Analytical Methods

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
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Cite this: *Anal. Methods*, 2025, **17**, 1265

Development of a novel and affordable point-of-care kit for rapid detection of urea and glucose adulteration in cow milk

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The increasing global population has raised the demand for cow milk, leading to its adulteration with harmful substances, including urea and glucose, that cause damage to humans when consumed regularly. Hence, this study started with predicting urea and glucose toxicity using ProTox-III software, wherein the results revealed that urea belongs to class IV with an LD_{50} value of 6350 mg kg^{-1} and glucose belongs to class VI with an LD_{50} value of 23 000 mg kg^{-1}. Then, a qualitative colorimetric kit and Fourier-transform infrared (FTIR) spectroscopy were used for the preliminary detection of urea and glucose in cow milk. The colorimetric kit confirmed the presence of urea and glucose by changing the sample colour. Based on these results, a point-of-care (PoC) kit was developed for urea and glucose detection in cow milk. The enzyme immobilization technique was used to coat urease and glucose oxidase/peroxidase on polystyrene strips to make PoC strips. The biochemical methods of the Berthelot assay and glucose oxidase/peroxidase (GOD/POD) assay were used to detect urea and glucose, respectively. The lowest detection limits of the developed microassay kit for urea and glucose were 1.5 and 3 μg from 300 μg of cow milk. The shelf life of the urease immobilized strip was ~30 days, with 15 times the reusability of a single well, and for the GOD/POD immobilized strip it was ~15 days, with 7 times the reusability, each with a detection efficiency of 85–90%. The strips provided results in ten minutes and were easily portable for on-site adulteration detection.

Received 20th October 2024
Accepted 23rd December 2024

DOI: 10.1039/d4ay01919a

rsc.li/methods

1. Introduction

Cow milk is considered a staple food for humans, a complete source of nutrition for all ages, primarily for growing children, and is consumed either as is or in different forms of dairy products. Hence, cow milk production keeps rising with increasing human population and demand. The global cow milk production in 2022 was 930 million metric tonnes (MMT), of which 203 MMT was produced by India alone.[1] Chemical milk manufacturing and adulteration are rising due to the rising demand for cow milk.[2] Long-term consumption of chemicals such as urea, detergents, and melamine, used as adulterants in cow milk, can harm human health.[3] The broad categorization of milk adulterants with typical examples is detailed in Fig. 1.[4] They are added mainly to attain the quality standards as defined by the Food Safety and Standards Authority of India (FSSAI):

(i) Nitrogenous chemicals to improve protein content (urea).

(ii) Sugars and carbohydrates to improve density and solid non-fat (SNF) content (glucose).

(iii) Preservatives and sterilizing agents to maintain shelf life (peroxides).

(iv) Neutralizers and detergents to maintain pH and prevent curdling during storage (buffer salts).

Cow milk naturally contains about 70 mg dL^{-1} of urea.[5] However, in some instances, urea is mixed with cow milk to elevate the non-protein nitrogen concentration, solid non-fat (SNF) value, and viscosity to provide the impression of thick milk.[6] About 4.8 and 10 mg dL^{-1} of lactose and glucose naturally present in milk are responsible for cow milk's sweet aftertaste.[7] The addition of water to milk by middlemen or vendors to increase the cow milk's volume, prior to distribution, causes dilution and changes its taste. To reinstate the natural sweetness and to increase the lactometer reading, glucose is added to the cow's milk, and hence it is treated as an adulterant.[8] Human-based studies have been summarised in Table 1 to help understand how added glucose affects organ systems.

Overall, the effects of chronic urea and glucose consumption are primarily observed in the human gastrointestinal tract on the blood glucose levels and insulin response. Fig. 2 summarizes the effects of glucose and urea consumed with cow milk on human health, which thus makes their detection from cow milk imperative.

Milk adulteration causes significant economic losses. Consumers pay for inferior products, while dairy farmers face

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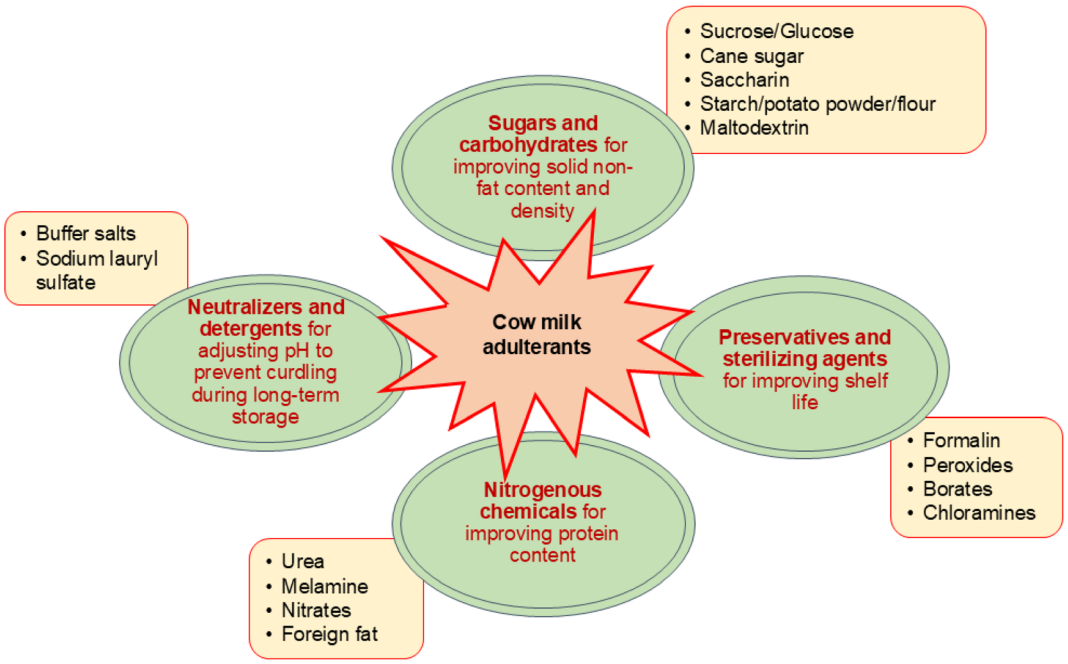

Fig. 1 Categories, roles, and examples of cow milk adulterants.

reputational damage and decreased sales. This results in the government incurring higher costs for testing and regulation enforcement. Ultimately, the entire economy suffers due to reduced consumer trust and market instability.

Since urea is one of the principal adulterants of milk, several methods have been devised to measure its concentration in milk samples. These methods include potentiometric biosensors,[12] nonlinear chemical fingerprinting technique,[13] reflectance spectroscopy,[14] voltamperometric discrimination,[15] urease nanoparticles for improved potentiometric urea biosensors,[16] Surface-Enhanced Raman Spectroscopy (SERS) detection,[17] a non-enzymatic method using a gold nanoparticle-based aptasensor,[18] liquid chromatography-tandem mass spectrometry (LC-TMS)[19] or Ultra High-Performance Liquid Chromatography (HPLC),[20] paper test cards[21] or micropads using *p*-dimethylaminobenzaldehyde (DMAB).[22] For detecting glucose in cow milk, methods such as paper card test,[21] colorimetric nanobiosensors,[23] and electrochemical analysis with immobilized enzymes enhanced by ultrasound have been developed.[24] Some of the other methods can be summarized as follows (Table 2).

However, the currently available methods for detecting urea and glucose in cow milk require high-precision instrumentation, expensive chemicals, and expertise to perform them. Acquisition of these expensive instruments might not be feasible, especially in rural areas, for regular testing of milk samples before dispatch or consumption. Some of these methods are easy to use. However, none of the devices explore the concept of the reusability of enzymes for visual colorimetric detection. Hence, this study highlights the development of a point-of-care (PoC) kit that requires minimal or no pretreatment of cow milk and allows on-site visual detection of adulterants. Enzymes offer a scientifically stable solution for developing a colorimetric adulterant detection technique. They bind only to specific substrates, thereby developing a unique colour, which can be detected visually and quantified using a spectrophotometer. Urease and glucose oxidase/peroxidase offer specificity in detecting urea and glucose and hence can be used for PoC kit development based on the characteristics of enzyme immobilization and colorimetry for detection.

The Berthelot method is a simple and generally performed colorimetric assay for the quantitative and qualitative determination of urea.[30] This assay utilizes the urease enzyme to break down urea into ammonia and carbon dioxide. Ammonia ions react with salicylate and hypochlorite in the presence of nitroprusside to give green-colored indophenol as the end product.

The green color increases in direct proportion to the urea concentration found in the sample. The reaction is summarized as follows:

$$\text{Urea} + \text{H}_2\text{O} \xrightarrow{\text{urease}} 2\text{NH}_4^+ + \text{CO}_2 \quad (1)$$

$$\text{NH}_4^+ + \text{salicylate} + \text{NaClO} \xrightarrow{\text{nitroprusside}} \text{indophenol(green)} \quad (2)$$

The efficacy of the Berthelot assay lies in using neutral pH and displaying a quick colour change for the adulterated sample to generate a colorimetric response within the shortest time.

The glucose oxidase/peroxidase (GOD/POD) method is most commonly used for the biochemical detection of glucose.[31] The

Table 1 Effects of consuming glucose-adulterated cow milk

Background of study	Type of study	Conclusions	Outcome	Reference
Effects of beverages consumed with an average meal on (postprandial glucose) PPG and insulinemic responses in overweight and obese adults	Breakfast beverages included (1) water, coffee sweetened with sugar, low-fat milk (LFM), and orange juice (OJ) with less energy; (2) whole, low-fat, and fat-free milk subjects ($n = 46$) (33F/13M), BMI = 32.5 ± 0.7 kg m^{-2} and age = 50 ± 1 years	(1) Coffee displayed a greater glucose AUC (area under the curve) than water, OJ, and LFM across diverse beverage kinds. Coffee and LFM had a greater insulin AUC than OJ and water (2) Water and milk samples had similar glucose AUCs; however, milk samples had a greater insulin AUC than water. In conclusion, it can be claimed that drinking water, reduced-calorie orange juice, or milk (regardless of its fat level) is preferred with breakfast	Although milk affected insulin AUC, it could not be considered a threat to diabetic individuals as long as it did not contain externally added glucose	9
The difference in impacts on Chinese men's plasma amino acid reactions and secretion of incretin hormones, based on the consumption of soy and cow milk	Twelve healthy Chinese men consumed cow milk and soy milk at random	After soymilk meals, plasma amino acids increased, particularly alanine, arginine, and GIP (glucose-dependent insulinotropic polypeptide), which may be involved in hyperinsulinemia. The decreased glycemia after consuming cow's milk may be attributed to branched-chain amino acids and (glucagon-like polypeptide) GLP-1 secretion	For controlling blood sugar levels, soy milk is an excellent substitute for cow milk	10
Fortification of milk with vitamin D3 has no effects on anthropometric measures, lipid profiling, or glycemic control in type 2 diabetic patients	Randomized triple-blind, placebo-controlled trial ($n = 102$) (34M/68F); 31–74 years of age with T2DM to receive 250 mL of unfortified or 250 mL of milk fortified with 1000 IU of vitamin D3 every day for nine weeks. Serum glucose, insulin, and fat levels were measured and examined, along with anthropometric features and blood pressure	HbA1C significantly dropped in both groups, but the plain milk drinkers' drop was more notable (7.5% *vs.* 3.1%), creating a significant between-group difference. Both groups displayed significant increases in serum calcium and significant decreases in systolic and diastolic blood pressure, total cholesterol, hip and waist circumference, and blood pressure. Furthermore, the body mass index of the fortified milk group was much lower. Serum 25-hydroxy vitamin D concentrations rose in the enhanced milk group	Milk fortified with vitamin D3 without the addition of glucose helped regulate anthropometric measurements in T2DM patients	11

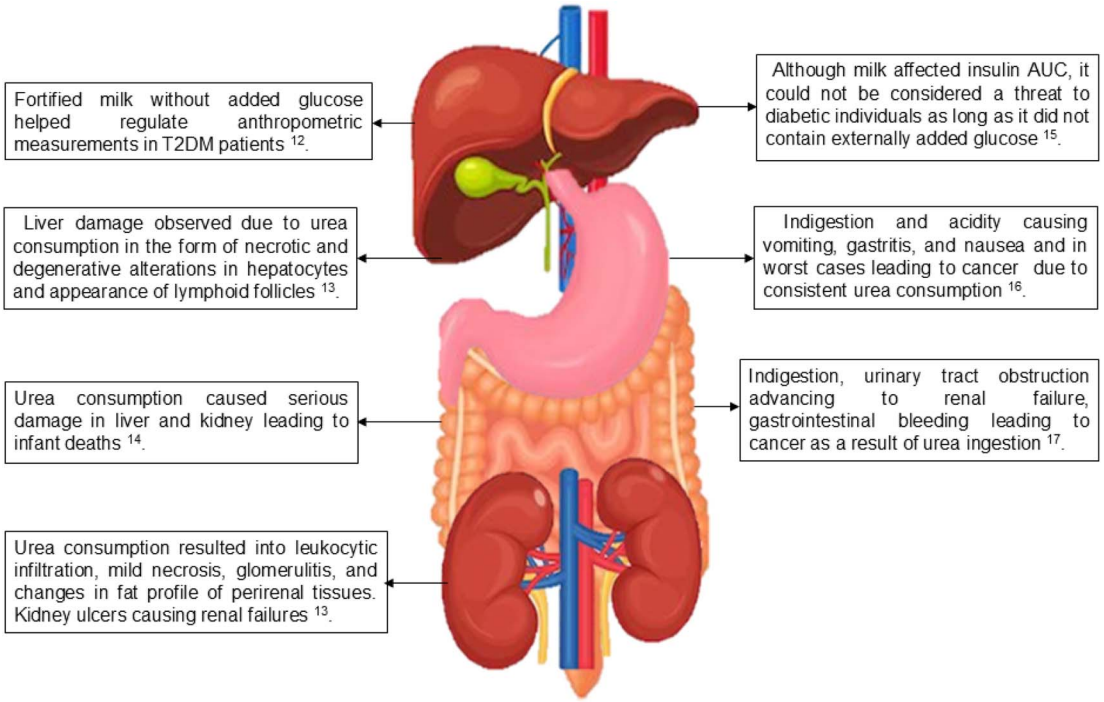

Fig. 2 Effects of cow milk adulterated with urea and glucose on human health.

assay consists of two steps. In the first step, glucose is broken down to gluconic acid and peroxide by the enzyme glucose oxidase (GOD). In the next step, peroxide is broken down to red-colored quinoneimine dye and water in the presence of aminoantipyrine and phenol by the action of the peroxidase (POD) enzyme. The intensity of the color developed is directly proportional to the concentration of glucose in the sample, as summarized in the following reaction:

$$\beta\text{-D-Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2 \quad (3)$$

$$H_2O_2 + \text{4-aminoantipyrine} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{quinoneimine(red)} + H_2O \quad (4)$$

The cross-linking and immobilization step would help maintain the enzyme's stability by retaining its structural and functional properties and reducing the loss of the enzyme and its function by forming stable inter- and intra-subunit covalent bonds.[32]

However, enzyme usage is expensive, so its reusability must be explored to salvage the cost. Enzyme immobilization offers a very reliable methodology, ensuring the best reuse of enzymes and improving their stability of catalytic activity at a reduced price.[33] A PoC kit developed using enzyme immobilization would assure dairy farm workers and homemakers of the quality of cow milk even in resource-poor settings. The PoC was optimized for using as low as 3–5 μL of cow milk sample to perform the assays efficiently. The cow milk sample requires no preprocessing or pretreatment and is directly used for the assay.

In light of the above, the current investigation focuses on developing a microassay-based PoC device using the concept of enzyme immobilization for urea and glucose detection in cow milk, both qualitatively and quantitatively in resource-poor settings.‡[34] The urease and glucose oxidase/peroxidase complex was immobilized on glutaraldehyde-treated 8 well polystyrene strips to detect urea and glucose in cow milk in micro-volumes. The immobilized enzymes were also tested for long-term usage and reusability based on different parameters. The use of this detection kit does not involve preprocessing of cow milk samples by techniques such as centrifugation and thus further reduces the cost of operation.

2. Materials and methods

2.1 Chemicals and reagents

Urease tablets (30 000 U L^{-1}) and reagent buffers R1 (phosphate buffer pH 6.7, EDTA, sodium salicylate, sodium nitroprusside) and R2 (sodium hypochlorite (NaClO) and sodium hydroxide) were obtained from Spinreact Pvt. Ltd. Urease enzyme (200 U mg^{-1}) was obtained from Sigma-Aldrich. The glucose oxidase/peroxidase enzyme complex (5000–1050 U L^{-1}) was obtained from Accurex India Pvt. Ltd. The diluent for GOD/POD assay was obtained from Accurex India Pvt. Ltd. Glutaraldehyde was obtained from Amresco Pvt. Ltd. 8 well non-coated polystyrene strips were obtained from NUNC Pvt. Ltd. Phosphate buffer (pH 7.2) and carbonate buffer (pH 9.2) were used in this investigation.

‡ An Indian patent with application no. 202311052491 incorporating parts of this report was filed on 4th August 2023.

Table 2 Rapid detection methods for detection of adulterants in milk

S. no.	Title	Summary	Detection limits	Citation
1	A point of care sensor for milk adulteration detection	A colorimetric technique, implemented on a paper-based sensor, is utilized to identify three specific adulterants commonly found in milk. Furthermore, a 3D design for a paper-based microfluidic device is proposed to facilitate the simultaneous detection of multiple milk adulterants	The limit of detection of the adulterants is found to be 0.2% (v/v) for boric acid and maltodextrin and 0.1% for hydrogen peroxide	25
2	Detection of adulteration in milk using capacitor sensor with especially focusing on electrical properties of the milk	Milk adulteration often involves the addition of substances such as detergents, ammonium sulfate, sodium hydroxide, sodium bicarbonate, salt, and fat. This research utilizes a capacitor sensor to detect these adulterants. The sensor operates by measuring the dielectric loss angle of milk samples, which varies depending on the presence of adulterants	By this sensor measurement system, adulterant detection in milk with different concentrations (from 5% to 20%) is studied	26
3	A novel inexpensive capacitive sensor for instant milk adulteration detection	The article proposes a non-contact method for quickly testing milk quality using a few drops of milk. This method involves using a capacitive sensor to measure the electrical properties of the milk sample. The system can detect adulteration with water, whey, or urea by analyzing these properties. The sensor is fabricated on a printed circuit board and connected to a CDC board to measure capacitance values. Experiments were conducted on various types of milk, and the results show that the system can accurately determine the level of adulteration	This sensor offers non-invasive droplet-based milk quality detection using only a few drops of test samples. It is handy, very compact, easy to fabricate, and highly economical. It is sensitive to the adulterants, and the response is precise with a repeatability index of 0.008%	27
4	Simultaneous determination of urea and melamine in milk powder by nonlinear chemical fingerprint technique	This paper proposed a nonlinear chemical fingerprint method for simultaneous determination of urea and melamine in milk powder using $H^+ + Ce^{4+} + Br^{O3-}$ + malonic acid as the reaction system. A multiple linear relationship was obtained between the adulterant content in milk powder and the inductive time of the corresponding mixed milk powder	The limits of detection for urea and melamine were 0.33 μg g^{-1} and 0.05 μg g^{-1}, respectively. The limits of quantification were 1.11 μg g^{-1} and 0.18 μg g^{-1}, respectively	28
5	Designing and prototyping a novel biosensor based on a volumetric bar-chart chip for urea detection	This article introduces a novel approach that eliminates the use of catalysts in V-chips and provides an efficient and simple path in the design of biosensors. The product of the enzymatic reaction of urease with urea is bicarbonate, which turns into CO_2 gas in an acidic environment. Therefore, the amount of gas produced is proportional to the amount of urea in the sample, and it can be quantitatively measured by visual detection from the amount of ink movement caused by CO_2 gas pressure	This biosensor has a linear response range of 0 to 1000 μg mL^{-1} and a detection limit of 3.6 μg mL^{-1} in raw milk	29

2.2 Toxicity prediction of urea and glucose using ProTox-III

It has been studied and verified that prolonged, consistent ingestion of urea and glucose could cause various health issues in the gastrointestinal tract, as depicted in Fig. 2. Hence, it becomes necessary to understand the details of the toxicity these chemical compounds induce in human organs and organ systems.[35]

ProTox-III software helps predict the probability of toxicity of a particular compound and its analogs to humans. The simplified molecular-input line-entry system (SMILES) was used to predict toxicity and provide the test molecules' structural data. Query input followed by the checklist gives the user a provision to select the toxicity prediction model based on four major categories:

(a) Organ toxicity.
(b) Toxicity endpoints.
(c) Tox21 nuclear receptor signalling pathways.
(d) Tox21 stress response pathways.

The prediction models are further divided into subcategories to specify the results further.

Urea and glucose were entered as the queries, and the SMILES was auto-updated to perform the toxicological analysis of these compounds and their analogs. Selection was further made on the toxicity models to be considered for these compounds, based on which detailed reports were obtained.[36]

2.3 Preliminary testing of cow milk using Fourier-transform infrared (FTIR) spectroscopy and the NDDB kit

For quantitative detection of adulteration in cow milk samples, preliminary tests are necessary to understand the presence or absence of urea and glucose as the first step of analysis. FTIR and the National Dairy Development Board (NDDB) kit were used for preliminary detection of urea and added glucose in cow milk samples. FTIR was used as the first step to analyze sample purity for the presence of urea and glucose. The sample spectrum obtained from FTIR profiling was analyzed for adulterant peaks at specific wavenumbers/cm. In the next step, when requisite reagents in the NDDB adulteration detection kit were added to cow milk, yellow and blue colours developed if the sample was adulterated with urea and glucose. However, the quantification of an adulterant was not possible with these methods.

2.3.1 FTIR spectroscopic analysis of cow milk for initial detection of urea and glucose. FTIR detection for urea and glucose was performed for cow milk samples locally obtained from Hyderabad and Pilani. 3–5 μL of the sample was pipetted on the detector plate of the FTIR unit (Bruker – Alpha II, Germany, Serial No. 211484). The probe was placed on the droplet surface, and the spectrum was obtained for each sample. The spectra obtained for cow milk samples for the wavelength range of 4000–900 cm^{-1} were checked for the peaks of urea and glucose. Packaged cow milk (Amul Taaza) was used as an unadulterated control to compare the spectra.

2.3.2 Testing for urea and glucose in cow milk using the NDDB kit. Fresh cow milk samples procured locally from Hyderabad and Pilani, India, were analyzed for urea and glucose using the NDDB adulteration detection method. The manual in the kit explains the qualitative method that generates a yellow colour, indicating the presence of urea in cow milk.[5] Packaged cow milk (Amul Taaza) was used as a control to test for urea, and the samples were analysed in duplicate. One set was tested as is, and the other was spiked with 35, 70, and 100 mg dL^{-1} of urea. 150 μL urea reagent in the kit was added to 150 μL of each of the control milk samples, cow milk samples, and urea stock and incubated for colour development.

The glucose reagents 1 and 2 from the NDDB kit were utilized to identify added glucose to cow milk qualitatively. As previously done for urea testing, packaged cow milk (Amul Taaza) was used as a control, and the other samples were analyzed in duplicate. One set was tested as is, and the other was spiked with 10, 20, and 40 mg dL^{-1} of glucose, which were used for qualitative analysis. The addition of glucose reagents, which results in the development of a blue colour, would confirm the glucose adulteration in cow milk.[5] 147 μL of glucose reagent 1 was added to 3 μL each of control, cow milk samples, and glucose stock solution. The samples were heated to 100 °C in a boiling water bath for three minutes, and then 150 μL of glucose reagent 2 was added to the samples.

2.4 Enzyme immobilization using the glutaraldehyde crosslinking method

The activation buffer solution was prepared by adding 50 μL glutaraldehyde to 950 μL carbonate buffer of pH 9.2 to activate the polystyrene strips. 120 μL of the activation buffer was added to each of the eight wells of the non-coated polystyrene strip. The activation buffer was discarded after incubating the strip at 4 °C overnight.[37] Phosphate-buffered saline (PBS) with a pH of 7.2 was used to wash the wells.

For urea detection, the urease solution for immobilization was prepared by dissolving one urease tablet in 2.5 mL of R1 buffer containing sodium salicylate and sodium nitroprusside. The strength of this enzyme solution was 20 times higher than the original concentration (600 U mL^{-1}). An activated polystyrene strip was taken, and 20 μL of urease solution (5×, 10×, 20× concentrates) was added to the two consecutive wells. These strips with enzyme solutions were left to dry at 30 °C for 60 minutes in a vacuum concentrator (Eppendorf, Hamburg, Model No. 5305). After drying, the enzyme-coated wells were washed with PBS and stored at 4 °C.

For glucose detection, GOD/POD enzyme combinations in a ratio of 5 : 1 were used in this investigation. 95 mg of GOD/POD enzyme was dissolved in 1 mL diluent buffer to make an enzyme solution with a 10 times higher concentration. Similarly, 10 μL of GOD/POD solution (1×, 2×, and 4× concentrates) were added to the respective wells of another glutaraldehyde-activated polystyrene strip. These strips with enzyme solutions were left to dry at 30 °C for 60 minutes in a vacuum concentrator (Eppendorf, Hamburg, Model No. 5305). After drying, the enzyme-coated wells were rinsed with PBS and preserved at 4 °C. In each of the strips, 2 wells were left blank, containing no enzyme for immobilization.

2.5 Qualitative and concomitant quantitative detection of urea and glucose from cow milk using enzyme-immobilized strips

The glutaraldehyde-based crosslinking method immobilized urease and GOD/POD enzymes on the non-coated, multiwell polystyrene strips. Considering the preliminary results obtained from the NDDB kit and FTIR, the Berthelot and GOD/POD methods were optimized for colorimetric qualitative and quantitative measurement of glucose and urea in cow milk. The efficiency of the kit was calculated using the basic equation:

$$\eta = (\text{number of positive results/total number of trials})$$

and further, the LOD for the strips was calculated using the following equation:

$$\text{LOD} = 3.3 \times \left(\frac{\text{standard deviation}}{\text{slope}}\right)$$

2.5.1 Urea detection using the Berthelot method. Enzyme assays were performed using 1× enzyme solution and varying concentration strengths for immobilized enzymes. For obtaining a standard graph, the Berthelot assay was performed using urease solution (1× strength).[38] 2 mL of 1× enzyme solution was prepared by adding 100 μL of 20× enzyme solution and making up the volume with R1 buffer. The assay was performed by adding 3 μL each of standard urea solutions (50, 100, 150, and 200 mg dL^{-1}) to 147 μL of 1× R1–urease solution. The strip was incubated for 5–7 minutes, and 150 μL of R2 buffer was added. The wells were observed for any colour change to green after 3–5 minutes. Absorbance readings were recorded at 580 nm.

For performing a standard assay using immobilized enzyme strips, 147 μL of R1 buffer and 3 μL of stock urea solution of 50 mg per dL concentration were added to the enzyme-immobilized wells and incubated at room temperature for 5–7 minutes. 150 μL R2 buffer was added, and the color change was observed after 3–5 minutes. Absorbance readings were taken at 580 nm. The same protocol was followed for detecting urea in cow milk samples using immobilized urease strips. The polystyrene strips were washed with PBS, covered with cellophane tape, and stored at 4 °C.

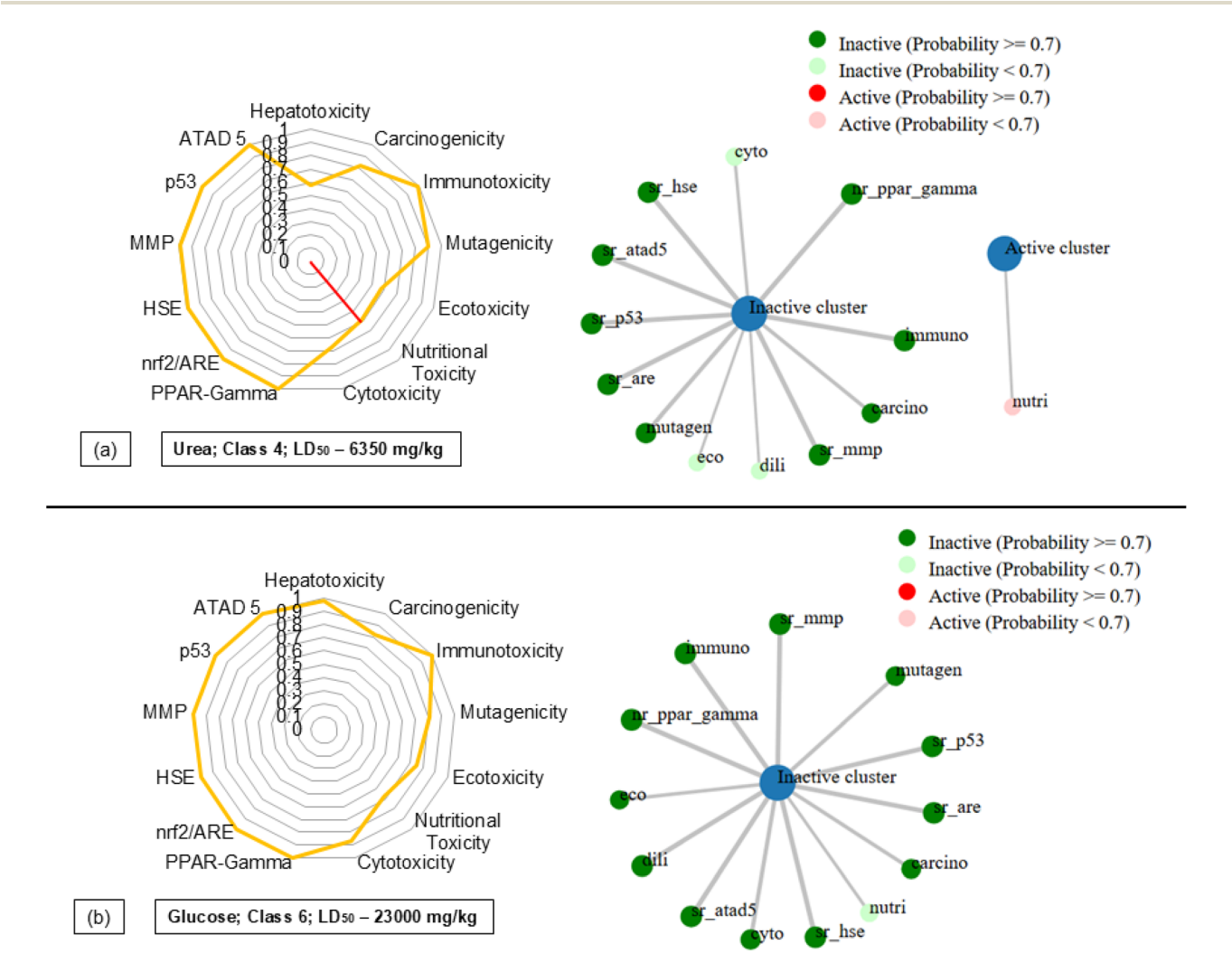

Fig. 3 Prediction of toxicity in humans for (a) urea and (b) glucose.

2.5.2 Glucose detection using the GOD/POD method. GOD/POD assay was performed to obtain a standard graph using GOD/POD solution (1× strength). 2 mL of 1× enzyme solution was prepared by adding 200 μL of 10× enzyme solution and making up the volume with diluent buffer.[39] The assay was performed by adding 3 μL each of standard glucose solutions (50, 100, 150, and 200 mg dL^{-1}) to 297 μL of 1× GOD/POD solution. The wells were observed for any colour change after 10 minutes. Absorbance readings were recorded at 505 nm.

For performing a standard assay using immobilized enzyme strips, 297 μL of diluent buffer and 3 μL of each stock glucose solution at a concentration of 100 mg dL^{-1} were added to the GOD/POD immobilized well and incubated for 10 minutes. The same protocol was followed for the cow milk samples. The colour changes were observed, and the absorbance readings were taken at 505 nm. After the glucose assay, the wells were washed with 200 μL PBS, covered with transparent cello tape, and stored at 4 °C. The sample-to-reaction volume ratio, here, too, was finalized to 1 : 100.

Quantitative studies for urea and glucose were divided into four parts as follows:

(1) Obtaining a standard graph for adulterant detection using enzyme solution (1×).

(2) Obtaining a standard graph for adulterant detection using polystyrene strips immobilized with varying enzyme concentrations.

(3) Repetitive assay using the immobilized enzyme strips over 15 days. The samples were incubated for 5, 10, 15, 20, 30, and 60 minutes.

(4) The functionality and efficacy of the immobilized enzyme on the polystyrene strip were understood by performing the Berthelot and GOD/POD assays repeatedly, every alternate day, for 30 days.

The procured samples were spiked using different volumes in the range of 1–10 μL from 50 mg per dL urea standard solution and 100 mg per dL glucose standard solution for respective assays.

In certain situations, based on the storage conditions of the strips, the incubation time might vary depending on factors such as enzyme efficiency and shelf life.

3. Results and discussion

The experiments for qualitative and quantitative measurements of urea and glucose using the developed kit have been performed in triplicate. These results were analyzed using IBM SPSS 29.0.2.0 software. The variance was calculated using ANOVA for two factors with replication.

3.1 Toxicity prediction of urea and glucose using ProTox-III

The toxicity prediction of a compound provides the results of specific organ-related and organ system-related effects. The report also consists of the toxicity class of a compound, ranging from 1 to 6, with 1 being the most toxic and 6 being the least. The details include the compound's molecular weight and the analogs' average molecular weights. LD_{50} values were included in the toxicity reports for both compounds. Further, the report provides options for additional models for

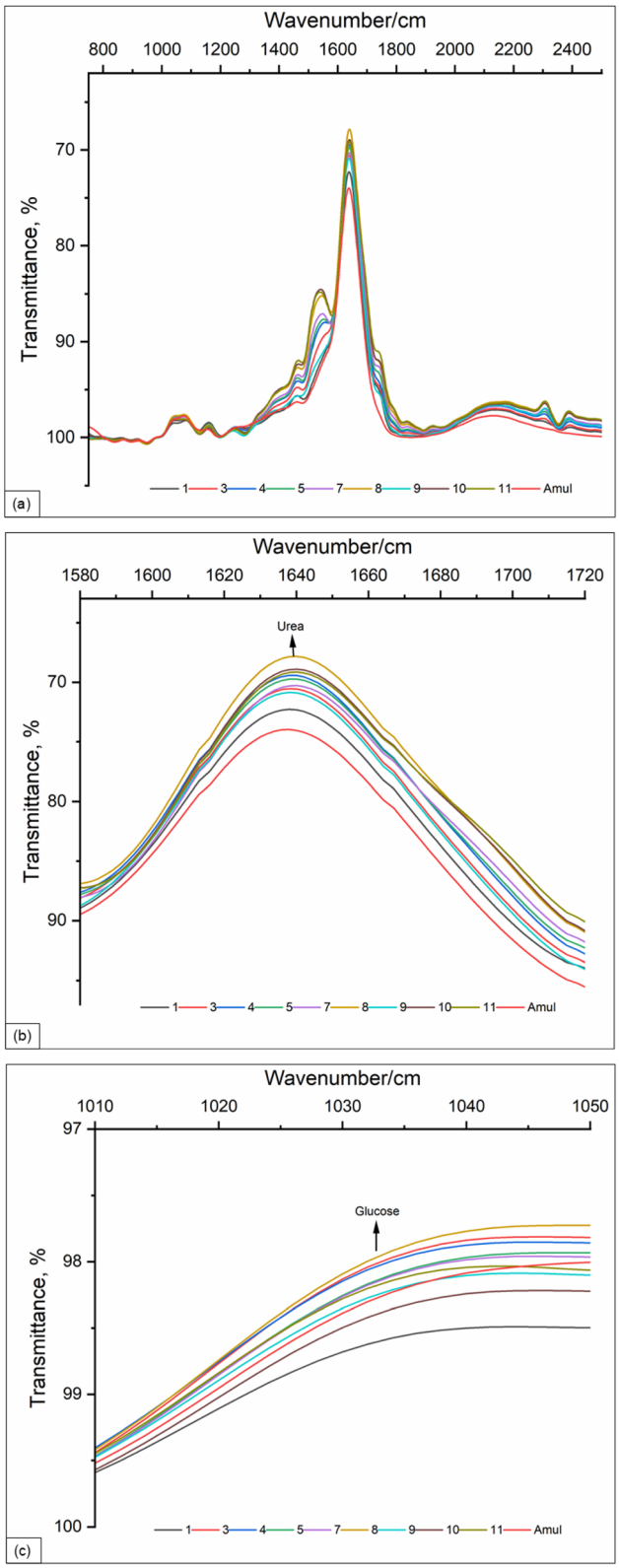

Fig. 4 FTIR spectra of (a) control and experimental milk samples, (b) urea, and (c) glucose.

predicting acute toxicity due to the selection made from the options. Based on the particular selections, reports were generated for urea and glucose and are presented in Fig. 3a and b, which depict their toxicity as observed in human organs and organ systems.[40]

The results revealed that urea belonged to class IV with an LD_{50} value of 6350 mg kg^{-1} and glucose belonged to class VI with an LD_{50} value of 23 000 mg kg^{-1}, respectively. From this result, it was concluded that no substantial damage occurs unless more significant concentrations of these compounds are ingested for a particular body weight. Further, the reports have been analyzed to understand the toxicities that could be elicited by urea and glucose in different human systems. With reference to Fig. 3a and b, which provide the details of toxicity as radar charts for urea and glucose, it was clear that none of these compounds exceeds the toxicity probabilities of the other compounds similar to them and hence do not contribute to hepatotoxicity, cytotoxicity, and immunotoxicity. The software has predicted nutritional toxicity for urea and glucose, probably due to active cardiotoxicity and nephrotoxicity. These results are calculated based on probability and structural similarity among toxicity components or molecules, and their effects vary based on human morphology and genetics. Also, the results differ when the compounds are ingested continuously and consistently over long periods, as summarized in Fig. 2.

3.2 Preliminary detection and confirmation of urea and glucose presence in cow milk using Fourier-transform infrared (FTIR) spectroscopy and the NDDB kit

The preliminary detection results for the presence or absence of urea and glucose in procured samples were obtained by FTIR spectroscopy, followed by testing of procured and spiked samples using the NDDB kit.

3.2.1 Confirmation of the presence or absence of urea and glucose using FTIR. In the first step of adulterant detection and confirmation, the packaged control milk sample (Amul Taaza) and procured samples were directly tested using FTIR spectroscopy. This step provides preliminary information about the presence or absence of urea and glucose in all samples. Fig. 4a shows a combined overall spectrum of all the samples. Fig. 4b depicts urea peaks in all the samples, as cow milk naturally contains urea. Sample 2 for urea and samples 2 and 6 for glucose detection underwent spoilage and were not included in the FTIR analysis. The presence of urea can be confirmed by analyzing the graph between the wavenumbers 1720–1580 cm^{-1}, specifically from the peak obtained at 1638 cm^{-1}.[41] Fig. 4c displays FTIR results for glucose in control and procured samples. It can be observed that no specific peaks are observed in the spectra for glucose at 1033 cm^{-1}, as lactose is the natural sugar found in cow milk. Hence, from the spectra obtained, it can be inferred that the procured cow milk samples do not contain any added adulterants.[42,43]

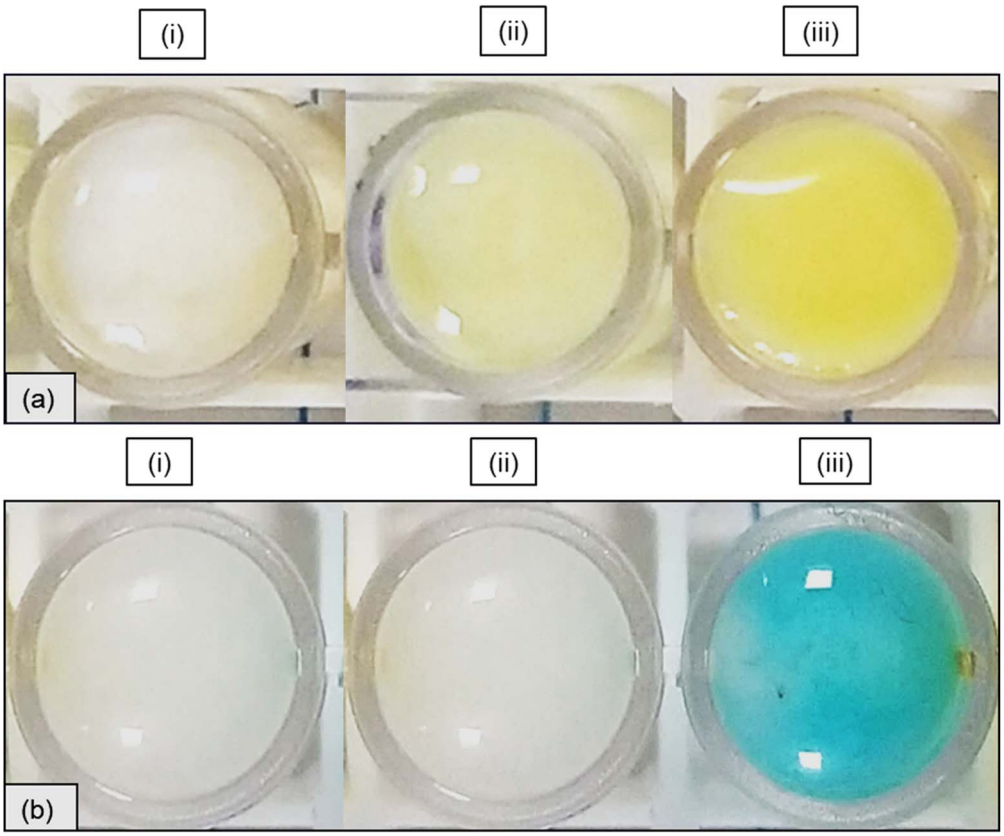

Fig. 5 NDDB results for adulteration detection: (a) for urea: (i) cow milk, (ii) cow milk + urea reagent and (iii) urea spiked cow milk + urea reagent; (b) for glucose: (i) cow milk, (ii) cow milk + glucose reagent and (iii) glucose spiked cow milk + glucose reagent.

3.2.2 Testing for urea and glucose using the NDDB kit. As a preliminary detection using the urea reagent provided in the kit by NDDB, the appearance of a very faint yellow colour for all the fresh cow milk samples confirmed that no added urea was present. Cow milk naturally contains urea, so the faint yellow colour development holds true. However, when the urea reagent was applied to spiked samples, different intensities of yellow colour were observed, signifying the presence of added urea.

Fresh cow milk samples procured locally from Hyderabad and Pilani, India, were analyzed for glucose using glucose reagents 1 and 2 in the NDDB kit. No development of colour was observed in the locally procured samples, whereas a gradient of blue colour development was observed in the spiked samples (Fig. 5).

3.3 Qualitative and quantitative measurement of urea in cow milk

To maintain the turnover number consistency over a more extended period for an immobilized enzyme, it becomes necessary to use a higher concentration than 1× over a polystyrene surface.[44]

Fig. 6a depicts the standard curve obtained using 1× enzyme solution to understand the efficacy of the Berthelot method. The standard baseline helped to understand the method's effectiveness and specificity for detecting urea (R^2 value is 0.998). Similarly, urease was immobilized for the Berthelot assay using the glutaraldehyde crosslinking method, and a standard curve was drawn for three concentration strengths of urease, with 5× being the most efficient ($R^2 = 0.981$), as observed in Fig. 6b. The detection efficiency increases with the increase in enzyme concentration, and beyond 20×, the rate of enzyme degradation also increases.[12] When a high concentration of enzyme is immobilized, although the detection efficiency is faster and more accurate, the degradation followed by reduction of strip shelf life is also faster due to the greater quantity of enzyme being washed off after every usage. This is reduced by using lower concentrations of enzymes for immobilization. The

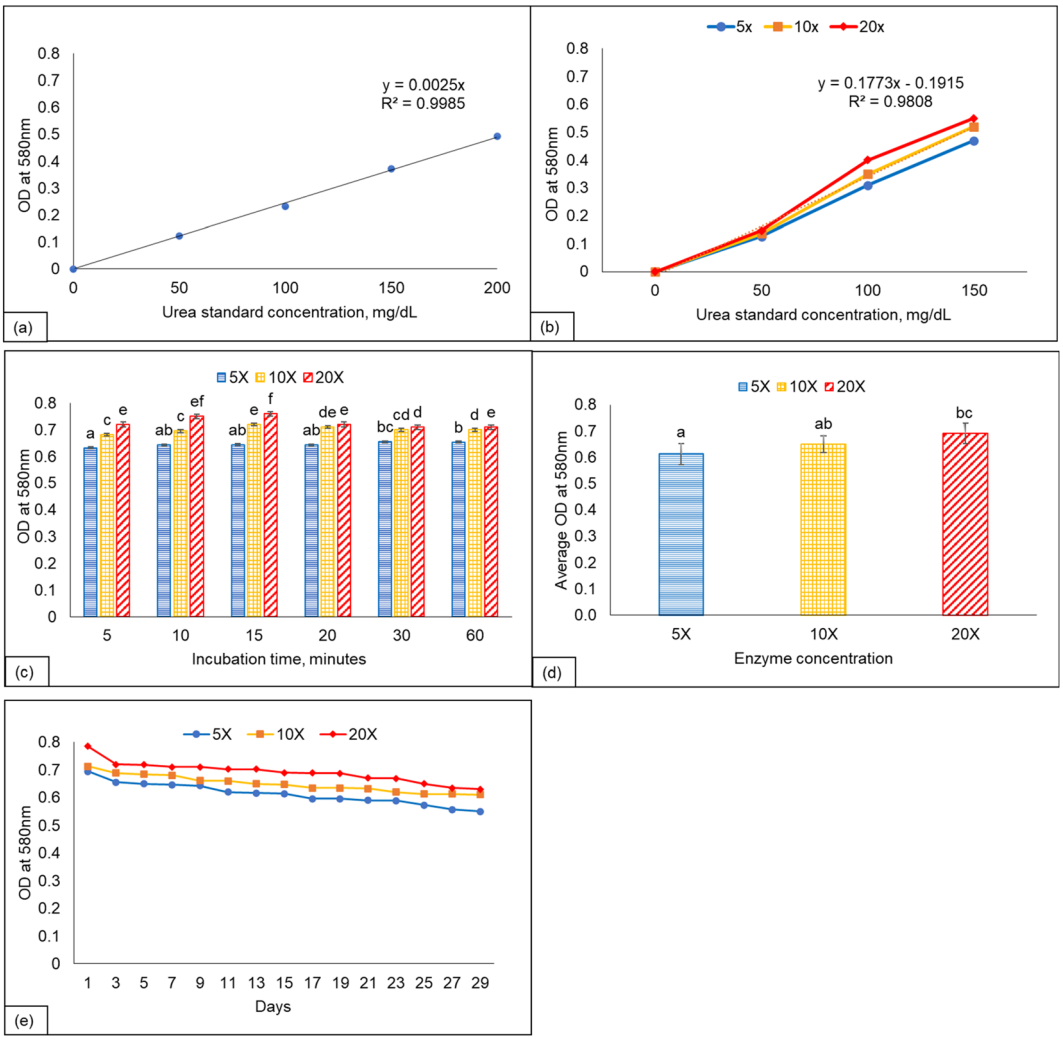

Fig. 6 Detection of urea using the Berthelot assay and strips immobilized with urease – results of the assay: (a) using 1× urease solution, (b) using urease immobilized at 5×, 10×, and 20× concentrations, (c) for understanding strip efficiency at various incubation periods, (d) for understanding strip efficiency using various urease concentrations and (e) for understanding the reusability and shelf life of immobilized urease. All results with $p < 0.05$ are significant.

detection is consistently possible using 5× and 10× urease concentrations. Hence, using 5× enzyme concentration would be favourable for immobilization and better for cost-effective detection.[45]

The wide range of time points helped in understanding the stability of the colorimetric response so that the colour did not disappear or fade away with an increase in the incubation period. The range of time points was obtained by performing these experiments in triplicate using 11 samples for ten consecutive days. Fig. 6c summarizes the direct variation between colorimetric assay and time of incubation. The green colour of indophenol stayed stable for approximately 60 minutes, which was also observed.[46] The stability of the colour thus developed is observed to be uniform for the 5× concentration of enzyme used for immobilization, whereas the colour intensity is observed to be reduced for the 20× immobilized enzyme. Hence, it would be conclusive to immobilize a lower enzyme concentration to keep the colour stable for longer. From the graph, we can conclude that the Berthelot assay is one of the most promising quick assays, which gives the result within 5–10 minutes, and the end colour thus developed stays stable for 60 minutes. This result proves valuable when the number of samples for detection is higher. The stability of the colour thus developed allows the user to document the results quickly.

Fig. 6d summarizes the results of the assays that compare the colorimetric assay and the enzyme strength. Increased enzyme concentrations are effective for quicker detections; hence, the standard deviation of the detections is also reduced with an increased specificity of detection. Although the fastest, best colorimetric response was observed with 20× urease enzyme strength, 5× is preferred, considering the reduced loss of the enzyme from wash-off and the consistency observed in the results.[47]

The functionality and efficacy of the immobilized enzyme on the polystyrene strip were understood by performing the assay repeatedly using the same well, every alternate day, for 30 days. During this period, 11 samples were used for analysis, alternately over a month. The absorbance readings were recorded during this period and analyzed for variable concentrations of immobilized urease. The detection efficiency reduced by 28%, 19%, and 25% in 30 days for 5×, 10×, and 20× enzyme concentrations, respectively, immobilized on the polystyrene strip, as observed in Fig. 6e.

The standard deviation (RSD) values for time point analysis, as depicted in Fig. 6c, range between 1.5 and 2.1% as determined by testing 11 samples in triplicate.[13] Meanwhile, RSD values for optimization of enzyme strength to be used for immobilization, as depicted in Fig. 6d, range from 3.1% to 4% as determined by testing 11 samples in triplicate.[48]

The results of the urea testing kit are summarized in Fig. 7.

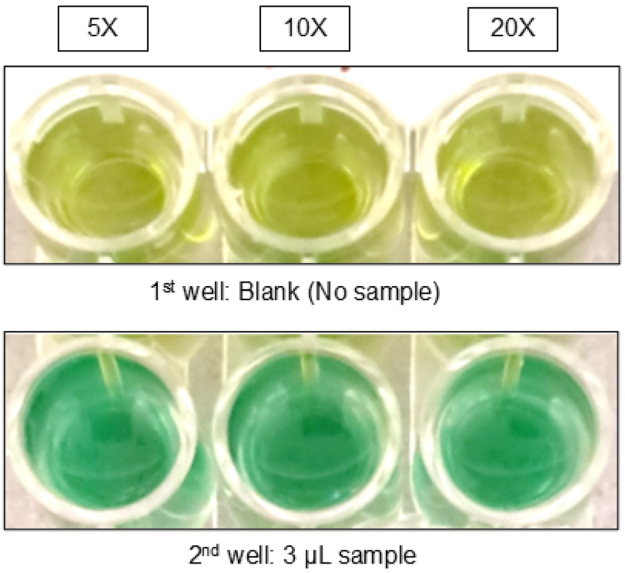

Fig. 7 Colorimetric results of the urea testing kit with 3 different enzyme concentrations.

3.4 Qualitative and quantitative detection of glucose in cow milk

As depicted in Fig. 8a, the graph was plotted using 1× enzyme solution concentration for GOD/POD combination for understanding the glucose detection specificity of GOD/POD enzyme combination in microvolumes, and Fig. 8b was plotted using enzyme immobilized strips of varying enzyme concentration strengths (1×, 2×, and 4×). The standard baseline helped to understand the method's efficacy and specificity for detecting glucose (the R^2 value is 0.997). Similar to urease, the enzyme combination of glucose oxidase/peroxidase was immobilized using the glutaraldehyde crosslinking method, and a standard curve was drawn for the three concentration strengths of GOD/POD, with 4× being the most efficient ($R^2 = 0.99$), as observed in Fig. 8b. The detection efficiency increases with the increase in enzyme concentration, and beyond 4×, the rate of enzyme degradation increases. The detection is consistently possible using 1× and 2× GOD/POD concentrations. However, using 1× enzyme concentration would be favourable for immobilization and better for reduced enzyme wastage and cost-effective detection.[45]

For colorimetric detection to be of use and reliance, it is essential that the colour must not disappear or fade away quickly. Hence, studying the assay by conducting repeats over different time points helps better understand the colour stability. The range of time points was obtained by performing these experiments in triplicate using 11 samples for ten consecutive days. The direct variation between the colorimetric assay and time of incubation is summarized in Fig. 8c. The red colour of quinoneimine dye stayed stable for approximately 20 minutes, followed by fading of the same. The trend in the colour development is observed to increase in direct variation with the enzyme concentration immobilized. However, the red colour faded quickly for 4× enzyme concentration, whereas the fading was slow for 1× enzyme concentration. Hence, it would be conclusive to immobilize a lower enzyme concentration to keep the colour stable for longer. The GOD/POD assay is one of the most commonly used and promising quick assays, which gives the result within 5–10 minutes, and the end colour thus developed stays stable for 20–30 minutes.[39,49]

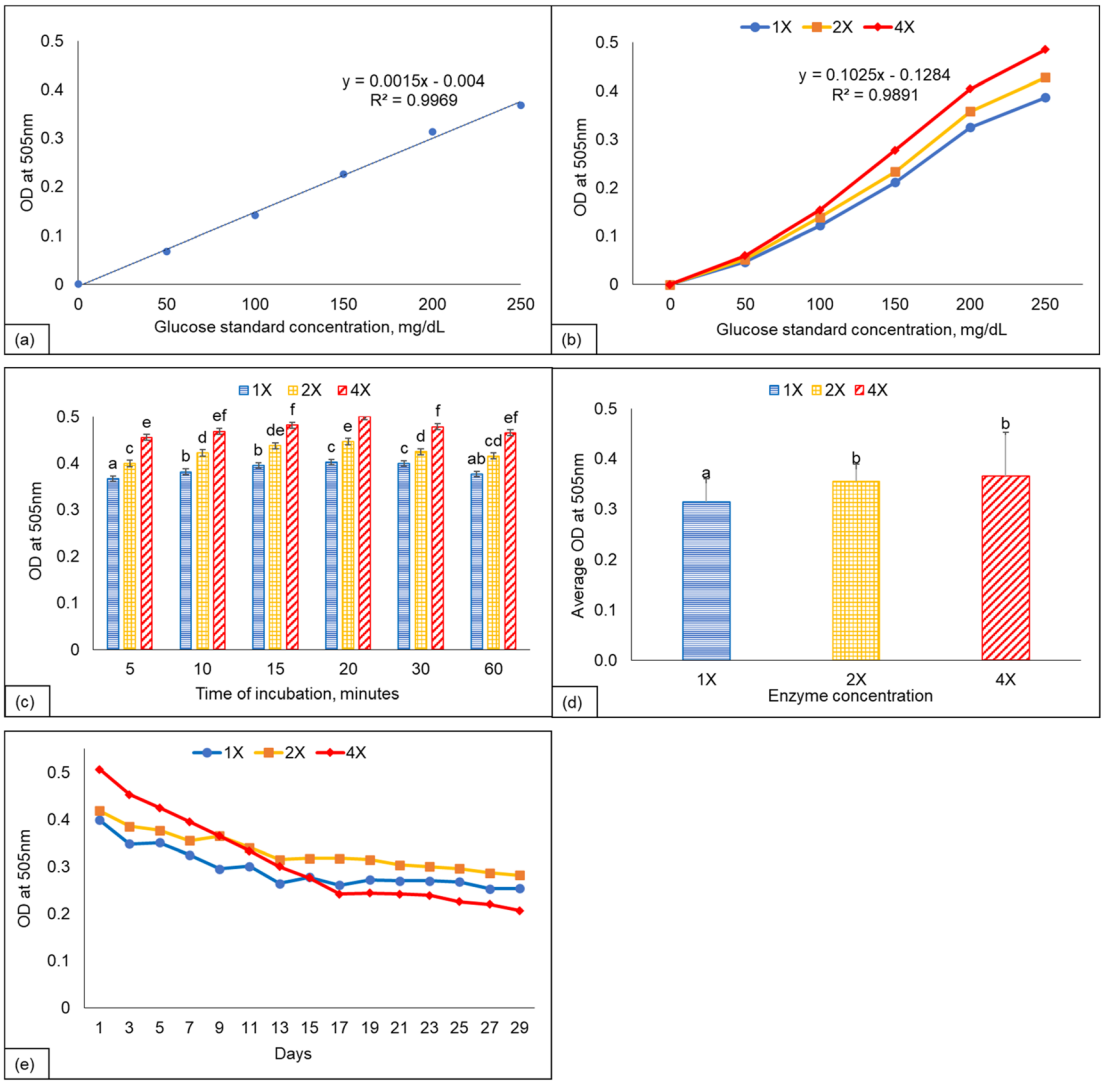

Fig. 8 Detection of glucose using GOD/POD assay and strips immobilized with GOD/POD enzymes – results of the assay (a) using 1× GOD/POD solution, (b) using GOD/POD immobilized at 1×, 2×, and 4× concentrations, (c) for understanding strip efficiency at various incubation periods, (d) for understanding strip efficiency using various GOD/POD concentrations and (e) for understanding the reusability and shelf life of immobilized GOD/POD. All results with $p < 0.05$ are significant.

A comparison of colorimetric assays based on different GOD/POD enzyme concentrations has been summarized in Fig. 8d. The best colorimetric response using the immobilized GOD/POD complex has been observed for 4× enzyme concentration. The 4× enzyme concentration provided the best response consecutively. Still, the greatest standard deviation infers the most difference in consecutive OD readings obtained by reusing the well immobilized with 4× enzyme concentration. However, the reuse of wells immobilized with 1× and 2× GOD/POD enzymes does not depict too much deviation, and hence, for cost efficiency, 1× concentration can be used.

For every alternate day, for 30 days, the GOD/POD assay was repeatedly performed using the same well to test the functionality and efficacy of the strip. During this period, 11 samples were used for analysis, alternately over a month. The absorbance readings were recorded during this period and analyzed for variable concentrations of immobilized GOD/POD. The efficiency was reduced by 36%, 32.5%, and 59% with each reuse for 1×, 2×, and 4× enzyme concentrations, respectively, immobilized on the polystyrene strip, as observed in Fig. 8e. From these observations, it can be concluded that GOD/POD degraded quickly, and higher concentrations than 2× cannot be immobilized for longer periods.[24]

The standard deviation (RSD) values for time point analysis, as depicted in Fig. 8c, range between 1.4 and 1.6% as determined by testing 11 samples in triplicate.[13] Meanwhile, RSD values for optimization of enzyme strength to be used for immobilization, as depicted in Fig. 8d, range from 4.6% to 8.1% as determined by testing 11 samples in triplicate.[48]

The results of the glucose testing kit are summarized in Fig. 9.

Hence, observing and comparing the results from the graphs concludes that the 1× or 2× concentration of the glucose oxidase/peroxidase enzyme combination is favorable, with

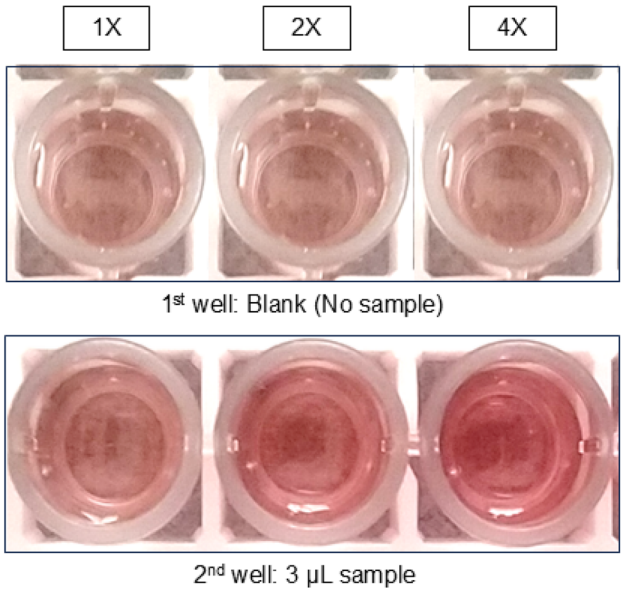

Fig. 9 Colorimetric results of the glucose testing kit with 3 different enzyme concentrations.

approximately 85–90% enzyme recovery. The recovery percentage was inversely proportional to the increase in enzyme concentration, as increased concentration of the immobilized enzyme caused quicker degradation over time.[23]

3.5 Comparison of test results – NDDB kit *vs.* immobilized enzyme strips

Conclusively, the results obtained using the NDDB kit for the detection of urea and glucose and those from the strip assay using immobilized urease and glucose oxidase/peroxidase for detection were compared.[50]

Lower sample volumes or errors in pipetting of lower volumes can lead to either false positive results or the absence of desired results, whereas the NDDB kit helped provide a result with just the presence or absence of an adulterant by testing substantial sample volumes with substantial reagent volumes. The strip exhibited about 90% accuracy of results against 100% detection of urea using NDDB reagents. In comparison, for understanding the strip's efficiency, it was found to be a bit lower for glucose detection at 82% against a precise 100% detection observed by using NDDB reagents. However, the strip was more practical, considering the safety of the reagents and identifying the presence of adulterants in minute concentrations for unknown samples, as low as 1.5 μg of urea and 3 μg of glucose, which represent the limit of quantification (LOQ) individually.

Furthermore, the cost estimate analysis of the strip provides details that indicate the cost reduction per assay when compared to commercially available kits for detecting urea and glucose, respectively, as summarized in Table 3.

Thus, from the cost estimate analysis, it is observed that immobilization of an enzyme offered a significant advantage of reusability and, therefore, can be put to repetitive use for

Table 3 Cost estimation/assay of the developed kit

	Commercially available detection kits					Kit developed in this study		
	Chemical-based		Enzyme-based			Enzyme-based		
Kit specifications	Urea	Glucose	Urea	Glucose		Urea	Glucose	
Enzyme used	DMAB reagent	Reagents 1 and 2	Urease (3 kU L^{-1})	GOD/POD (5 kU : 1 kU L^{-1})		Urease (3 kU L^{-1})	GOD/POD (5 kU : 1 kU L^{-1})	
Reaction volume/assay	4 mL	3 mL	2 mL	1 mL		300 μL	300 μL	
Time of reaction	2–5 minutes	2–5 minutes	10–15 minutes	10–15 minutes		10–15 minutes	10–15 minutes	
Number of assays/kit	100	100	250	500		330	330	
Cost/assay, USD	0.028$	0.052$	0.12$	0.048$		0.025$	0.045$	

multiple samples, thus reducing the overall cost of urea or glucose detection by almost 2–4 times when compared with commercially available kits.[51] The strips with urease and glucose oxidase/peroxidase immobilized on the surfaces were used for colorimetric analysis of numerous samples, proving their cost efficacy.

4. Conclusion

Milk adulterated with urea and glucose was detected using the Berthelot and GOD/POD assays. These reactions not only enable qualitative detection of the adulterants by color change but also determine the concentration of the adulterants with an absorbance reading of the color intensity at specific wavelengths. The intensity of color development in the end step of the reaction was compared with the control sample, which provided a visual idea of added urea and glucose in the milk. The NDDB kit helped understand the presence or absence of urea and glucose by change of colour in the samples, while the urease and GOD/POD immobilized PoC strips had limits of detection (LOD) for adulterant concentrations as low as 0.5 μg of urea and 1 μg of glucose from the cow milk defined using spectrophotometers. The method was optimized for the following factors: (i) enzyme concentration immobilized on polystyrene strips, (ii) time of incubation for quick detection, and (iii) days for which the strips can be stored and reused. Overall, these strips with immobilized enzymes ensured quick adulterant detection using microlitres of samples, reducing wastage. The strips were robust enough to support the detection of urea for over 30 days with 15 times the reusability of a single well, and that for the GOD/POD immobilized strip was ~15 days, with the reusability of each well up to 7 times. The detection efficiency of both strips was 85–90%. These properties of the strips make them user-friendly, providing them with the safety of reagents and ease of usage. Being colorimetric, the results can be seen with the naked eye and understood. The results are obtained within a short period of time without the requirement of any sophisticated instruments to read them. Although immobilization ensured the reusability of wells for assays, under certain conditions, *e.g.*, washing off of the well with phosphate buffer after use, improper storage of the strips at inappropriate temperatures, blocking off the enzyme active sites if the strip is used for detecting adulteration of skimmed milk, and change in the pH of reagents used for detection were some of the most common reasons due to which enzyme activity might get inhibited.

However, the development of these strips for detecting urea and glucose would be helpful to a lot of people to understand the quality of milk they have been consuming. It can be of great assistance to check for milk quality at various transport stages from farm to home, thus achieving the end goal of food safety.

Consent for publication

The authors of the manuscript agreed to submit the work to the RSC Analytical Methods journal.

Data availability

The required data that support the findings of this study have been included in the article.

Author contributions

SK and HB conceptualized this research work. HB performed the experimentation, formally analysed the data, validated the conclusions, and wrote, reviewed and edited the original manuscript draft. SGP reviewed and edited the manuscript draft, supported with the required resources and data curation.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

HB thanks the Indian Council of Medical Research (ICMR) for funding this research work through grant no. 3/1/2/210/2021-NUT. HB and SGP thank BITS Pilani, Hyderabad Campus, for supporting this research work. The authors immensely thank late Prof. Suman Kapur for her valuable input during the initial period of this research work.

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