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Ahsan, J., Riaz, K., Samad, H. et al. (2 more authors) (2025) Simultaneous multi-adulterant detection in milk using an in-house developed multiplexed microfluidic paper-based analytical device (M-µPAD). Sensing and Imaging, 26 (1). 63. ISSN 1557-2064

https://doi.org/10.1007/s11220-025-00580-y

Sensing and Imaging (2025) 26:63
https://doi.org/10.1007/s11220-025-00580-y

RESEARCH

Simultaneous Multi-Adulterant Detection in Milk Using an In-house Developed Multiplexed Microfluidic Paper-Based Analytical Device (M-µPAD)

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Received: 9 January 2025 / Revised: 20 March 2025 / Accepted: 28 March 2025

Abstract

Milk adulteration poses a significant challenge in developing countries, impacting food safety and public health. This study introduces a multiplexed microfluidic paper-based analytical device (M-µPAD) for rapid and simultaneous detection of multiple milk adulterants. The M-µPAD, fabricated using readily available materials like filter paper and wax crayons, eliminates the need for specialized equipment. The device demonstrates linear relationships between colorimetric response and adulterant concentration for urea (10–200 mg/dL), starch (20-1000 mg/dL), and detergent (100–3000 mg/dL), with detection limits of 11.1, 22.4, and 120.8 mg/dL, respectively. Requiring only 40 µL of reagents and 80 µL of milk sample, the M-µPAD achieves over 95% accuracy and reproducibility with RSD values below 4%. This affordable, in-house fabricated platform offers a promising solution for milk quality assessment in resource-limited settings, addressing the need for accessible and efficient adulterant detection methods.

Keywords Multiplexed µPADs · Colorimetric Analysis · Milk adulteration detection · Resource-limited diagnostics · Low-cost fabrication

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Published online: 15 May 2025

1 Introduction

Milk and dairy products have played a fundamental role in human nutrition for millennia, serving as a primary source of essential nutrients across all age groups [1, 2]. However, the increasing global demand for milk, coupled with rising production costs, supply-demand imbalances, and the complexities of supply chain management, has rendered milk a prime target for economically motivated food fraud [3–6]. This issue is particularly prevalent in developing countries, where regulatory infrastructure remains deficient, exacerbating the challenge of ensuring milk authenticity and safety. Among various forms of food fraud, adulteration—the intentional addition of non-milk substances—has emerged as a critical public health concern, as it compromises both nutritional value and consumer safety [7].

Milk adulteration involves the introduction of a diverse range of contaminants, including starch, urea, detergents, formalin, melamine, hydrogen peroxide, sucrose, sodium chloride, oils, water, antibiotics, and other harmful substances [4, 7]. The quality of milk is generally assessed based on factors like fat percentage, Solid Not Fat (SNF) value, protein content, and other considerations [8]. These inexpensive and widely accessible adulterants are often employed to maintain or improve these parameters by manipulate milk's physical and chemical properties. For instance, water is commonly added to increase volume, while urea and melamine elevate non-protein nitrogen content, mimicking higher protein levels [7]. Detergents and soaps enhance whiteness and emulsify added oils, while sugar and starch increase density in diluted milk [7]. Preservatives such as hydrogen peroxide, sodium carbonate, and formalin prolong shelf life by inhibiting microbial growth [7]. Despite guidelines established by the World Health Organization (WHO) and other food safety authorities regarding safe chemical consumption limits in milk—such as 70 mg/100 mL for urea, 0.05% v/v and 0.15% v/v for hydrogen peroxide and starch, respectively, and less than 0.002 mg/kg for detergent and soap [9–11]; these adulterants continue to pose significant health risks. Chronic exposure to contaminated milk has been linked to severe health complications, including renal failure, gastrointestinal disorders, respiratory distress, ulcers, vision impairment, neurological issues, and even carcinogenesis [4, 7, 12].

Ensuring milk quality necessitates robust analytical techniques capable of detecting adulterants with high sensitivity and specificity. Traditional laboratory-based methods such as microbial assays, the Gerber fat test, lactometer density test, and the Kjeldahl protein test have been widely employed for milk quality assessment [13–15]. However, these methods often fail to detect a broad spectrum of chemical adulterants, as adulterers may introduce additional substances specifically designed to circumvent standard testing procedures. Consequently, several advanced instrumental techniques have been developed for the precise detection of milk adulteration [16], including gas chromatography [17], high-performance liquid chromatography (HPLC) [18], capillary electrophoresis [19], mass spectrometry [20], UV-visible spectroscopy [21], infrared (IR) and Raman spectroscopies [22, 23], Enzyme-Linked Immunosorbent Assay (ELISA) [24], osmometry [25], and electrochemical sensors [26]. Although these techniques provide high accuracy and reliability, their widespread application remains limited due to the high costs, need for sophisticated lab-

oratory infrastructure, and reliance on skilled personnel. These constraints pose a significant challenge, particularly in developing countries, where milk production and distribution often occur through decentralized networks of small-scale vendors, making quality enforcement a daunting task [27]. As a result, there is an urgent need for affordable, field-deployable and domestic testing methods and kits that can be implemented at various points within the supply chain and at homes to safeguard public health and enhance regulatory compliance.

At present, field-deployable and domestic milk adulteration testing kits rely on chemical spot tests, which require handling hazardous reagents such as sulfuric acid, sodium hydroxide, and trichloroacetic acid to induce colorimetric changes in test vials, tubes or paper strips [28, 29]. While these tests offer on-site detection, their reliance on corrosive chemicals presents safety concerns especially for domestic users. In contrast, microfluidic paper-based analytical devices (μPADs) have emerged as a promising alternative for rapid, cost-effective, and portable food quality assessment [30]. These devices utilize capillary-driven fluid flow within hydrophilic cellulose or nitrocellulose matrices, eliminating the need for external pumps and complex instrumentation. In their comprehensive review, Kulkarni et al. (2023) highlight the significant advancements and versatility of μPADs in biochemical sensing applications [31]. The authors emphasize that μPADs have gained traction due to their low cost, ease of fabrication, and suitability for point-of-care testing. They discuss various fabrication techniques, noting that while wax printing has been popular, newer methods like 3D printing offer promising alternatives. The review underscores the importance of fluid handling and control in μPADs, detailing both active and passive methods. In terms of detection, the authors point out that colorimetry, electrochemistry, and fluorescence are widely used techniques, each with its own advantages in sensitivity and ease of use. Notably, the paper illustrates the broad applicability of μPADs across healthcare, food safety, and environmental monitoring sectors [31].

Microfluidics, a rapidly advancing field focused on the manipulation of fluids within microscale channels, offers numerous advantages, including reduced reagent consumption, enhanced sensitivity, and shorter analysis times [32]. The fundamental principles governing microfluidic systems rely on fluid behavior at the microscale, where surface tension and capillary forces dominate over gravitational effects [32]. Among various microfluidic platforms, paper-based microfluidics has gained substantial attention due to its affordability, simplicity, and compatibility with colorimetric detection techniques [30–32]. μPADs employ hydrophilic networks to transport liquid samples, facilitating rapid, user-friendly assays with minimal operational requirements [30–32]. μPADs have demonstrated its effectiveness in colorimetric detection of micro-organisms, contaminants, antibiotics residues, and adulterants in milk such as E. coli and Staphylococcus aureus with their antibiotic-resistant strains [33], tetracyclines [34], sulfonamides [35], salt [36], hydrogen peroxide [37], glucose and sucrose [38], starch [39], urea [39, 40], and detergents [41].

Paper test cards are fabricated through wax printing to perform spot tests for detecting urea, different starch types, and sugars like glucose using hydrophilic detection spots [38]. Each test card is prepared to detect a specific adulterant by applying and drying appropriate colorimetric reagent before spotting milk on these paper test cards. Another study demonstrated the integration of a μPAD wax-printed onto a car-

ton with calibration color bars, enabling on-site qualitative detection of urea, protein, and nitrite in milk for milk quality test [42]. Authors discussed that their wax-printed μPAD are limited to offering qualitative visual assessments only. Guinati et al. (2023) present a μPAD for the detection of multiple adulterants including urea, hydrogen peroxide (H_2O_2), and pH in milk samples [43]. Their study demonstrated reproducibility, accuracy (91–102%), and strong agreement with reference techniques, making this μPAD a viable tool for rapid, and on-site milk quality screening. To fully realize the potential of μPADs for milk quality assessment, there is a critical need for developing accessible, low-cost, in-house fabrication methods. Additionally, μPADs should be designed to simultaneously detect and quantify multiple adulterants within a single test, streamlining the analysis process.

This study addresses these challenges by introducing an in-house fabricated, multiplexed microfluidic paper-based analytical device (M-μPAD) that leverages readily available materials such as filter paper and wax crayons. Unlike previous μPADs that often require specialized wax-printing technology, our approach significantly enhances accessibility and scalability by using a simple, low-cost fabrication method. This M-μPAD allows for the simultaneous detection of multiple milk adulterants (urea, starch, and detergent) with high accuracy and reproducibility, making it particularly suitable for resource-limited settings where conventional analytical techniques are inaccessible. Furthermore, the M-μPAD's ability to achieve comparable detection limits and linear response ranges to more complexly fabricated devices underscore its potential as a transformative tool for combating milk adulteration.

2 Materials and Methods

2.1 Materials and Reagents

AutoCAD was used to design the devices while HP LaserJet printer (Pro 400) was used to print the design outlines. For the μPAD, Whatman qualitative filter paper grade 1, sourced from Sigma, was chosen as the substrate for the M-μPAD due to its high purity, uniform pore size, and excellent hydrophilic properties. These characteristics are crucial for ensuring consistent capillary-driven fluid flow and reliable colorimetric detection in the device. Crayola wax crayons coloring was used for wax deposition and patterning hydrophobic barriers to form microfluidic channels. Hot plate (Stuart CB162) was used to heat the wax-colored filter paper to form hydrophobic boundaries of microfluidic channels. Ethanol (Merck), 4-Dimethylaminobenzaldehyde (4-DMAB) (Merck), Hydrochloric acid (Merck), Iodine (Sigma), Potassium iodide (Sigma), De-ionized water (Milli-Q), and Phenolphthalein (TCI) were used as reagents for adulteration testing. Laboratory-grade Urea (Sigma), commercial-grade urea sold as plant fertilizer, Rice Starch (Fauji Foods), and commercially available detergent were used as adulterants in ultra-heat-treated (UHT) milk (Nestle) for preparing standard sample solutions. While preparing samples, all the measurements were made using AL Electronic Analytical Balance model 2104. HP ScanJet Pro 3000 scanner was used for scanning and digital image acquisition after colorimetric

reactions. Table 1 shows the list of equipment and consumables used in the study, including vendor, model/type, and country of origin.

2.2 Fabrication of M-µPADs

Devices were designed using AutoCAD, as shown in Fig. 1 (a). Design outlines were printed on Whatman filter paper using LaserJet printer, as shown in Fig. 1 (b). For the calibration of an adulterant, µ-PAD consisting of 5 × 5 spot arrays with 0.9 cm spots were designed. For simultaneous detection of adulterants, multiplexed-µPADs (M-µPAD) with three detection zones are designed (Fig. 1 (e)). For wax deposition, the paper was colored using Crayola wax crayons as shown in Fig. 1 (c). For microfluidic channels preparation, the outlined filter paper was colored using wax crayons and heated on a hot plate at a temperature of 100 °C for two minutes for wax penetration into the filter paper micro-pores to form hydrophobic boundaries (Fig. 1 (d)). The paper was then dried for 5 min at room temperature to ensure no further spreading of wax. Figure 1 (f) shows the facile and affordable in-house fabricated multiplexed-µPAD for the simultaneous detection of adulterants.

2.3 Reagent Application on Detection Zones

The 4-DMAB reagent was prepared by dissolving 1.6 g of DMAB in 100 mL of ethyl alcohol and adding 10 mL of concentrated hydrochloric acid to detect urea. Iodine 0.01 N reagent was prepared by dissolving a mixture of 2.6 g of iodine and 3 g of potassium iodide (KI) in 200 mL of distilled water to detect starch. Phenolphthalein reagent was prepared by dissolving 0.05 g of phenolphthalein in 50 mL of 95% ethanol for detergent detection. The solution was then diluted to 100 mL with de-ionized

Table 1 List of equipment and consumables used in the study, including vendor, model/type, and country of origin

Consumable	Vendor	Model/Type	Country
AutoCAD Software	Autodesk	CAD	USA
LaserJet Printer	HP	Pro 400	USA
Filter Paper	Sigma	Grade 1	UK
Wax Crayons	Crayola	Commercial	USA
Hot Plate	Stuart	CB162	UK
Ethanol	Merck	Lab Grade	Germany
4-Dimethylaminobenzaldehyde	Merck	Lab Grade	Germany
Hydrochloric Acid	Merck	Lab Grade	Germany
Iodine	Sigma	Lab Grade	USA
Potassium Iodide	Sigma	Lab Grade	USA
De-ionized Water	Milli-Q	Lab Grade	USA
Phenolphthalein	TCI	Lab Grade	Japan
Urea (Lab-grade)	Sigma	Lab Grade	USA
Urea (Commercial)	Engro	Plant fertilizer	Pakistan
Rice Starch	Fauji Foods	Food Grade	Pakistan
Detergent	Commercial	Commercial	Pakistan
Electronic Balance	AL	2104	USA

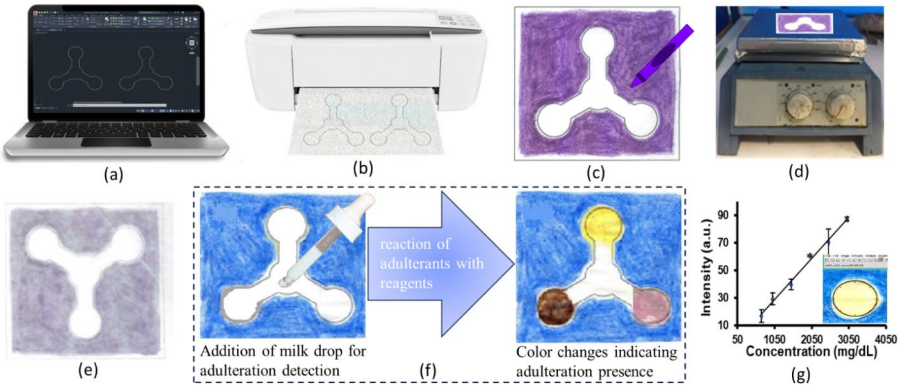

Fig. 1 The fabrication and working process of M-µPAD, (**a**) designing of M-µPAD in AutoCAD, (**b**) printing of microfluidic channel outlines on filter paper using LaserJet printer, (**c**) wax deposition through crayon colouring to create hydrophobic barriers around microfluidic channel, (**d**) heating of wax deposited on filter paper for strong hydrophobic barriers, (**e**) facile and in-house fabricated M-µPAD, and (**f**) simultaneous adulterants detection by simply dropping a milk drop on M–µPAD through colour changes after reaction of adulterants with reagents on different detection zones, and (**g**) analysing colorimetric images to determine different adulterant concentrations

(DI) water. Ethanol was distilled before its usage to make solutions. Fresh solutions were prepared when needed. 40 µL of each reagent was spotted on the respective detection zone/spot with the help of a micropipette with extra care wearing protective equipment. Once the reagent was applied, the devices were allowed to dry for 5 min and kept in airtight bags till usage to increase the shelf life.

2.4 Sample Preparation

UHT milk was chosen for the sample preparation due to its stability and extraordinary shelf life [44]. Standard laboratory grade and commercial urea solutions ranging from 10 to 7000 mg/dL were mixed with milk at room temperature to prepare milk adulteration with urea. Similarly, milk adulteration with detergent were prepared by mixing detergent solutions ranging from 100 to 9000 mg/dL with milk at room temperature. However, as gelatinization occurs between 55 °C and 85 °C [45]; therefore, the starch solutions ranging from 20 to 3000 mg/dL were boiled for 4 min using a heat-stir hotplate with milk to prepare milk adulteration with starch.

2.5 Colorimetric Reactions

For each milk sample, 80 µL was dispensed onto three separate spots using a micropipette to obtain an average reading for each adulterant at a specific concentration. A colorimetric reaction occurred between the reagent and the adulterant, resulting in a color change. These spots were allowed to dry for 5 min to ensure color saturation before image analysis. The spots spiked with different adulterants were allowed to dry for 5 min for color saturation before image analysis. The colorimetric reactions are schematically illustrated in Fig. 2. Urea detection relied on a reaction with DMAB

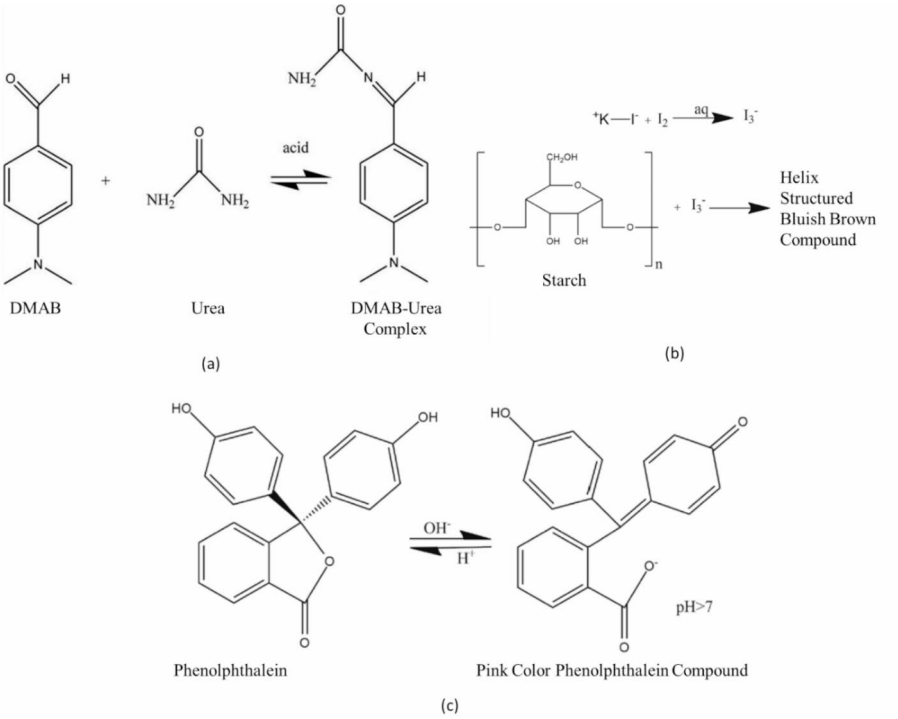

Fig. 2 Schematic illustration of colorimetric reactions of different adulterants with corresponding reagents, (**a**) urea with DMAB [46], (**b**) starch with iodine [47], and (**c**) detergent with phenolphthalein [48]

in an acidic environment (HCl), where the protonation of DMAB generates a charge-deficient carbonyl carbon. Urea, acting as a nucleophile, attacks this site to form a yellow urea-DMAB Schiff base complex $Urea + DMAB \xrightarrow{HCL} imine\ complex$, with color intensity increasing proportionally to urea concentration due to greater complex formation as presented in Fig. 2 (a) [46]. Spot tests with varying urea concentrations confirmed that higher concentrations correlated with increased yellow color intensity. For starch detection, iodine reagent (prepared with iodine and potassium iodide) interacted with the helical amylose component of starch, forming a charge-transfer complex where iodine molecules (I2) are encapsulated within the helix. This complex absorbs light at 600–620 nm, producing a bluish-brown precipitate (Fig. 2b) [47]. Interactions with milk lipids or proteins may slightly modulate the observed hue. In detergent detection, phenolphthalein was used as a pH-sensitive indicator. Detergents introduce hydroxide ions (OH-), increasing milk's alkalinity (pH > 8.2). This triggers phenolphthalein's structural transition from a colorless lactone form to a pink phenolate ion $Phenolphtahlein \xrightarrow{OH^-} Phenolate$, with intensity reflecting detergent concentration (Fig. 2c) [48]. When phenolphthalein ewas added to milk containing detergent, it turned pink, indicating the presence of hydroxide ions. The spots spiked with adulterants were allowed to dry for 5 min to ensure colour stabilization and saturation prior to image analysis.

2.6 Image Analysis

The μPADs were scanned after application of samples and colorimetric reactions after drying for 5 min. The quantitative analysis of the μPADs was performed using ImageJ software, and the average intensity of the desired areas was calculated using its circular area selecting tool. This average/mean intensity was the mean gray value of all the pixels, and the grey value of each pixel depicts the pixel's brightness. There was a negative control spotted for all three adulterants with pure milk, and the average intensity of negative control was subtracted from all measured sample values.

2.7 Calibration Curves and Blind Sample Quantification

Calibration curves for all the adulterants are plotted using the colour intensities vs. the varying concentrations of adulterant. Samples of known concentrations were used to develop the calibration curves. Negative control intensity was subtracted from all sample colour intensities. Parameters such as limit of detection (LOD), sensitivities and linear ranges were calculated. Quantitative analysis was conducted using classical linear regression fit curves. The colour intensity curves were generated from three repetitions of each concentration and taking the average, and the error bars indicate the standard deviation of the three repeated experiments for each concentration. The blind samples were quantified using these calibration curves. Blind samples could be visually identified if adulterated or not, qualitatively by comparing them with negative control. To quantify the level of adulteration: the background-subtracted mean intensity of a blind sample was compared to the calibration curve.

2.8 Quality Assurance and Data Analysis

The determination of the limit of detection (LOD) for the colorimetric assays on μPADs involved utilizing the residual standard deviation of the calibration curve and the y-intercepts of regression lines [39, 49]. Standard deviation and slope of the calibration response curve were derived through residual analysis. LOD values were computed using the equation $LOD_{calibration} = 3 \times (\sigma/m)$ [39, 49], where σ and m represent the standard deviation and slope of the calibration curve, respectively. All experiments were conducted in triplicate, and the results are presented as a mean ± standard deviation.

3 Results and Discussion

3.1 Quantification of Adulterants

The quantification of adulterants added to milk is a crucial aspect of ensuring its safety and quality. Calibration curves serve as fundamental tool in this process, allowing for the determination of the concentration of adulterants present in milk samples. We constructed these curves by systematically adding known quantities of adulterants to milk samples, establish a relationship between the concentration of

the adulterant and color intensity due to reaction between adulterants and reagents on μ-PAD arrays. These curves enable the subsequent quantification of unknown adulterant concentrations in blind milk samples by comparing their measured color intensities to the calibration curve.

The data obtained is then used to generate calibration curves for different adulterants (Figs. 3, 4 and 5) for quantitative analysis, conducting three experiments for each concentration of adulterants. The error bars in our results represent the standard deviation across these multiple experiments. For each adulterant color intensity curve, data points are included in fitting using classical linear regression model with $R^2 > 0.95$ as shown in insets of Figs. 3, 4 and 5. This gave the linear ranges for different adulterants to establish robust quantification metrics. Additionally, the limit of detection (LOD) for adulterants is determined from these curves, identifying the lowest detectable concentration.

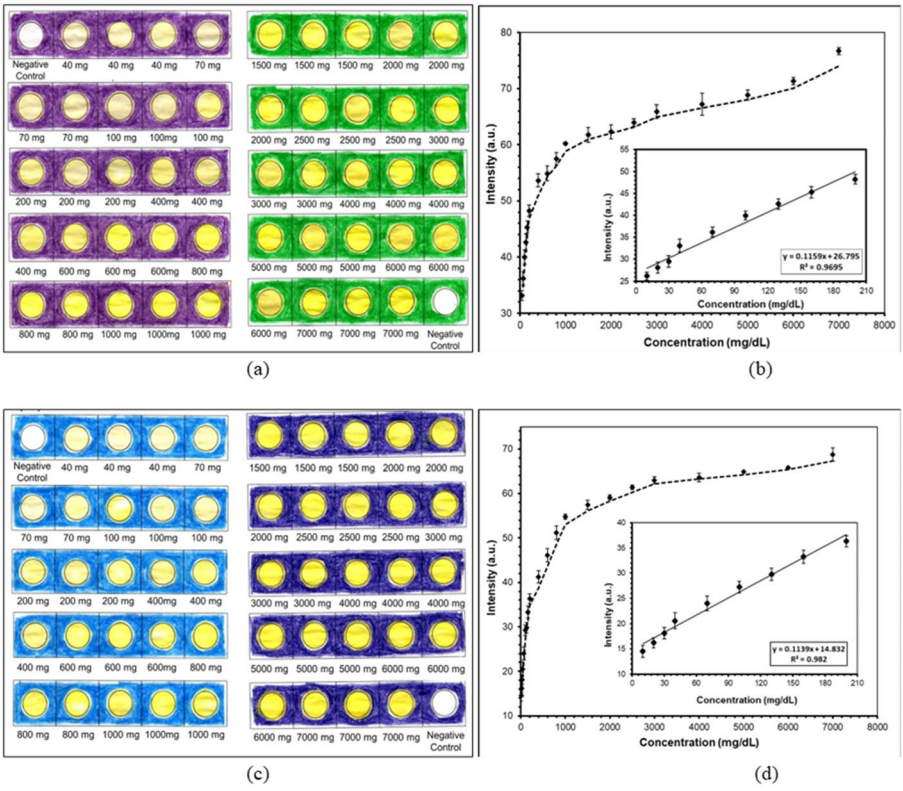

Fig. 3 Quantification of commercial- and laboratory-grade urea adulterated milk using DMAB based reagent, (**a**) spot test images of milk spiked with commercial-grade urea carried on μ-PAD spot arrays (5 × 5), (**b**) calibration curve prepared from spot tests of milk adulterated with commercial-grade urea, (**c**) spot test images of milk spiked with laboratory-grade urea carried on μ-PAD spot arrays (5 × 5), and (**d**) calibration curve prepared from spot tests of milk adulterated with laboratory-grade urea. Each data point is the average of 3 colour intensities for an adulterant concentration and the error bars indicate the standard deviations

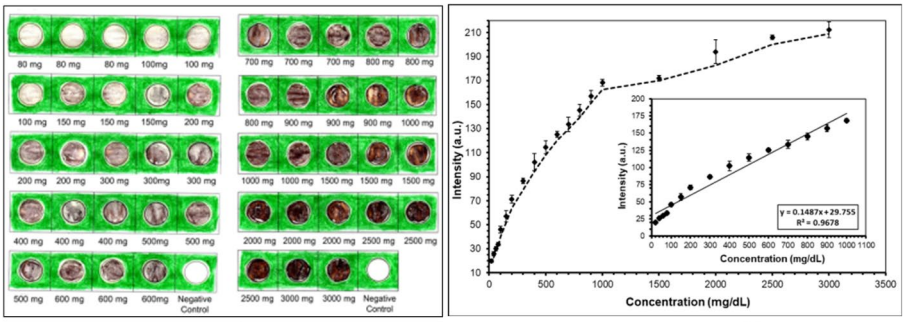

Fig. 4 Quantification of starch adulterated milk using iodine reagent, (**a**) spot test images of milk spiked with starch carried on µ-PAD spot arrays (5 × 5), and (**b**) calibration curve prepared from spot tests of milk adulterated with starch. Each data point is the average of 3 color intensities for an adulterant concentration and the error bars indicate the standard deviations

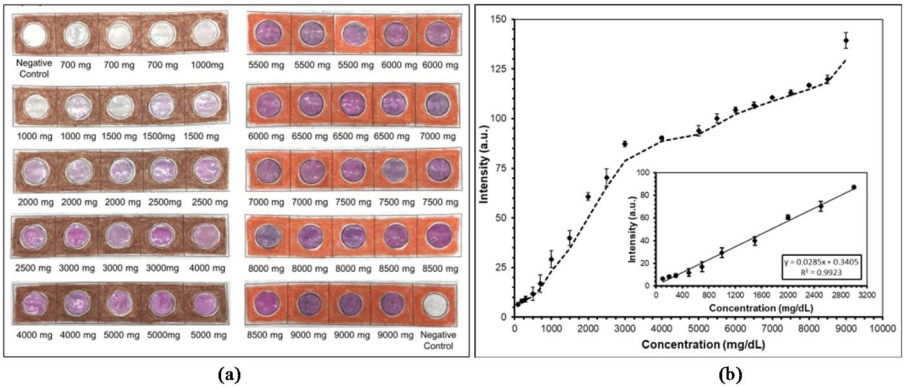

Fig. 5 Quantification of detergent adulterated milk using phenolphthalein reagent, (**a**) spot test images of milk spiked with detergent carried on µ-PAD spot arrays (5 × 5), and (**b**) calibration curve prepared from spot tests of milk adulterated with detergent. Each data point is the average of 3 color intensities for an adulterant concentration and the error bars indicate the standard deviations

3.1.1 Quantification of Urea

Protein constitutes 95 to 97% of the nitrogen content in milk, while approximately 3 to 5% of the milk's nitrogen comes from non-protein nitrogen sources like urea, uric acid, and creatine. Of this non-protein nitrogen, almost 50% is comprised of urea [38]. Nitrogen-rich urea is frequently added to milk to mask the addition of water, increase whiteness and non-protein nitrogen content and to regulate the Solid Not Fat (SNF) content [7]. Therefore, urea is a natural component of milk, and the amount naturally found in milk can be up to 70 mg/dL [9, 29]. Therefore, it is fine if there is up to 70 mg/dL of urea detected in milk. The urea concentration above this threshold would indicate that the milk has been adulterated and is unsafe for human consumption. Milk Urea Nitrogen test method is employed for the quantification of urea in milk on µ-PAD with 5 × 5 spot arrays. DMAB based reagent was used for the

colorimetric detection of urea. Urea forms a visible yellow complex with DMAB based reagent at room temperature. μ-PAD arrays spotted with DMAB based reagent were used for quantification of milk adulterated with urea concentration from 10 mg to 7000 mg. The concentration of 40 mg/dL of commercial-grade, and lab-grade urea resulted in a pale-yellow colour observable with the naked eye. There was a gradual increase in the intensity of the colour from pale yellow to yellow as the concentration of urea in the samples increased, indicating a proportional relation between the two. Such inspection can be used for semi-quantitation of urea with naked eye. Figure 3 shows the observational change in pale yellow to bright yellow intensity for spot tests of commercial-grade urea and laboratory-grade urea adulterated milk samples. ImageJ was used to perform intensity analysis of the μ-PAD spots after colorimetric reactions and preparation of concentration intensity calibration curve. Figure 3 (b) gives the calibration curve of commercial urea in milk which shows the colour intensity curve with changing concentrations of commercial urea. For the commercial urea, a linear behaviour was observed for 10–200 mg/dL range of the concentration of the commercial urea in milk as shown in Fig. 3 (b) inset. For commercial urea the sensitivity, resolution, and LOD were 0.12 a.u./mgdL^{-1}, 3.932 mg/dL, and 12. 8 mg/dL respectively. Figure 3 (c) shows the spot tests observational changes of colours for laboratory-grade urea. For the laboratory-grade urea, a linear behaviour was observed for 10–200 mg/dL range of the concentration of the laboratory-grade urea in milk as shown in Fig. 3 (d) inset. For lab grade urea the sensitivity, resolution, and LOD were 0.12 a.u./mgdL^{-1}, 2.270 mg/dL, and 11.1 mg/dL respectively.

3.1.2 Quantification of Starch

Starch, unlike urea, is not a natural constituent of milk, therefore, any level of starch is considered unacceptable [50]. Starch is added in milk to elevate the solid-not-fat content, consequently boosting the density of the diluted milk [7]. Consuming excessive starch can result in gastrointestinal issues such as diarrhea, as undigested starch accumulates in the colon [4, 7]. Starch is a biopolymer found in foods that we regularly consume, such as rice, vegetables, grains, cereals, etc. For our analysis, we used rice starch which is popular for adulteration as it is easily available commercially. To identify the presence of starch, individuals can employ iodine by boiling milk with an iodine solution [29]. Iodine forms a triiodide ion complex in the presence of iodide; triiodide is soluble in water, prevent the loss of iodine through sublimation and can detect very low concentrations of starch [50]. Triiodide forms a complex with the helical coil structure of starch, and result in bluish brown precipitate [50]. μ-PAD spot arrays with iodine reagent were used for quantification of milk adulterated with starch concentration ranging from 20 mg to 3000 mg. The concentration of 80 mg/dL of starch resulted in a light brown color observable with the naked eye and there was a gradual increase in the intensity of the color from light brown to brown and dark brown as the concentration of starch in the milk samples increased as shown in Fig. 4 (a). Figure 4 (b) gives the calibration curve of starch which shows the color intensity with changing concentrations of starch. For the starch, a linear behavior was observed for 80–1000 mg/dL range of the concentration of the starch in milk as shown in Fig. 4 (b) inset. By using ImageJ, calibration curves were plotted and the

calculated values of sensitivity, resolution, and LOD were 0.15 a.u./mgdL^{-1}, 3.13 mg/dL, and 9.39 mg/dL, respectively.

3.1.3 Quantification of Detergent

Detergent is a completely foreign substance commonly added to milk as an adulterant to enhance its appearance and mask the addition of other adulterants [7]. Consumption of detergent in milk can lead gastrointestinal problem, food poisoning and many other related diseases [4, 7]. For its detection, the phenolphthalein indicator was used as a reagent. A colorimetric reaction resulted in the formation of pink color in the presence of detergent in milk. µ-PAD spot arrays with phenolphthalein reagent were used for quantification of milk adulterated with detergent concentration ranging from 100 mg to 9000 mg. The concentration of 700 mg/dL of detergent is visual LOD as it resulted in a light pink color observable with the naked eye and there was a gradual increase in the intensity of the color toward dark pink as the concentration of detergent in the milk samples increased as shown in Fig. 5 (a). Figure 5 (b) gives the calibration curve of detergent which shows the color intensity curve with changing concentrations of detergent. For the detergent, a linear behavior was observed for 100–3000 mg/dL range of the concentration of the detergent in milk as shown in Fig. 5 (b) inset. By using ImageJ, calibration curves were plotted and the calculated values of sensitivity, resolution, and LOD were 0.03 a.u./mgdL^{-1}, 2.270 mg/dL, and 120.8 mg/dL, respectively.

3.1.4 Specificity Test

The specificity of the M-µPAD was rigorously assessed by evaluating its capacity to selectively detect urea, starch, and detergent without interference from other milk components. This involved utilizing specific reagents for each adulterant: 4-DMAB for urea, iodine for starch, and phenolphthalein for detergent. We evaluated the analytical response for each analyte in the presence of the other target compounds. This was achieved by spiking milk samples with individual analytes at three different concentrations and subsequently adding the other two adulterants at fixed levels as shown in Fig. 6. By comparing the colorimetric readouts of these samples with those containing only the target analyte, we determined if the presence of other compounds affected the analytical response. Statistical analysis showed no significant differences between the means, indicating that no cross-reactions occurred between the colorimetric reactions and confirming the high specificity of our device.

3.2 Simultaneous Analysis of Blind Samples for Multiple Adulterants

The individual calibration curves of urea, starch, and detergent adulteration provide a standard for analyzing blind samples of multiple adulterants simultaneously on M-µPAD with three detection zones. The capability of the M-µPAD to simultaneously quantify three adulterants in milk was tested by analyzing eight blind milk samples spiked with all three adulterants together with different concentrations shown in Table 2. The blind milk samples were spotted on the sampling zone of the

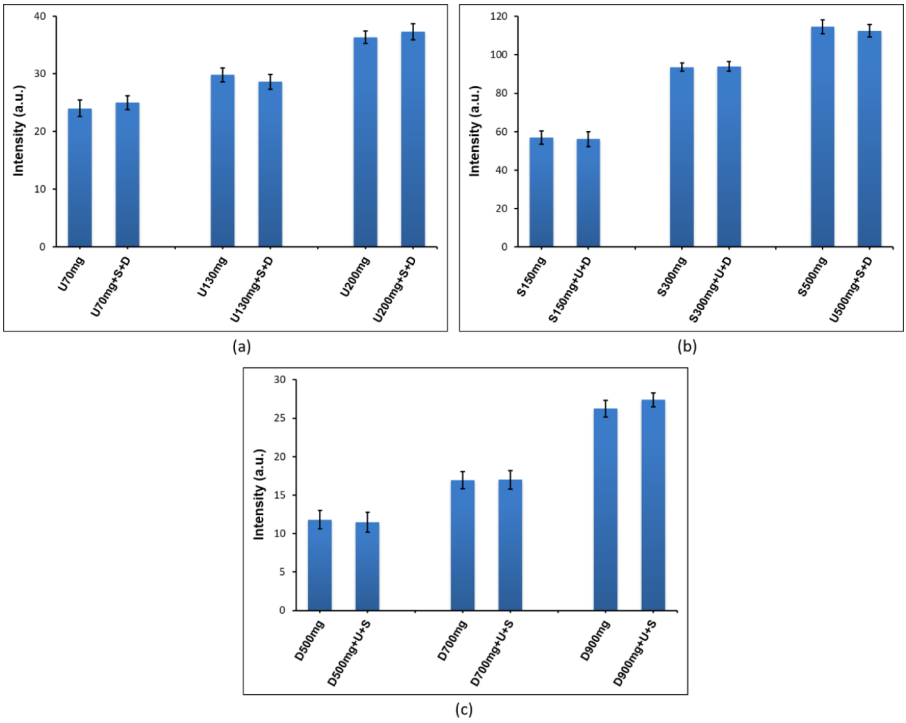

Fig. 6 Evaluation of M-µPAD specificity for milk samples spiked with (**a**) urea, (**b**) starch, and (**c**) detergent at three concentrations, and then with the other two adulterants at fixed concentrations

M-µPAD, allowing the milk to flow through microfluidic channels towards the detection zones by capillary action. Within these detection zones, the adulterants present in the milk reacted with specific reagents, displaying colorimetric changes as shown in Fig. 7. Quantitative analysis of the adulterant in the blind samples was conducted using ImageJ software by utilizing scanned images of detection zones to measure the colour intensity. To determine the extent of adulteration, the background-subtracted intensity of each adulterant in the blind sample was compared to the corresponding calibration curve for the specific adulterant.

Table 2 shows the accuracy of the simultaneous adulteration detection on M-µPAD by comparing the actual added adulterant quantities with the detected adulterant quantities from the calibration curve. Accuracy is determined through the recovery rate (RT) which is calculated as RT = (Recovered amount/ Added amount) × 100% [34, 43]. Each blind sample is tested on 5 M-µPAD devices having same dimensions and fabrication conditions under same protocol. The adulterant recovered/detected amount reported in Table 2 is the mean of adulterant recovered amount determined on 5 M-µPAD devices with same blind sample. The multiplexed device demonstrated recovery rates of 99.7–105.6% for urea, 95–104.3% for starch and 95.75–104.8% for detergent in blind milk samples. We have achieved accuracy of 95% and more for simultaneous detection of adulterants on M-µPAD by employing colorimetric assay.

Table 2 Quantitative detection of adulterants simultaneously in blind milk samples on M- MPAD

Blind Samples		Added Amount (mg/dL)	Recovered/Detected Amount (mg/dL)	Recovery Rate (RT) (%)	Relative Standard Deviation (RSD) (%)
S1	Urea	7000	7045	100.6	0.53
	Starch	500	521	104.2	3.87
	Detergent	500	524	104.8	2.67
S2	Urea	4000	4098	102.4	2.16
	Starch	600	626	104.3	2.72
	Detergent	400	383	95.75	1.19
S3	Urea	2000	2113	105.6	2.43
	Starch	1000	1010	101.0	3.56
	Detergent	300	308	102.7	2.04
S4	Urea	600	598	99.7	1.42
	Starch	1500	1466	97.7	2.39
	Detergent	200	210	105	3.44
S5	Urea	10	10.3	103	1.99
	Starch	50	48	96	4.95
	Detergent	7000	7189	102.7	1.55
S6	Urea	20	20.8	104	2.2
	Starch	100	96	96.0	3.76
	Detergent	6000	5991	99.8	0.20
S7	Urea	30	30.1	100.3	1.5
	Starch	150	144	96.0	2.18
	Detergent	5000	4852	97.0	1.76
S8	Urea	40	41	102.5	2.05
	Starch	200	190	95.0	2.98
	Detergent	3000	2958	98.6	1.50

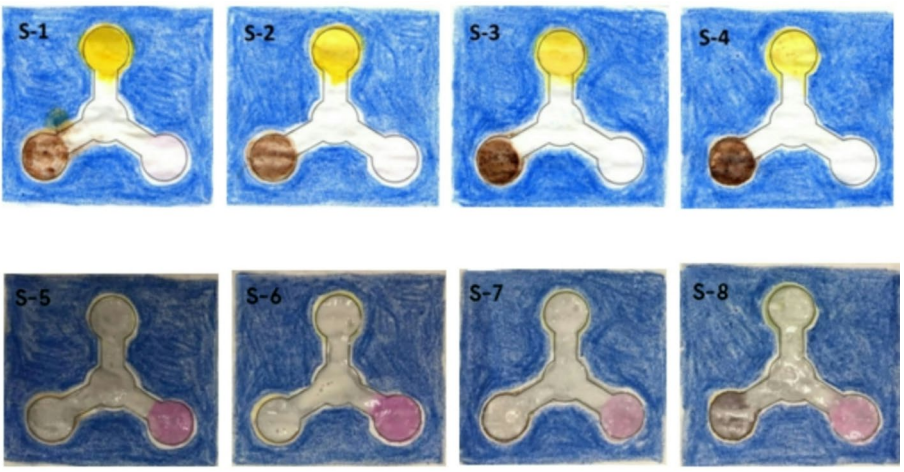

Fig. 7 Images showing simultaneous colorimetric assays conducted on M-µPADs for urea, starch, and detergent in eight blind milk samples with concentrations shown in Table 2

To investigate the reproducibility, each simultaneous colorimetric assay with a blind sample is conducted on five M-μPAD devices with same dimensions under same assay conditions. The reproducibility is investigated through relative standard deviation (RSD) % obtained through colorimetric assays conducted on five M-μPAD devices for a blind milk sample under same assay conditions. The M-μPAD devices showed good reproducibility from 0.20 to 3.87% for adulterants in blind milk samples across multiple experiments as shown in Table 2.

The storage stability of the M-μPAD is a critical factor for its practical application. Our preliminary results show that devices stored at room temperature maintain over 90% of their initial detection accuracy after one week, while refrigeration helps retain over 95% accuracy for two weeks. However, for long-term storage, further evaluations are necessary to ensure the device's reliability. This would involve assessing performance over extended periods and exploring packaging strategies to enhance shelf life.

Our study compares the performance of the M-μPAD with existing μPADs that employ colorimetric assays for milk adulterants, as summarized in Table 3. While the literature showcases μPADs with promising capabilities, many involve complex and costly fabrication methods, such as PDMS stamping and wax printing. In contrast, our M-μPAD is fabricated using a facile, affordable, in-house approach, eliminating the need for specialized equipment. A critical limitation of many reported μPADs is the absence of detailed information regarding linear detection ranges, hindering quantitative analysis. Our study addresses this gap by providing comprehensive calibration curves and identifying linear detection ranges for each adulterant. Moreover, the M-μPAD excels in simultaneous detection and quantification of multiple adulterants by sample application on a single device in single test surpassing the capabilities of many existing μPADs that often require separate devices or tests for different adulterants. Moreover, the M-μPAD exhibits comparable performance in terms of accuracy, reproducibility, and resource efficiency compared to more complex μPADs. Future directions for this research include addressing several key challenges. Firstly, enhancing the sensitivity of the M-μPAD to detect trace levels of adulterants is essential. Additionally, studies should focus on minimizing interference from milk components and improving reagent stability. Developing user-friendly training materials and exploring scalable manufacturing techniques will also be important. Finally, comparative studies with advanced analytical methods will help establish the reliability and accuracy of the M-μPAD in a broader analytical context.

4 Conclusion

In this study, we have successfully developed a facile and affordable multiplexed microfluidic paper-based analytical device (M-μPAD) for the rapid and simultaneous detection and quantification of multiple milk adulterants. By leveraging readily available materials and a simple fabrication process, the M-μPAD overcomes the limitations of traditional μPADs, which often require specialized equipment and complex procedures. The device demonstrates high accuracy, reproducibility, and sensitivity in detecting milk adulterants simultaneously, making it a valuable tool for

Table 3 Comparison of the performance of different (μ-PADs) using colorimetric detection of multiple adulterants in milk

Ref.	Fabrication Method	Device Reproducibility (RSD%)	Reagent Per Spot/Zone	Milk Sample Per Spot/Zone	Colorimetric Rection Time	Adulterants Quantification	Simultaneous Detection	Blind Samples Accuracy (%)
[36]	PDMS Stamping	NR	NR	NR	5 days	Yes	No	NR
[38]	Wax Printing	NR	7.5–35 μL	115–130 μL	40 min	Yes	No	≥83%
[39]	Wax Printing	NR	3–15 μL	5–10 μL	45–60 min	Yes	No	NR
[42]	Wax Printing	NR	20–25 μL	30–50 μL	10 min	No	Yes	NR
[43]	Cutter Printer– Laminated Paper With EVA-Coated Polyester	≤6%	0.5 μL	10 SμL	5 min	Yes	Yes	≥91%
[51]	Wax Printing	NR	0.5-1-25 μL	10–20 μL	NR	No	Yes	NR
This Work	Wax Crayons Coloring	<4%	40 μL	80 μL	5 min	Yes	Yes	≥95%

ensuring milk quality and safety, particularly in resource-constrained settings. The M-µPAD's ability to provide quantitative results and its potential for further optimization highlight its significance in addressing the critical issue of milk adulteration. Future research can explore the expansion of the M-µPAD's capabilities to include additional adulterants and contaminants, as well as the integration of other analytical techniques for enhanced performance.

Acknowledgments Open Access funding provided by the Qatar National Library (QNL).

Author Contributions Jannat Ahsan and Hudair Samad worked on devising Methodology, Fabrication, carry on Investigation and Analysis and Writing– original draft; Kashif Riaz worked on Conceptualization, Resources, Supervision, and Writing– review & editing; Muhammad Qasim Mehmood worked on Validation, and Writing– review & editing; Abdelkrim Khelif worked on Project administration, Supervision, and finalizing manuscript.

Funding Open Access funding provided by the Qatar National Library.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing Interests The authors declare no competing interests.

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