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Development of a Plant-Based Dual-Zone Colorimetric Strip for Rapid Screening of Urea and Alkaline Adulteration in Milk

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Milk adulteration represents a critical global food safety issue, as illegal additions of synthetic chemicals and neutralizers compromise nutritional quality and pose severe human health risks. Among common adulterants, urea and alkaline neutralizers (such as caustic soda, sodium bicarbonate, or carbonate) are added to artificially inflate apparent protein content and prevent souring. To address the need for simple, low-cost point-of-care testing, this study developed a plant-based dual-zone colorimetric paper strip for the simultaneous screening of urea and alkaline adulterants in milk. The screening device utilizes Whatman filter paper partitioned by a paraffin wax barrier to prevent cross-contamination. Zone A is functionalized with crude soybean (Glycine max) extract as a natural source of urease enzyme integrated with turmeric-derived curcumin (Curcuma longa), while Zone B contains curcumin alone as a pH-responsive indicator. Enzymatic hydrolysis of urea in Zone A generates ammonia, causing local alkalinization and a distinct visual color change from yellow to reddish-brown. Zone B responds directly to elevated pH (> 8.0) resulting from alkaline adulteration. Under optimized conditions, the test strip exhibited a rapid visual response within 30–60 seconds using a sample volume of 20-30 μL. Evaluation across 30 field and commercial milk samples demonstrated presumptive positive screening in 13 samples (43.3%), with no false-positive color changes observed in negative pure milk controls. While the curcumin indicator effectively screens for alkaline conditions, it does not distinguish specific sodium hydroxide from other basic adulterants. This eco-friendly, equipment-free paper strip provides a promising preliminary screening platform for dairy quality monitoring, though quantitative validation against gold-standard laboratory methods (such as HPLC/FTIR) and broader matrix interference testing remain necessary before field deployment.

Keywords: Alkaline adulteration, Curcumin pH indicator, Dual-zone colorimetric sensor, Milk adulteration screening, Paper-based analytical device, Soybean urease.

Background

Milk is an essential nutrient-dense food consumed worldwide, providing high-quality proteins, lipids, vitamins, and essential minerals. However, intentional milk adulteration remains a persistent food safety problem, particularly in developing and resource-limited regions. Unscrupulous suppliers frequently add synthetic chemical compounds, neutralizers, detergents, and volume expanders to maximize profit margins and extend shelf stability. Among these, urea is

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commonly added to artificially elevate non-protein nitrogen (NPN) readings during routine Kjeldahl or Dumas protein testing, while alkaline substances—such as caustic soda (sodium hydroxide, NaOH), sodium carbonate, or sodium bicarbonate—are added as neutralizers to mask souring caused by bacterial acid production.[3,7]

Chronic exposure to these chemical adulterants carries substantial health consequences. Excess urea intake disrupts renal physiology and metabolic homeostasis, while caustic soda and alkaline neutralizers can cause severe gastrointestinal irritation, mucosal erosion, and systemic electrolyte imbalance. Consequently, rapid, reliable, and accessible analytical screening tools are urgently needed to monitor milk quality across dairy supply chains.[2,5]

Problem Statement & Novelty

Standard laboratory techniques for detecting milk adulteration—including High-Performance Liquid Chromatography (HPLC), Fourier-Transform Infrared Spectroscopy (FTIR), Near-Infrared (NIR) spectroscopy, and electrochemical sensors—offer high sensitivity and/or specificity depending on the analyte and method. However, these approaches generally require instrumentation, trained personnel, controlled analytical conditions, and, in many cases, sample preparation, which can limit routine decentralized testing. Recent work on dairy-safety sensors and paper-based platforms likewise emphasizes the continuing need to balance analytical performance with portability, simplicity, and field usability.[4,5,6,8,18]

Although various paper-based analytical devices (μPADs) and colorimetric dipsticks have been developed for food and dairy screening, their analytical scope, reagent integration, multiplexing capability, and field-readout requirements vary considerably. Multi-analyte μPAD platforms have demonstrated the feasibility of simultaneous adulterant screening, while recent reviews of dairy food-safety sensors emphasize continuing challenges related to selectivity, matrix effects, robustness, and practical field deployment.[16,18] Other paper-based urea assays further support the feasibility of rapid colorimetric screening approaches for urea-related analysis.[17,20] These developments highlight the value of a simple, visually interpretable platform that combines more than one screening function while remaining suitable for decentralized use. To address these considerations, the present study establishes a simple, plant-based dual-zone colorimetric paper strip utilizing crude soybean (Glycine max) urease and turmeric-derived (Curcuma longa) curcumin. The primary novelty of this work lies in the green integration of plant-derived enzymatic and colorimetric reagents onto a single wax-partitioned filter-paper substrate, enabling simultaneous preliminary screening of urea and alkaline adulterants without electrical equipment or instrument-based readout.[1,12,13,15]

Study Objectives

The specific objectives of this study were to:

• Develop a low-cost, eco-friendly dual-zone paper strip for milk adulteration screening.

• Utilize natural soybean (Glycine max) extract as a bio-source of urease and turmeric (Curcuma longa) curcumin as a pH-responsive colorimetric indicator.[10,11]

• Implement a paraffin wax hydrophobic barrier to achieve effective zone isolation and prevent reagent cross-contamination.

• Evaluate the visual response time, qualitative detection capability, and preliminary performance on commercial and raw field milk samples.

MATERIALS AND METHODS

Reagents and Plant Materials

Whatman No. 1 filter paper was used as the paper substrate for strip fabrication. Fresh turmeric rhizomes (Curcuma longa) and dry soybean seeds (Glycine max) were procured from local agricultural suppliers. Analytical-grade ethanol (99.5%), urea (NH_2CONH_2), sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$), and paraffin wax were purchased from standard chemical suppliers. All aqueous solutions were prepared using distilled water.

Fabrication of Dual-Zone Colorimetric Strips

Step 1: Preparation of Turmeric Curcumin Indicator Paper

Fresh turmeric rhizomes were cleaned, shade-dried, and finely pulverized. Curcuminoid-rich extract was prepared by dissolving 10 g of pulverized turmeric powder in 50 mL of 99.5% ethanol under magnetic stirring for 2 hours at

room temperature. The extract was filtered through Whatman No. 1 paper. Sheets of filter paper were immersed in the ethanolic extract until uniformly saturated, air-dried in a dust-free, light-protected chamber, and cut into individual strips (5.0 cm × 0.8 cm). The use of turmeric ethanol extract immobilized on filter paper is consistent with previously reported turmeric-based pH-indicator paper preparation.[10]

Step 2: Preparation of Crude Soybean Urease Extract

Soybean seeds (Glycine max) were ground into a fine powder. A 20% (w/v) crude enzyme suspension was prepared by dispersing 1.0 g of soybean powder in 5.0 mL of distilled water. The mixture was stirred thoroughly for 15 minutes and filtered to yield a crude aqueous urease extract. Soybean is a recognized biological source of urease, supporting its use as the enzyme source in this preliminary strip format.[11] To improve reagent adhesion and sample wicking, a small proportion of lentil protein/starch extract was incorporated as a natural biopolymeric binder. Because this binder formulation was developed experimentally in the present study, its proportion and performance should be regarded as formulation parameters rather than as a standardized literature method.

Step 3: Hydrophobic Partitioning and Zone Functionalization

A central hydrophobic barrier line (2 mm width) was applied across the middle of each curcumin paper strip using melted paraffin wax to divide the strip into two distinct testing zones (Zone A and Zone B). Hydrophobic patterning and physical compartmentalization are established design principles in paper-based analytical devices for controlling fluid movement and minimizing inter-zone interference.[13,16] Zone A (Urea Screening Zone) was functionalized by applying 10 µL of the crude soybean urease extract onto the curcumin paper substrate and drying thoroughly. Zone B (Alkaline Adulteration Zone) was left as plain curcumin paper without enzyme modification. The prepared dual-zone strips were stored in sealed amber containers with silica-gel desiccants at 4–8 °C until use. The selected application volume and storage conditions were the experimental conditions used in this study; they should not be interpreted as fully optimized or stability-validated parameters.

Sample Collection and Testing Protocol

A total of 30 milk samples were collected from various local sources: raw farm milk (n = 10), packaged commercial milk (n = 10), reconstituted commercial milk powder (n = 5), and human mother's milk (n = 5, utilized as an unadulterated biological reference control). For testing, a single 20–30 µL droplet of milk sample was deposited onto Zone A and Zone B simultaneously. Color changes were observed visually under ambient light within a standardized readout window of 30–60 seconds. Rapid visual urea screening on paper has been reported previously, supporting the use of a short visual readout format in preliminary testing.[17,20] The present sample volume and 30–60-second observation window were the conditions selected for this study and were not established as universal operating specifications.

Chemical Detection Mechanisms

Alkaline Adulteration Detection Mechanism (Zone B)

Curcumin exists in a keto-enol tautomeric equilibrium and exhibits pH-dependent optical behavior. In acidic or near-neutral media, such as normal unadulterated milk (pH ~6.6), turmeric-derived curcumin remains predominantly in its yellow form, whereas alkaline conditions produce a visible shift toward reddish-brown coloration. Turmeric-based filter-paper pH indicators and recent curcumin-based colorimetric sensor studies support the use of this response for qualitative alkaline screening.[9,10,14] When an alkaline adulterant (e.g., NaOH, $NaHCO_3$, Na_2CO_3) is present, the elevated pH promotes deprotonation of curcumin and alters its electronic structure, producing the observed color transition.[9,14]

Scientific Clarification: Because curcumin responds to elevated pH in general, Zone B functions as an indicator of general alkaline adulteration (or neutralizing agents) rather than providing chemical specificity exclusively for sodium hydroxide (NaOH).

Urea Detection Mechanism (Zone A)

Zone A contains both active soybean urease and curcumin indicator. When milk containing urea is applied to Zone A, urease catalyzes the hydrolysis of urea into ammonia and carbon dioxide according to Reaction (1). The use of soybean urease as a biological source of urease is well documented,[11] and paper-based urea detection

has been demonstrated in previous strip-format studies.[17,20]

$$NH_2CONH_2 + H_2O \text{ — (Urease) —> } 2\ NH_3 + CO_2$$

The generated ammonia dissolves in the sample matrix to form ammonium hydroxide (NH_4OH), raising the local micro-environmental pH above 8.0. This localized pH rise triggers the deprotonation of adjacent curcumin molecules, resulting in a yellow-to-reddish-brown color change in Zone A. In unadulterated milk lacking added urea, no ammonia is generated, and Zone A remains yellow.

RESULTS

Colorimetric Response and Kinetics

The performance of the dual-zone paper strip was evaluated by monitoring visual color development following sample application. In unadulterated control milk samples (pH ~6.6), both Zone A and Zone B maintained their original yellow appearance throughout the observation period. In contrast, milk samples containing alkaline adulterants or spiked urea demonstrated rapid visual color transitions from yellow to reddish-brown within 30–60 seconds. The paraffin wax barrier effectively restricted liquid migration between zones, preventing cross-contamination during testing.

Real-Sample Screening Analysis

A total of 30 field and commercial milk samples were screened using the dual-zone paper strips. Out of 30 samples tested, 13 samples yielded positive colorimetric reactions, representing a 43.3% presumptive positive screening rate within the collected sample set. Table 3 presents the qualitative colorimetric responses and sample classification for representative milk categories.

Method Controls and Repeatability

To evaluate method fidelity, negative controls (unadulterated milk) and positive controls (milk spiked with 100 mg/dL urea and 0.05 M NaOH) were tested in triplicate. No false-positive color changes were observed in any negative control samples under the experimental testing conditions. Repeatability trials across five distinct strip batches showed consistent visual

Table 1. Comparative overview of standard instrumental methods versus the proposed plant-based dual-zone screening strip.

Parameter	Standard Instrumental Methods (FTIR / HPLC / Sensors)	Developed Dual-Zone Screening Strip
Primary Purpose	Quantitative verification & definitive analysis	Rapid qualitative screening at point-of-care
Analyte Capability	Multi-component quantitative spectrum	Dual screening (Urea & Alkaline adulterants)
Reagent Source	Synthetic reagents & certified standards	Plant-derived bio-reagents (Soybean & Turmeric)
Capital Cost	Generally high; instrument-dependent	Low-cost consumables; exact unit cost requires formal costing
Instrumentation Needed	Complex spectroscopic/chromatographic units	None (Equipment-free visual readout)
Technical Expertise	Requires trained laboratory technicians	Simple; usable by farmers and field operators
Analysis Time	Method- and batch-dependent	30–60 seconds under study conditions
Portability	Stationary / limited field portability	Highly portable, lightweight paper strips
Environmental Footprint	Chemical waste generation	Biodegradable, eco-friendly green materials
Field Applicability	Centralized laboratory settings	Direct on-site and field screening
Detection Principle	Absorbance / Chromatographic retention	Colorimetric pH & enzymatic reaction on paper

Table 2. Reagents, biological sources, and their specific functional roles in the dual-zone screening strip

Component / Material	Chemical / Biological Property	Functional Role in Dual-Zone Strip
Turmeric (Curcumin)	Natural polyphenolic pH indicator (keto-enol tautomer)	Colorimetric sensor for alkaline pH (Yellow to Reddish-brown)
Soybean Extract (Glycine max)	Rich source of active urease enzyme	Catalyzes urea hydrolysis to produce alkaline ammonia (NH_3)
Lentil Protein / Starch Matrix	Natural biopolymer & protein binder	Enhances reagent adhesion to paper fibers and aids capillary flow
Ethanol (99.5%)	Organic solvent	Extracts active curcuminoids from pulverized turmeric rhizomes
Filter Paper (Whatman No. 1)	High porosity & capillary wicking	Solid substrate and carrier for visual colorimetric reactions
Paraffin Wax	Hydrophobic water-repellent barrier	Forms physical boundary line preventing cross-talk between Zone A & B

Table 3. Qualitative screening results of representative milk samples using the dual-zone colorimetric paper strip

Sample Category	Zone A (Urea Zone)	Zone B (Alkaline Zone)	Observed Color Response	Presumptive Status
Packaged Commercial Milk	Negative (-)	Positive (+)	Zone A: Yellow | Zone B: Reddish-brown	Presumptive Adulterated (Alkaline)
Raw Dairy Farm Milk	Positive (+)	Negative (-)	Zone A: Reddish-brown | Zone B: Yellow	Presumptive Adulterated (Urea)
Human Mother's Milk (Control)	Negative (-)	Negative (-)	Zone A: Yellow | Zone B: Yellow	Pure / Unadulterated Control
Reconstituted Milk Powder	Negative (-)	Negative (-)	Zone A: Yellow | Zone B: Yellow	Pure / Unadulterated Control

color development, demonstrating satisfactory fabrication reproducibility for qualitative screening.

DISCUSSION

Critical Interpretation of Specificity & Matrix Effects

A critical scientific consideration in evaluating paper-based colorimetric sensors is distinguishing general chemical reactions from analyte-specific detection. Multi-analyte μPADs demonstrate that paper platforms can support simultaneous screening, but the interpretation of each signal remains dependent on the underlying chemistry and selectivity of the detection reagent.[16] In this study, Zone B utilizes curcumin, which responds to elevated pH. Therefore, a positive response in Zone B indicates elevated alkalinity or the presence of basic neutralizers (e.g., NaOH, Na_2CO_3, $NaHCO_3$, detergents) rather than proving exclusive specificity for sodium hydroxide. Describing this response as 'alkaline adulteration screening' accurately reflects the underlying chemical mechanism.[14,18]

Similarly, while Zone A employs soybean urease to hydrolyze urea into basic ammonia, potential matrix interferences must be acknowledged. Natural milk variations—including buffering capacity, protein composition, and endogenous nitrogenous constituents—can influence the magnitude and speed of color development. Previous work on milk-adulterant detection and paper-based urea assays supports the need for appropriate controls and further selectivity evaluation before analytical claims are generalized.[2,17,20] Future studies must evaluate potential cross-interferents, including nitrogenous compounds and non-target salts, to define a comprehensive selectivity profile.

Reagent Stability and Environmental Sensitivities

Curcumin is susceptible to photodegradation under prolonged light exposure, which can reduce the chemical stability of turmeric-derived colorimetric materials.[19] Furthermore, crude plant enzymes such as soybean urease may be affected by temperature, moisture, and storage conditions. To mitigate these potential environmental sensitivities, the developed strips were stored in light-shielded amber containers containing desiccant packs at 4–8 °C. However, the present study did not establish a formal shelf-life, accelerated-stability profile, or residual enzyme-activity study. Therefore, these storage conditions should be considered interim preservation conditions rather than a validated shelf-life specification. Formulation optimization, including protective biopolymeric matrices or enzyme stabilizers, will be important for future room-temperature stability studies.

Method Scope and Future Directions

While plant-derived urease sensors have theoretical relevance in clinical diagnostic screening (e.g., salivary urea monitoring) or environmental testing (e.g., soil/groundwater fertilizer runoff), the experimental scope of this study was strictly restricted to qualitative screening of milk adulteration. The observed 43.3% presumptive positivity rate among collected real samples represents localized preliminary screening data and should not be extrapolated as a population-level prevalence estimate for commercial dairy supply chains.

To transition this proof-of-concept strip into a fully validated analytical method, future research must focus on: (1) Determining quantitative analytical limits of detection (LOD) and quantification (LOQ); (2) Integrating smartphone-based image analysis (RGB/HSV colorimetry) for objective color quantification; (3) Performing rigorous cross-validation against gold-standard laboratory reference methods such as HPLC and FTIR; and (4) Conducting large-scale field validation studies across diverse milk matrices.

CONCLUSION

This study demonstrated a plant-based dual-zone colorimetric paper strip for rapid preliminary screening of urea and alkaline adulterants in milk. By integrating crude soybean urease extract with turmeric-derived curcumin on a wax-partitioned filter-paper substrate, an equipment-free visual screening format was achieved. The test strip produced a visual color response within 30–60 seconds under the study conditions, with no false-positive responses observed in the tested negative controls. The curcumin indicator screens for general alkaline

conditions resulting from basic adulterants, while Zone A provides a qualitative enzymatic response associated with urea. Overall, the platform represents a proof-of-concept approach for accessible preliminary milk screening; quantitative validation, broader interference testing, stability studies, and comparison with validated reference methods are required before routine field or regulatory use.

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Conflict of interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This study was primarily an in vitro laboratory-based evaluation of milk quality. No animal experimentation or human clinical intervention was performed.

Informed Consent Statement

Human mother's milk was used as a reference control sample; therefore, the authors should ensure that its collection and use complied with applicable institutional requirements and donor-consent procedures.

Clinical Trial Registration

This research does not involve any clinical trials.

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Not Applicable.

Author Contributions

Priyanka Gaikwad: Conceptualized the project, designed the methodology, performed experiments, analyzed the data, and drafted the original manuscript; Roma Sharma: Supervised, reviewed, and critically revised the manuscript; Tanvi Kisan Dond: Sample collection, reagent preparation, experimental work, and data organization; Bushra Sayyed: Sample collection, reagent preparation, experimental work, and data organization; Snehal Dhananjay Jadhav: Sample collection, reagent preparation, experimental work, and data organization

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