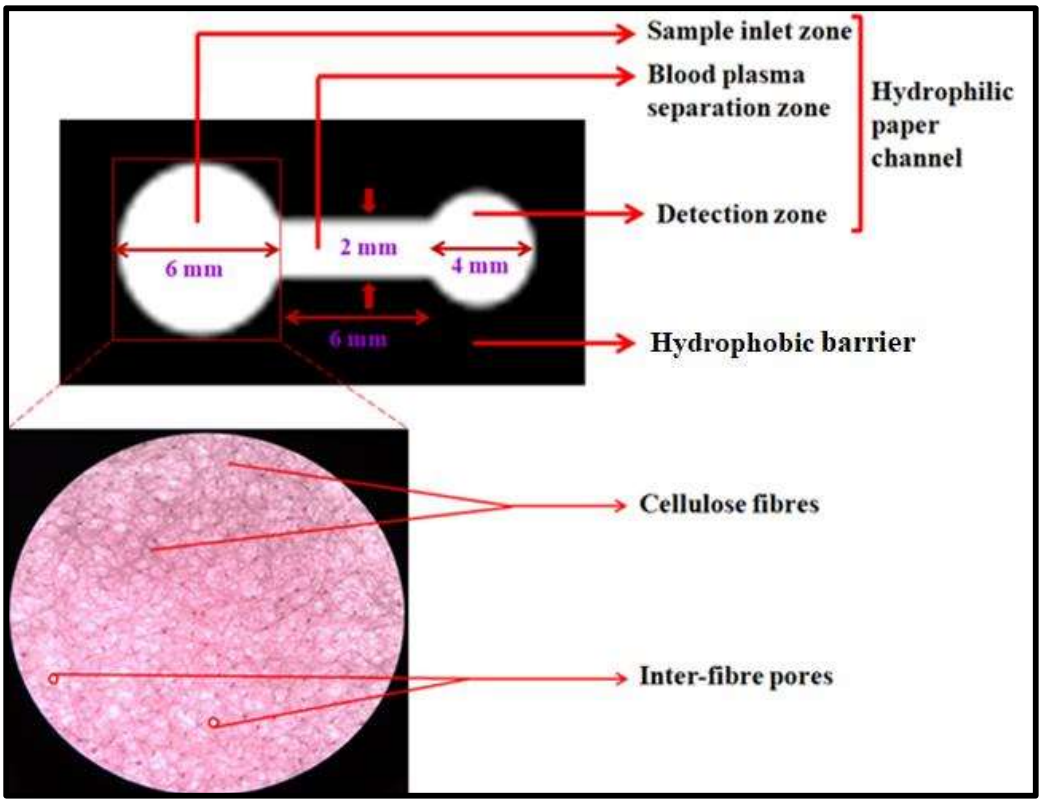

Fig. 3.2 Schematic representation of the printed paper device with the hydrophilic channel containing a sample inlet zone, blood-plasma separation zone, and detection zone, and the patterning of hydrophobic barriers by LaserJet printer. The magnified view shows the microscopic image of the Whatman cellulose paper (Grade-4) matrix. The paper matrix consists of random distribution of cellulose fibers and forms non-uniform, microscopic inter-fiber pores for liquid flow.

3.2.4 Method of estimation

The effect of salt concentration on the plasma separation method, which is an essential pre-requisite for the present analytical procedure, was studied first, in an effort to standardize this critical step without any complex microfluidic intervention. The dispensing zone of the paper strip was functionalized with 1.5 µL of different concentrations of $CaCl_2$ solution (10, 20, 40, 50, and 60 mM), whereas 1 µL alkaline picrate reagent was dispensed in the

detection zone of the paper strip and dried 3-5 minutes. Later 10 µL of the whole blood sample, which was gently dispensed on the source pad (sample insertion zone), progressively undergoes a simple in-situ plasma separation procedure as triggered by the spontaneous reactions leading to an intrinsic control over the RBC aggregation mechanisms. On reaching the detection zone of the paper strip by capillary action, creatinine in blood plasma reacts with alkaline picrate to form a creatinine-picric acid complex rendering a color that is visible on µPADs. An image of the region-of-interest (ROI) of the µPAD was captured by a smartphone and the color intensity of the ROI is correlated to the creatinine concentration. The overall clinical assay is summarily illustrated in Fig. 3.3**.**

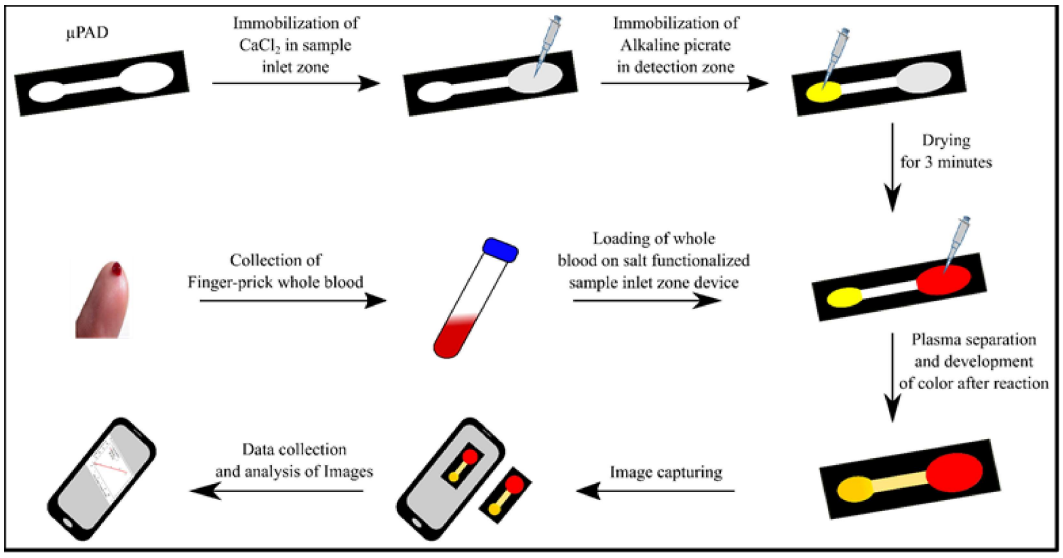

Fig. 3.3 Schematic illustration of paper-strip-based creatinine estimation. After immobilization of the sample inlet zone with $CaCl_2$, the detection zone is immobilized with alkaline picrate. Further, after collection, the loading of whole blood in the salt-functionalized sample inlet zone of the paper-based device is carried out. Separation of plasma from the whole blood occurs due to aggregation of RBCs in presence of calcium ions and the separated plasma travels to the detection zone due to capillary effects and is resisted by viscous forces. Creatinine present in separated plasma is reacted

with reagent (alkaline picrate) in the detection pad, leading to the formation of color at the detection zone. The image is captured in a smartphone followed by an image analysis using an in-house developed smartphone app (CREA-SENSE) for quantitative estimation of creatinine.

3.2.5 Smartphone app (CREA-SENSE) development and image analysis

An android based smartphone app, CREA-SENSE, was developed for real-time detection and quantitative estimation of the creatinine concentration. The integrated process of the smartphone app development and image analysis is illustrated in Fig. 3.4. Python 3.7.9 and Android Studio 4.1 were used for algorithm and software package development. After image acquisition, image processing involves a series of image operations to improve the quality of a digital image, by undergoing the process of distinguishing the object (ROI) from the background, leading to the generation of quantitative information. The image was analyzed in an automated digital environment, obviating manual intervention and keeping the provision of cloud integration as well as integration with a medical decision-making system. Several stages were executed by the developed smartphone app (i.e. segmentation of ROI, background correction, pre-processing, extraction of image features, and analysis) as shown in Fig. 3.4 (a). Images were recorded before and after 2 minutes of reaction using the Redmi 4A smartphone (Xiaomi, Beijing, China) based on a 10.5 cm-height support to maintain the focal length constant. The image was read by the CREA-SENSE app and converted to grayscale. Once the grayscale image is obtained, the spatial image filtering technique was applied for modifying or enhancing an image. The mean intensity of the detection zone was determined by identifying a region of interest centered around the detection spot. For all the analyses, the mean intensity of the detection zone before the reaction is subtracted from that of the same zone after 2 minutes of reaction to avoid undue artifacts due to inevitable variabilities in the ambient light conditions. Finally, the mean

grayscale intensity feature of the captured images was calibrated with the known creatinine concentration, and the calibration curve was used for prediction of unknown creatinine concentration. The screenshots of the step-by-step operation of the smartphone app for the overall detection process are delineated in Fig. 3.4 (b). All tests were conducted by non-specialized personnel outside the laboratory environment.

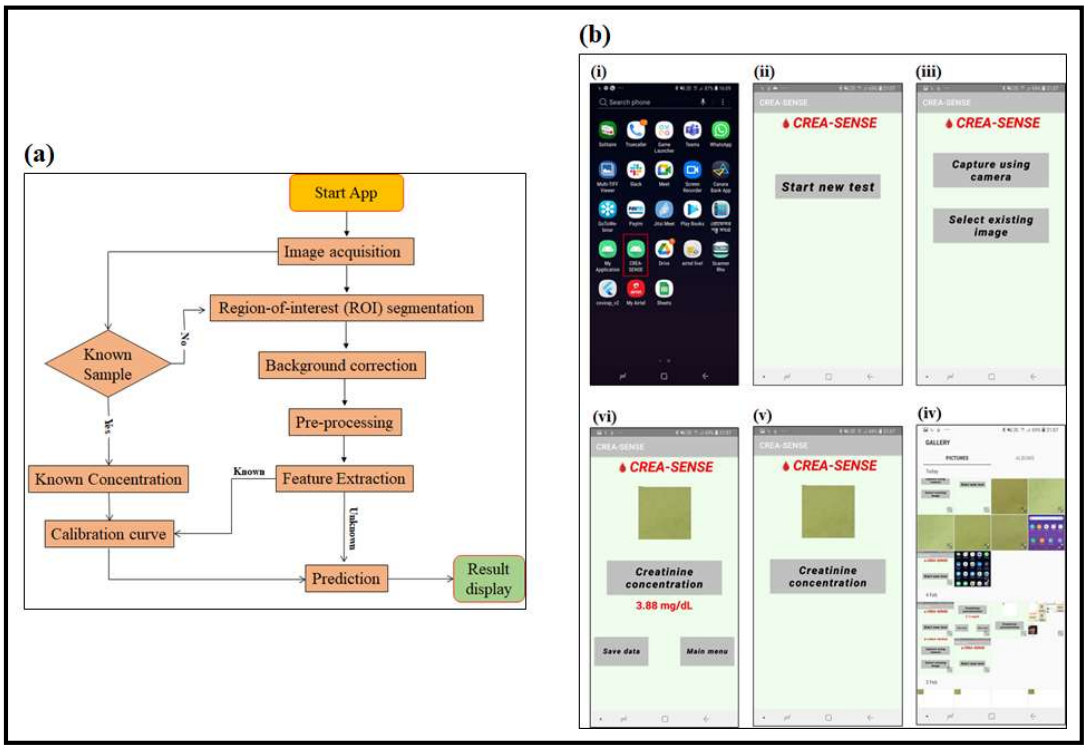

Fig. 3.4 The smartphone-based image analysis for quantitative estimation of creatinine. (a) Flowchart for image processing and implemented in the "CREA-SENSE" app. (b) Screenshots of sequential operation of the app are (i) open the app (ii) start new test (iii) capturing the image (iv) select the image for analysis (v) view of the image from the gallery where it stored and (vi) displaying the outcome.

3.3 Results and discussion

3.3.1 Effect of salt concentration on the plasma separation efficacy

The use of salt-functionalized μPADs for the separation of plasma from whole blood is delineated in Fig. 3.5. The addition of EDTA to fresh human whole blood outside the vascular system (*in vitro*) chelates Ca^{2+} and, hence, prevents aggregation of RBCs and therefore whole blood may easily penetrate through the fibrous networks on the paper by capillary driving force as an integrated medium (Fig. 3.5(a)-(i)). However, by adding various concentrations of $CaCl_2$ solution into EDTA-treated human blood, calcium ions can reduce the charge repulsion between RBCs. In this situation, RBCs are aggregated and are unable to wick with the plasma in the fiber network and are separated from the plasma phase. The separated plasma continues to travel forward into the detection zone of the paper device and the wicking distance of plasma gets also increased when the salt concentration increases. As shown in (Fig. 3.5(a)-(ii)), the RBC aggregation and therefore separation of plasma are conspicuous when the whole blood is dispensed on a 50 mM $CaCl_2$ pretreated paper. The curve (distance of plasma traveled versus concentrations of $CaCl_2$ solution) depicts that the optimum concentration of $CaCl_2$ solution is 50 mM to separate plasma sufficiently from whole blood (Fig. 3.5(b)). Such a separation is adequate for the estimation of creatinine with a low sample volume of blood which enables the kit to be used under extreme POC settings without compromised accuracy.

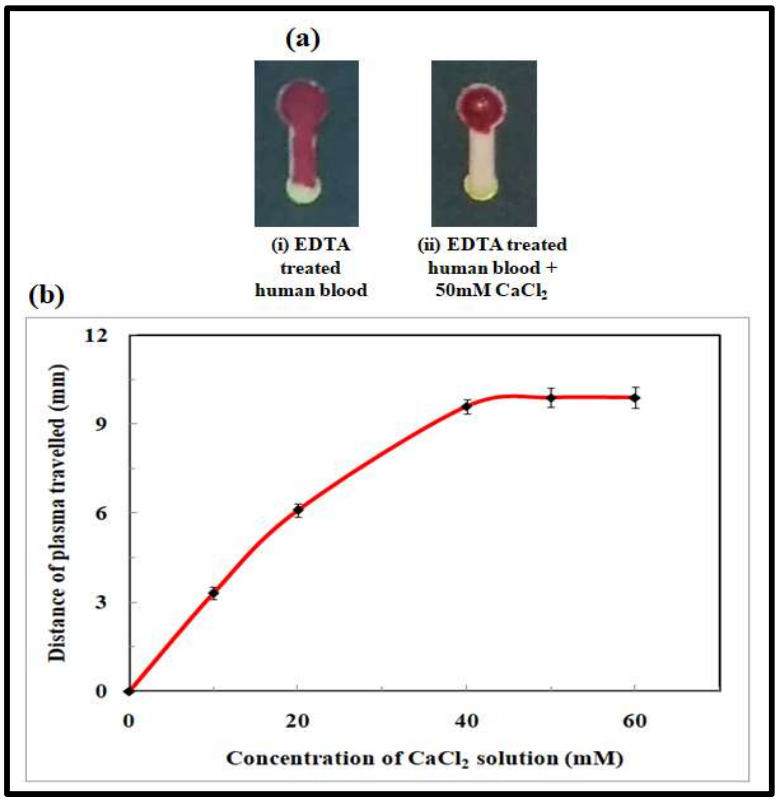

Fig. 3.5 Effect of $CaCl_2$ concentrations on plasma separation. (a) Separation of plasma from EDTA-treated whole blood on µPADs functionalized without and with 50 mM $CaCl_2$ solutions (optimum), respectively. (b) The curve shows the distance of separated plasma traveled from the RBC front (mm) vs. concentrations of $CaCl_2$ solution. Every data point represents the mean values and error bars show the standard deviation among three independent experimental results.

3.3.2 Principle for plasma separation and colorimetric reaction based on time-dependent phenomena

Before the experiments, 1.5 µL 50 mM $CaCl_2$ solution and 1 µL alkaline picrate reagent are applied in the sample dispensing zone and detection zone of the paper strip, respectively. The µPAD is allowed to dry for 3 min. Images are captured (as shown in Fig. 3.6) by microscope (Olympus CX23) with 10 × resolution at different time intervals during the entire experimentation. This facet of image capturing is not a part of the test protocol

but is essentially demonstrative of the essential physical premises of blood plasma separation as a precursor to the colorimetric reaction. First, 10 μL of the whole blood is dripped into the salt functionalized sample inlet zone (t = 0). The salt is dissolved in plasma, facilitating the suppression of the electric double layer on the surface of RBCs by the counter ion valence charge of the calcium ion. This leads to the aggregation of the RBCs, and therefore the aggregated RBCs are unable to travel in the interlaced fibrous network of the paper matrix (see the blow-up in Fig. 3.2). As a result, due to capillary imbibition through the paper channel, plasma starts to filter out through the porous membrane and blood cells are retained in the sample inlet zone after 30 s. The viscosity of the separated plasma is less than the viscosity of the whole blood, and therefore, the velocity of the separated plasma becomes higher. According to Darcy's law, the rate of flow in a paper-based channel is inversely related to the viscosity, and therefore, the flow rate of the separated plasma enhances and the same is transported through the hydrophilic channel which reaches the detection zone of the paper strip approximately after 90 s. There, the creatinine present in the plasma reacts with alkaline picrate reagent to form a creatinine-picric acid complex that produces color in the detection zone after about 2 minutes. Thereafter, images of the region-of-interest are captured by a smartphone, and the color is analyzed for the estimation of creatinine concentration.

3.3.3 Technology translation to the extreme point-of-care application using modified Jaffe reaction

The transfer of the pathological lab-standard technique for the estimation of creatinine to extreme point-of-care applications is one of the critical and challenging propositions. In traditional laboratory-based approaches, at first, the whole blood is centrifuged to separate plasma. The separated plasma is then collected in a test tube and NaOH solution is added. After that, the picric acid solution is mixed into the resulting solution. The final solution is

vortexed thoroughly and left for 15 minutes at room temperature (20-25 °C) to allow the development of color according to the Jaffe reaction (Toora and Rajagopal 2002). Thereafter, the absorbance of the solution is measured and the unknown blood plasma creatinine is estimated.

Although Tseng et. al. and Fu et. al. (Fu et al. 2018; Tseng et al. 2018) previously transferred a traditional lab-based creatinine level detection procedure to a miniaturized platform for POC-based applications, an exclusive heating step at 37 °C for 5 min remained to be a mandatory requirement for the same, in an effort to obtain discernable color signal as triggered by the Jaffe reaction. In sharp contrast, the present implementation of the modified Jaffe reaction could be executed in normal ambient temperature conditions without demanding any separate environmental control, in a time span of only 2 minutes, via executing a single-step sample-to-result integration. Such unprecedented reduction in the reaction time could be achieved, when, instead of picric acid and NaOH being dispensed to the detection zone one after the other, a premixed solution of NaOH and picric acid was dispensed altogether in the detection zone at once for the analytical purpose. Notably, the elimination of any heating step and complex plasma separation methodology renders the present test extremely user-friendly to unskilled frontline healthcare workers for deployment in community healthcare centers.

3.3.4 Performance evaluation compared to the conventional approach

We evaluated the performance of the present device to estimate the creatinine concentrations vis a vis the conventional measurement results obtained using a commercially available biochemical auto-analyzer (EM360). A collage of the corresponding data analysis is provided in Fig. 3.7, with the details provided in the figure caption.

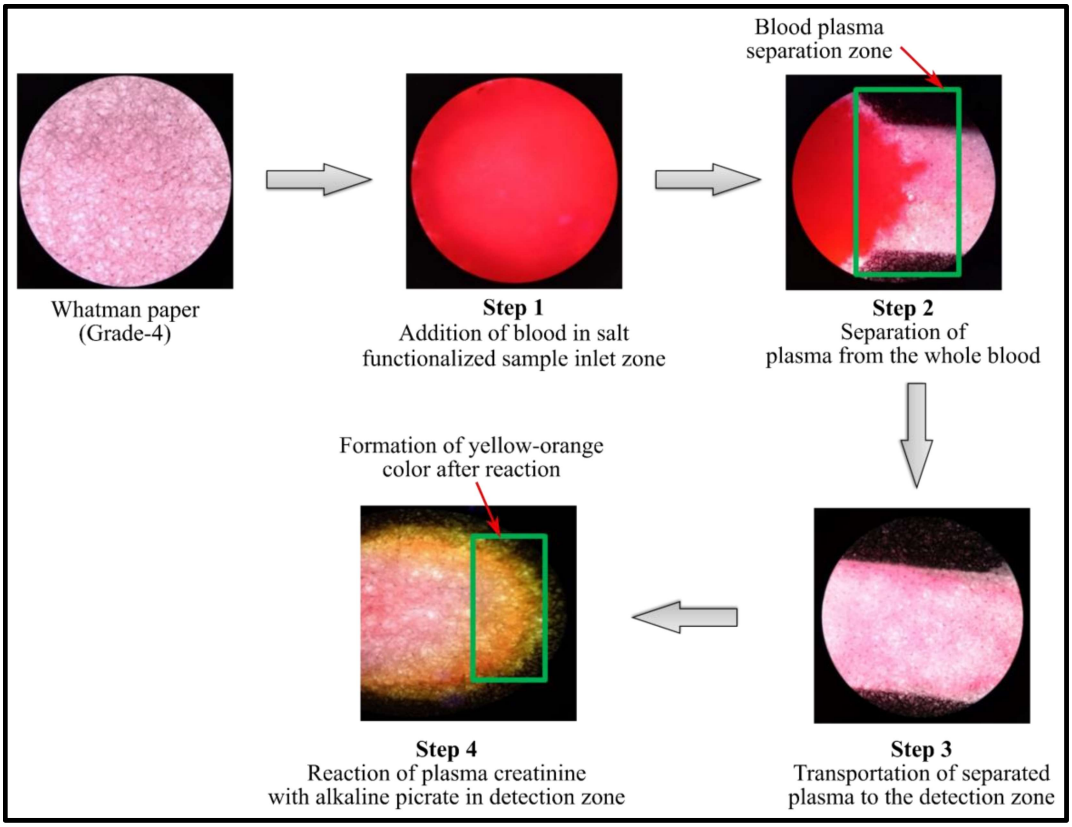

Fig. 3.6 Real-time images captured by microscope showing the separation of plasma from whole blood and transportation of the separated plasma to the detection zone for colorimetric assay at different time intervals. Step 1: loading of blood in salt functionalized sample inlet zone (t = 0 sec), Step 2: start separating plasma from whole blood after 30 seconds (t = 30 sec), Step 3: transportation of separated plasma through the hydrophilic channel and arriving at the detection zone (t = 90 sec), Step 4: reaction of creatinine in plasma with alkaline picrate takes place in the detection zone and formation of color (t = 120 sec) for quantitative estimation of creatinine.

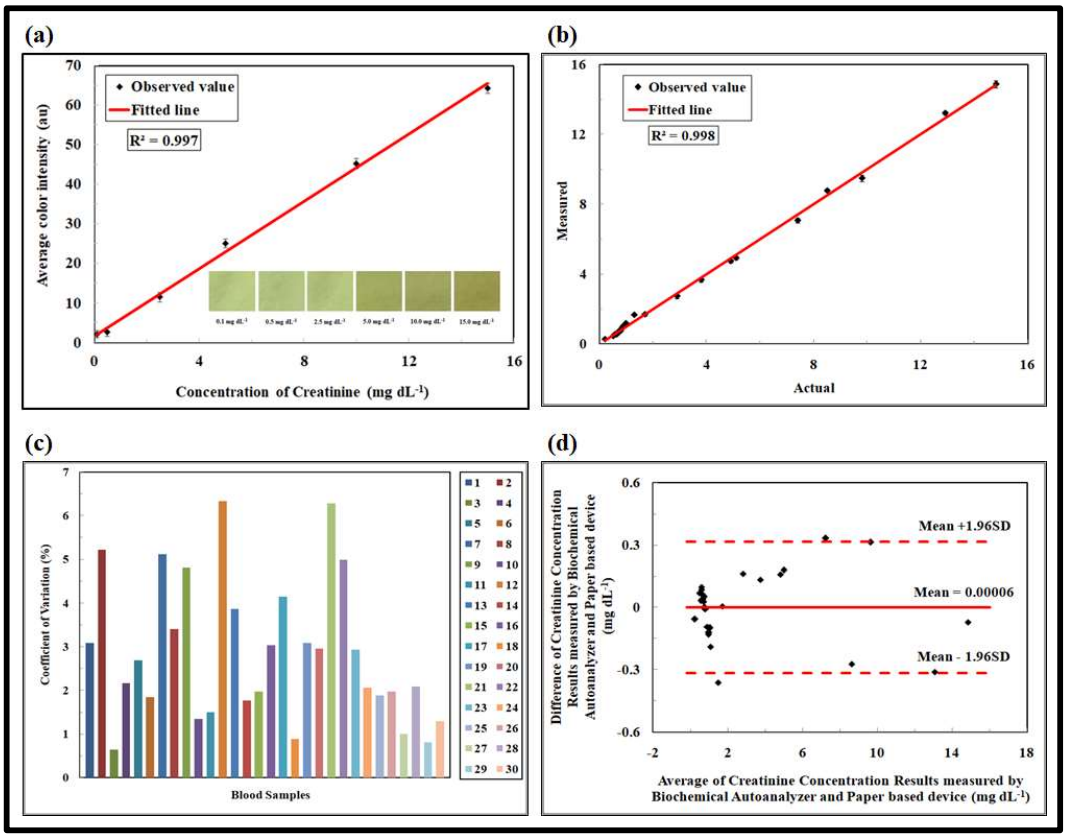

Fig. 3.7 Results obtained from the integrated paper-based device. (a) Calibration curve of average color intensity with the concentration of commercial creatinine in the range of 0.1 to 15 mg/dl in the paper-based microfluidic device. (b) Comparison of creatinine measurement results obtained using the paper-based microfluidic device (Measured) and biochemical auto-analyzer (Actual) for 30 different blood samples. (c) Coefficient of variation of creatinine measurement data for 30 blood samples using the paper-based microfluidic device. (d) The Bland–Altman plot shows the mean of against difference between actual and measured creatinine concentration to evaluate the accuracy of the paper-based microfluidic device. The thick red line indicates the mean. Dotted red lines show 95% limits of agreement (LOA), (average difference ± 1.96 SD).

To calibrate the paper-based microfluidic device, the average color intensity is plotted with different concentrations of commercially available creatinine (mg/dl) which is shown in Fig. 3.7(a). Average color intensities obtained with the present method show a

strong linear correlation with creatinine concentrations (R^2 = 0.997). To evaluate the accuracy of our paper-based creatinine measurement, we perform tests with 30 blood samples. Fig. 3.7(b) presents a scatter-plot of the results obtained by the two methods. All samples are measured three times. The experimental result with creatinine concentrations ranging from ~ 0.2 to 15 mg/dl demonstrates an excellent agreement between the two methods with a correlation coefficient of ~0.9992.

To evaluate the repeatability of the measurement, we calculate the percentage of the coefficient of variation (CV). CV values of creatinine of 30 blood samples where each sample creatinine has been measured three times using the present device are displayed in Fig 3.7(c). The analysis reveals the CV for every sample ranges from 0.64-6.4% which indicates a very good precision among the measurements. The Bland-Altman plot for these two methods is shown in Fig. 3.7(d), revealing a bias (mean difference) of 0.00006 mg/dl with 95% LOA between - 0.316 (lower limit) and 0.316 mg/dl (upper limit). It is obvious from Fig. 3.7(d) that most of the data points falls between limit of agreements, therefore, the both method can be used interchangeably for the estimation of creatinine concentration.

3.3.5 Analytical performance of the proposed method

The limit of detection (LOD) and limit of quantification (LOQ) of the integrated paper-based microfluidic device (proposed method) are 0.679 mg/dl and 2.056 mg/dl respectively. The device-to-device and day-to-day reproducibility of the method for the five measurements of creatinine has been calculated, which are found to be 2.5% and 4.9% respectively. Table 3.2 depicts the accuracy measurement of the proposed method (in comparison with the standard method) for creatinine estimation in blood samples. The median accuracy of our proposed method (paper-based microfluidic device) is 94%.

Table 3.2 Accuracy measurement of proposed method (paper-based microfluidic device)

Serial No.	Concentration of creatinine in blood samples using the proposed method (mg/ dL)	Concentration of creatinine in blood samples using the standard method (mg/ dL)	Accuracy of the proposed method	Median accuracy of the proposed method
1	0.27	0.21	72.94	
2	0.54	0.64	84.67	
3	0.69	0.72	96.03	
4	0.78	0.77	98.90	
5	0.61	0.67	90.52	
6	0.95	0.86	89.06	
7	0.57	0.64	89.56	
8	0.57	0.65	87.14	
9	0.73	0.78	93.17	
10	1.18	0.99	80.81	
11	1.07	0.97	89.72	
12	0.45	0.52	86.50	
13	0.55	0.58	94.36	
14	0.64	0.68	93.90	94.13
15	0.81	0.80	99.08	
16	0.75	0.75	99.89	
17	1.03	0.90	85.47	
18	1.66	1.30	72.07	
19	1.10	1.00	90.18	
20	1.04	0.92	86.92	
21	1.69	1.70	99.64	
22	2.74	2.90	94.43	
23	3.67	3.80	96.52	
24	4.47	4.90	96.74	
25	4.93	5.10	96.49	
26	7.07	7.40	95.51	
27	8.77	8.50	96.79	
28	9.49	9.80	96.80	
29	13.21	12.90	97.59	
30	14.87	14.80	99.52	

A comparison between the performances of the developed App with the scanner detection mode is presented in Table 3.3 that shows a good similarity between the two different methods.

Table 3.3 Comparison of estimation of creatinine concentration between developed app and scanner detection mode

Serial No.	Actual creatinine concentration (mg/dl)	Creatinine concentration with developed App (mg/dl)	Creatinine concentration with Scanner detection mode (mg/dl)
1	0.5	0.47	0.52
2	2.5	2.05	1.96
3	5	5.01	5.07
4	10	9.92	10.80
5	15	15.12	14.53

3.3.6 Chi-square test

A chi-square test is performed, where the null hypothesis is used to assess the agreement between the two methods (Measured and Actual) to determine creatinine concentration. The level of significance (p-value) is considered as 0.05 and the degree of freedom (DOF) for this test on our paper-based device is found to be 29. The value of chi-square (χ^2) for 30 observations is found as 0.342 which is significantly less than the χ^2 value from the table (42.557) with this p-value and DOF. Therefore, with this level of significance, the null hypothesis can be considered true.

3.3.7 Reagent stability and selectivity

The reagent stability test is performed with an alkaline picrate modified salt functionalized paper-based device that is stored at room temperature (24-30°C). The curves are

constructed by plotting average color intensities of the creatinine-picric acid complex obtained from the present device versus the number of days and are depicted in Fig. 3.8.

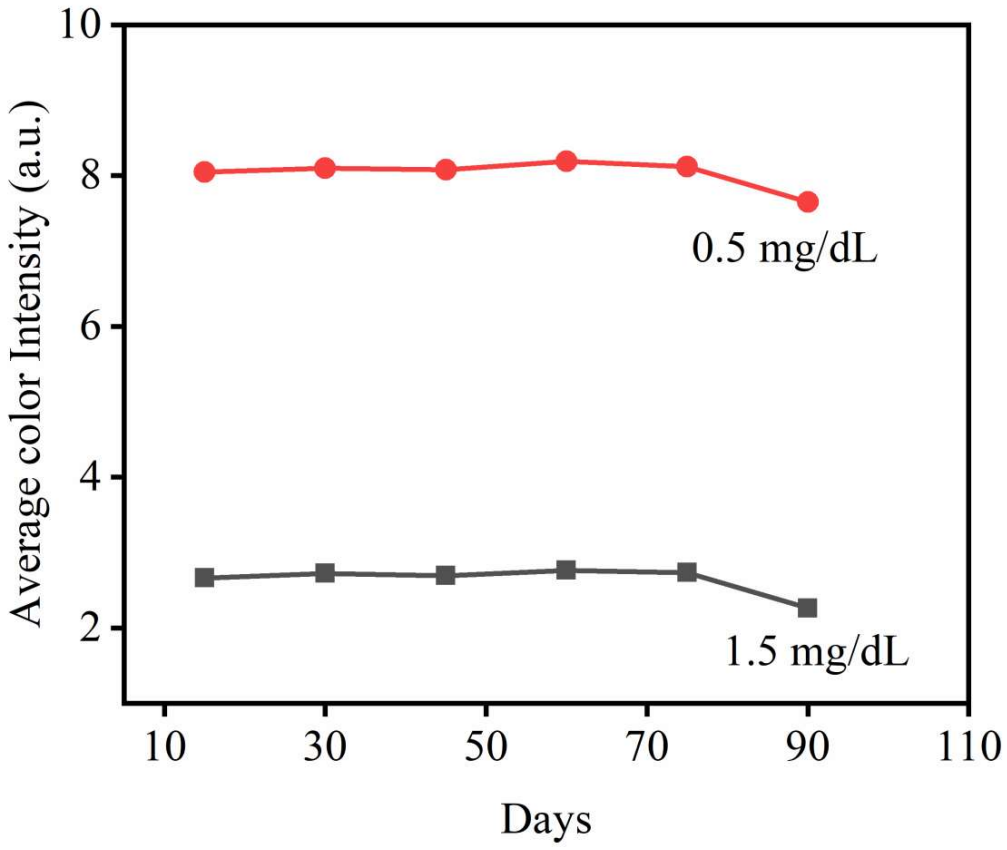

Fig. 3.8 Performance of storage stability of reagent (alkaline picrate) on salt functionalized paper with two different concentrations of commercial creatinine (0.5 and 1.5 mg/dl) at room temperature. The curves show that color intensities of the creatinine-picric acid complex are reduced very little after approximately 75 days.

It is observed that, for two different concentrations of commercial creatinine (0.5 and 1.5 mg/dl), intensities are found to remain unaltered during the first 75 days and are slightly reduced after that duration. Therefore, it is clear that the device is used for a long time and

preserved at room temperature. It can be stored in a transparent plastic box and no protection from light is required.

To investigate the selectivity, each of the interfering substances such as acetone, glucose, bilirubin, and ascorbic acid which have been described as pseudochromogens is added in large excess, and the response is monitored. The effect of interfering compounds is found to have virtually no influence on this assay method, premised on their virtual non-interference with the backbone colorimetric reaction considered for the present assay.

3.3.8 Estimation of cost for creatinine test

We next provide an estimated cost per test to realize the commercial competitiveness of the POC device, based on the test kit components as itemized in Table 3.4. The entire procedure involves single-time remittance of a smartphone (~$130) (*fixed costs*) and the recurring costs of paper strips and chemical reagents. Considering that users will have a pre-owned smartphone in which the CREA-SENSE app could be installed for free of cost, the average cost per test is estimated to be around ~$0.02 (*variable costs*) (including overhead expenses for pilot-scale manufacturing and commercial adaptation). The overall cost can be reduced further if up scaled to mass-scale manufacturing.

Finally, we summarize the significant advantages of the present kit as compared to some other recently reported methods, as presented in Table 3.5. As evident, this work has established improvements in different accounts such as the required volume of blood, the method of detection and sensing efficacy, simplicity and user-friendliness, the limit of detection, validation, duration of analysis, price, and setting (laboratory or field), which proves its potential to be used as an extreme point-of-care device engaging the involvement of minimally trained frontline workers.

Table 3.4 Cost-component break-up per test

Components/Reagents used	**Quantity**	**Cost of unit (INR)**	**Cost (INR)**
Fixed Costs			
Smartphone	1	10000	10000
Installation of CREA-SENSE App	NA	0	0
	Total Cost		10000 INR ≈ $130
Variable Costs			
Whatman paper	1 for 10 detections	1000.00 (100 pcs.)	1.000
Picric acid	0.83 mg	4500.00 (100 gm)	0.03735
Sodium hydroxide	17 µg	1100.00 (500 gm)	0.000037
Calcium chloride	8.3 µg	2760.00 (100 gm)	0.000229
DI water	2.5 µl	120 (1000 ml)	0.0003
Ancillary cost	-	-	0.472
	Total Cost		1.509 INR ≈ $0.02

Table 3.5 Assay performance comparison with other representative reported methods

Serial Number	Detection method	Description	Sensing principle	Sample type	Required sample volume (µL)	Detection range (mg/dl)	Analysis time (minutes)	Validation	Limit of Detection (mg/dl)	Approximate Price of cartridge/ strip	Setting	References
1	Colorimetric	Automatic biochemistry analyzer Pentra C200 (Horiba)	Reaction with picric acid in alkaline solution (Jaffé)	Serum or plasma	9	0.14–18	8.5	—	—	~$25	Laboratory	(Gbinigie et al. 2015)
2	Colorimetric	AgNPs-based sensor	AgNPs coated with picric acid (Jaffé)	Serum	100	0.0-0.01	4	Correlation $R^2 = 0.9998$	0.0001	Not Reported	Potentially adaptable for field	(Parmar et al. 2016)
3	Colorimetric	Integrated SIMPLE-based biosensor	Enzymatic reaction	Plasma	5	0.67–20	5	—	0.76	~$5	Laboratory	(Dal Dosso et al. 2018)
4	Colorimetric	3-D µPAD integrated with CMOS camera and Wifi chip	Reaction with picric acid in alkaline solution (Jaffé)	Whole blood	5	0.19–7.64	6	Correlation $R^2 = 0.9920$	0.19	Not Reported	Field / Laboratory	(Tseng et al. 2018)
5	Colorimetric	Paper-based chip integrated with CMOS camera and USB connection	Reaction with picric acid in alkaline solution (Jaffé)	Serum	3	0.2–8.0	5	Correlation $R^2 = 0.9994$	0.9	Not Reported	Field / Laboratory	(Fu et al. 2018)
6	Colorimetric	simply-fabricated paper strip integrated with image analysis	Reaction with alkaline picrate solution (Modified Jaffé)	Whole blood	10	0.1–15	2	Correlation $R^2 = 0.9992$	0.679	~$0.02	Field / Laboratory	Present study

3.4 Conclusion

In this chapter, we have presented a simple yet accurate clinical assay for plasma creatinine measurement on an easy-to-fabricate paper based microfluidic strip using the minimally invasive technique (finger pricked). Besides, a simple method of image analysis is coupled with this microfluidic device that facilitates colorimetric analysis. The results bear an excellent correlation with traditional gold-standard measurements achieving an extremely high correlation coefficient (0.9992) and showing an extremely low CV (<6.5%) indicating excellent precision among the measurements. The robustness and efficacy of the clinical assay have also been established. Further, the test results reflect a high level of sensitivity and specificity. The simple paper strip appears to be convenient to use in home comfort as an alternative to venepuncture-based blood examination, typically for those patients who require frequent monitoring of their kidney function. In addition, either through digital dissemination or through a smartphone app, the patients may themselves keep a record of their creatinine values, very much akin to the manner in which diabetics record their plasma glucose results, offering a simple foundation to patients of taking control of their own health monitoring.