RSC Advances

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2025, **15**, 1577

Rapid detection of hydrogen peroxide and nitrite in adulterated cow milk using enzymatic and nonenzymatic methods on a reusable platform

Harsha Bharwani, Suman Kapur† and Sankar Ganesh Palani *

Cow milk is readily adulterated due to its complex properties that can emulsify many adulterants. Among the commonly used adulterants in cow milk are hydrogen peroxide (HP) and nitrite. Commercially available HP is added to extend cow milk's shelf life, while nitrite enters through the tap or pond water added to increase cow milk's volume. HP disrupts human free radical balance, while nitrite can cause methemoglobinemia. This study aims to rapidly detect these adulterants on-site by developing a point-of-care kit. A modified streptavidin–horseradish peroxidase (Strep–HRP)–tetramethylbenzidine (TMB) assay was used for designing the biosensor for HP detection. The Strep–HRP complex was immobilized on 8-well polystyrene strips with glutaraldehyde crosslinking. TMB was used as the substrate to detect HP at concentrations of 0.04% (v/v) and higher. Nitrite was detected using a modified Griess assay, wherein for the biosensor, the Griess reagent was coated on polystyrene strips with polyethylene glycol (PEG) used as the stabilizer to identify nitrite concentrations of 32 μg mL^{-1} and above. The Strep–HRP and Griess assay strips prepared in this investigation were stable for 25 and 10 days, with three times reusability for HP and twice for nitrite detection. Both strips were accurate, up to 95%, for detecting HP and nitrite in cow milk samples.

Received 21st September 2024
Accepted 10th January 2025

DOI: 10.1039/d4ra06816e

rsc.li/rsc-advances

1 Introduction

Cow milk is a nutrient-rich complex emulsion consumed by most people globally, constituting an integral part of their diets from childhood to adulthood. Milk is defined as 'the normal mammary secretion derived from complete milking of a healthy milch animal without either addition thereto or extraction therefrom'.[1] Over time, global milk and dairy consumption has gradually increased. Milk is a white-colored complex emulsion and, hence, susceptible to easy adulteration. Prolonged consumption of adulterated cow milk, containing harmful chemicals, poses health risks. Ensuring the safety and quality of cow milk is crucial worldwide due to its significant role in the diet. To confirm its safety, thorough testing for adulterants is essential. As cow milk demand increases, adulteration becomes a tempting means to meet demand and gain profit quickly.[2] On a global scale, the Food Safety and Standards Authority of India (FSSAI) has defined most commonly added adulterants in cow milk, some added to extend shelf life, and some added to comply with quality standards.[3]

One of the adulterants added to cow milk to maintain its shelf life and quality while transporting it is hydrogen peroxide. When consumed, hydrogen peroxide (HP) rapidly diffuses into the oral cavity and upper GI tract cells, thus triggering the onset of oxide radical imbalance. In excess concentrations, HP causes an imbalance in the free radical concentration of the organ systems, thus causing irregularity in their functions, which might result in cancer and genotoxicity.[4] Hence, the action of enzymes, including peroxidases, is essential to maintain balance. The activity of peroxidases is catalyzed by metal ion cofactors, and their sequestration helps in preventing the toxicity of HP in the cell by decreasing Fenton chemistry. Failure of the metal ion sequestration can damage tissues or all organs of the body.[5]

There are some reports on the adverse effects of HP exposure on various human cells. Human lymphocytes were exposed to tannic acid and gallic acid, which reduced formamidopyrimidine-DNA-glycosylase (FPG)-sensitive sites, while ellagic acid inhibited the formation of EndoIII-sensitive sites. It was observed that the exposure resulted in enhanced lymphocyte resistance to DNA breakage induced by HP.[6] Significant DNA damage was observed in HepG2 cells that were directly proportional to the concentration of HP and time of exposure/incubation.[7] The damage reduced over time, which might be due to DNA repair mechanisms and antioxidant enzymes. Exposure of human lymphocytes to HP caused an increase in apoptosis and necrosis. Comet assay and FPG

Environmental Biotechnology Laboratory, Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, Hyderabad Campus, Jawahar Nagar, Kapra Mandal, Medchal District, Hyderabad 500078, India. E-mail: sangan@hyderabad.bits-pilani.ac.in

† Deceased as on 11th November 2022.

confirmed the genotoxicity caused by exposure to HP, whereas exposure to grape juice reduced DNA damage and necrosis.[8]

The HP detection is carried out using some advanced or complex techniques, such as using a chromogenic strip with an integrated QR code,[9] Fourier Transformation Infrared (FTIR) Spectroscopy,[10] sensors based on Fibre Optic Displacements (FODS),[11] flexible substrates such as carbonaceous materials, polymers, paper, glass, and silicon wafers enhanced with metal oxide-based nanostructures for improved performance that have detection limits ranging from 100 nM to 1 mM, suitable for health, food, and environmental monitoring,[12] membrane-based sensors,[13] for on-site detections using the portable ratiometric fluorescent probe[14] are some of the notable examples. However, the idea of developing a cost-effective qualitative and quantitative sensor for hydrogen peroxide is still under development.

Tap or pond water containing nitrites and nitrates is another common adulterant in cow milk distributed mainly in unorganized sectors. Regular consumption of such adulterated cow milk causes gut issues. Fig. 1 describes how nitrites and nitrates in effluents discharged from small-scale industries enter water resources such as lakes and ponds and, from there, into public taps. When milk from the cow is less than the regular volume, the milkman adds pond water or water from public taps to satisfy consumer demand. In this process, nitrites directly enter the cow milk. Cows consume the pond water, and excess nitrate is either excreted in the urine or enters into the gut and is converted to nitrite by the gut bacteria.[15] This signifies indirect addition and indicates the presence of nitrites in cow milk. Cow milk usually contains less than 1 ppm of nitrite. When humans consume cow milk adulterated with tap or pond water, the nitrites combine with haemoglobin and are converted into methemoglobin. This process impairs the oxygen-carrying capacity of red blood cells and can potentially be fatal.[16]

Nitrite is most commonly determined using the Griess assay, wherein nitrite in the presence of *N*-1-naphthylethylenediamine dihydrochloride (NED) and sulfanilamide undergoes diazotization to form a magenta-colored azo dye recorded at 540 nm.[17] Some of the detection devices designed based on the Griess assay include hydrogels for simultaneous detection of nitrite and nitrate,[18] in combination with 3,3,5,5-tetramethylbenzidine (TMB) and glucosamine hydrochloride (GAH).[19] However, none are reusable; hence, they are not cost-effective for wide use in the field.

Surface drying of enzymes and reagents is a long-explored area of interest for the development of PoC devices being developed for the detection of small molecules. Some of the research has been highlighted in the following table (Table 1):

In comparison with spray drying and freeze-drying methods, vacuum drying is suitable for different enzymes that are stable over a wide range of temperatures. Freeze drying is an expensive procedure that involves the use of liquid nitrogen.[25] Post-drying care for such enzymes is extremely important after freeze drying. Spray drying is a commonly used method for large enzyme volumes. Hence, vacuum drying is the most suitable method for working with micro volumes of enzymes.

With the above-mentioned gaps, the current research focuses on developing a microassay-based kit to detect hydrogen peroxide and nitrite in cow milk. The kit has been developed using enzyme immobilization and reagent stability, which help ensure ease of adulterant detection, affordability and accessibility for common people, and safety during use. The unique characteristics of the developed kit are reusability and the absence of sample preprocessing. This approach addresses the immediate health risks of these adulterants and supports broader efforts to maintain food safety and consumer trust in dairy products.

2 Materials and methods

2.1 Chemicals and reagents

The following chemicals and reagents of analytical grade were used in this investigation: streptavidin tagged horseradish peroxidase (HRP) enzyme (RD Systems, Minneapolis) (20× stock), 3,3,5,5-tetramethylbenzidine (TMB) substrate (Himedia) (10× stock), sulfanilamide (SRL Chemicals), *N*-(1-naphthyl) ethylenediamine dihydrochloride (NED) (Sigma Aldrich), glutaraldehyde (Amresco Pvt. Ltd) polyethylene glycol-400 (PEG-400) (SRL Chemicals), 30% hydrogen peroxide (500 mL) (Avra Synthesis Pvt. Ltd) 8-well non-coated polystyrene strips (NUNC Pvt. Ltd) phosphate buffer (pH 7.2), carbonate buffer (pH 9.2), citrate-phosphate buffer (pH 4.8–5).

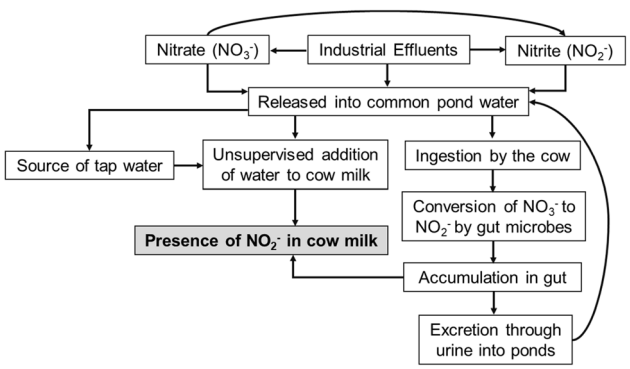

Fig. 1 Influx of nitrites in cow milk.

2.2 Toxicity prediction of urea and glucose using ProTox – III

Adulterants, ingested by consuming adulterated cow milk, carry risks of developing health issues, which, in the worst cases, might be fatal. Hence, it becomes imperative to understand these chemical compounds' toxicity and be aware of their effects on human organs and organ systems.[26] The rate at which an adulterant can cause toxicity or the effects of its analogues on humans can be predicted using the ProTox – III software. Forecasting the toxicity and presenting the structural data of the molecule under consideration can be achieved by the simplified molecular-input line-entry system (SMILES).[27] The model prediction for toxicity of an adulterant is created when the user provides a query followed by a selection in the checklist based on four major categories:

Table 1 Summary of methods used for surface fixation of enzymes and small molecules, their reuse, and stability

S. no	Title	Summary	Advantages	Disadvantages	Reference/ citation
1	Enzyme drying process	The patent describes a method for drying enzymes by spraying an aqueous solution onto a heated fluidized bed of inert particles. The enzyme-containing solution is dried rapidly and efficiently, resulting in a dry, free-flowing powder that retains its enzymatic activity	The process is simple, inexpensive, and effective, and it can be used to dry a wide variety of thermally unstable materials. Other advantages include a rapid drying process, moderate processing conditions, and retention of a substantial percentage of the total enzymatic activity initially present	May result in loss of enzymes during spraying action. Might not be suitable for micro/nano surfaces	20
2	Method for immobilizing and drying enzymes	The patent describes the vacuum drying method as particularly well suited for immobilizing lipases, especially those from *Candida antarctica*	The vacuum drying method is efficient and can be carried out on an industrial scale. The method is particularly well suited for immobilizing lipases. The method can be used to immobilize a variety of proteins and enzymes as the drying can be done over a wider temperature range of 0 °C to 40 °C. For certain heat-stable enzymes, the temperature can be increased, thus ensuring quicker drying with the retention of a substantial enzyme activity percentage. The vacuum drying activity also allows the reuse of immobilized enzymes	The method is not suitable for heat-sensitive enzymes	21
3	Drying of enzyme immobilized on eco-friendly supports	This article discusses the use of different drying technologies and agricultural by-products as supports for immobilizing lipase produced by the endophytic fungus *Cercospora kikuchii*. The study found that spray drying was the most effective drying technology, and microcrystalline cellulose and rice husk were the best supports for retaining lipase activity	Eco-friendly supports are readily available, nontoxic, and resistant to mechanical stress. Spray drying is a mild and cost-effective drying method. The immobilized lipase showed good stability and reusability	Spray drying might not be effective for immobilizing nano volumes of enzymes	22
4	Optimizing drying conditions for the microwave vacuum drying of enzymes	This study aimed to use experimental planning for the optimization of microwave vacuum drying of enzymes using α-amylase as a model. A factorial in star design was used to optimize the microwave vacuum-drying process, and the variables were power output and vacuum pressure. This technique analyzed the material dehydrated for its enzymatic activity, water activity, and moisture content. Response surface	The immobilized derivatives had decreased enzyme activity during storage and reuse. The water content in the immobilized derivatives could affect their stability. The dehydrated product showed high enzymatic activity and low water activity	The method cannot be generalized for all kinds of enzymes or sensitive proteins	23

Table 1 (*Contd.*)

S. no	Title	Summary	Advantages	Disadvantages	Reference/ citation
		methodology was used to estimate the main effects of vacuum pressure and power on the enzymatic and water activities			
5	Immobilization of α-acetolactate decarboxylase (ALDC) in hybrid gelatin/alginate support for application to reduce diacetyl off-flavor in beer	The objectives of the article were to develop low-cost support using gelatin to immobilize the ALDC enzyme for the removal of off-flavor in beer during maturation. The yield, efficiency and activity recovered, and the stability of free and immobilized enzymes at different temperatures and pH were evaluated for this	The enzyme drying was performed using vacuum drying method on the gelatin and alginate supports. The enzyme was immobilized using the glutaraldehyde cross-linking method. Immobilized enzyme showed greater stability at temperatures of 50 °C and 60 °C. The immobilized derivative showed adequate reuse capacity of 30 cycles of 2 hours each, and its dehydrated form had excellent activity retention of upto 90% after long periods of storage	The entire procedure for reducing off-flavor in beer involves higher cost due to enzyme immobilization using polymer beads	24

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

Different types of prediction models were categorized to specify results more precisely. Queries for hydrogen peroxide and nitrite were inserted, which got auto-updated in the SMILES to acquire toxicological analysis for these compounds and their analogs. Specific toxicity models were then chosen for hydrogen peroxide and nitrite, generating detailed reports based on the selection.[28]

2.3 Preliminary testing of cow milk for hydrogen peroxide and sodium nitrite

To detect adulteration in cow milk samples, preliminary tests are necessary to understand the presence or absence of hydrogen peroxide and sodium nitrite as the first step of the analysis. The National Dairy Development Board (NDDB) kit was used for the preliminary detection of hydrogen peroxide and nitrite in cow milk samples. However, the quantification of the adulterants is not possible with this kit. Fresh cow milk samples procured locally at Hyderabad and Pilani, India, were tested for hydrogen peroxide and sodium nitrite using the NDDB kit.[29] Packaged cow milk (Amul Taaza) was used as a control, and the samples were divided into three sets and then tested in triplicates. The first set was used as a control, the second set was spiked with 0.02–0.08% (v/v) of peroxide, and the last set was spiked with 0.2–1 mM of sodium nitrite.

To perform the NDDB test for HP, 20 μL hydrogen peroxide reagent from the kit was added to 280 μL of each of the control cow milk samples, locally procured cow milk samples, and 0.2% hydrogen peroxide stock and incubated at 26–28 °C for 10 min for color development. Similarly, for testing nitrite, 60 μL of nitrite reagent was added to 240 μL each of the control cow milk samples, locally procured cow milk samples, and 1 mM sodium nitrite stock solution and incubated at 26–28 °C for 10 min to observe color development.

2.4 Detection of hydrogen peroxide using enzyme-immobilized strips

2.4.1 Streptavidin–horseradish peroxidase (Strep–HRP) immobilization using 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 uncoated polystyrene strips. 120 μL of the activation buffer was added to each of the eight wells. The activation buffer was discarded after incubating the strip at 4 °C overnight. The wells were washed with phosphate-buffered saline (PBS) (pH 7.2).

For hydrogen peroxide detection, the streptavidin-tagged HRP solution 20× was diluted for immobilization. It was highly specific due to streptavidin tagging and, hence, was diluted with PBS in different ratios, *i.e.*, 50×, 100×, 200×, 400×, 800×, and 1600×. An activated polystyrene strip was taken, and 100 μL of each dilution was added to the 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, covered with cello tape, and stored at 4 °C.

2.4.2 Testing varying HRP dilutions for strip reusability. HRP dilutions, 50 to 1600×, were immobilized on polystyrene strips. These strips were used to optimize the best possible enzyme dilution ratio for detection specificity, reuse, and strip longevity. A standard HRP–TMB–hydrogen peroxide detection assay was performed to analyze the color development observed with each immobilized enzyme dilution. 100 μL of PBS was added to the wells containing immobilized enzyme, and the strip was incubated for 10 minutes. 100 μL TMB substrate was added, and the strip was incubated again for 10 minutes. 3 μL of 0.04% (v/v) hydrogen peroxide was added to each of the wells, and the strip was incubated for 10–12 minutes for the color to develop. Absorbance readings were recorded at 630 nm. The wells were washed and reused for the same assay three times further to assess the reusability of the immobilized enzyme and to determine the final optimized Strep–HRP dilution for kit development.

2.4.3 HRP–TMB detection method for hydrogen peroxide. Enzyme assays were performed using varying HRP dilutions. An HRP–TMB assay was performed using varying concentrations of hydrogen peroxide to obtain a standard graph using 400× diluted HRP. To 100 μL of 1 : 400 Strep–HRP dilution, 100 μL TMB was added, and the strips were incubated for 5 min 2–8 μL of 0.04% hydrogen peroxide was added to each of the wells, and the strip was incubated for 10–12 minutes for the color to develop. Absorbance was recorded at 630 nm.

For performing a standard assay using immobilized HRP enzyme strips, 100 μL of PBS was added to the wells with 1 : 400 diluted and immobilized HRP enzyme, and the strip was incubated for 10 minutes. 100 μL TMB was added, and the strip was incubated again for 10 minutes. 2–8 μL of 0.04% hydrogen peroxide was added to each of the wells, and the strip was incubated for 10–12 minutes for the color to develop. Absorbance readings were taken at 630 nm. The same protocol was followed for detecting hydrogen peroxide in cow milk samples using immobilized Strep–HRP strips. The polystyrene strips were washed with PBS, covered with cellophane tape, and stored at 4 °C. Standard curves obtained using HRP solution and immobilized HRP were compared and analysed.

The developed method for the colorimetric HP sensor has been summarized in the following schematic (Fig. 2):

2.5 Detection of nitrite using Griess assay strips

2.5.1 Nitrite detection strip preparation. 5× NED solution was prepared in distilled water, and 5× sulfanilamide solution was prepared in 5% hydrochloric acid. 5× Griess reagent was prepared by mixing NED and sulfanilamide solutions in a ratio of 1 : 1. 6% PEG was used as a stabilizer for the study.

For nitrite detection, two eight-well strips were taken. For the first strip, reagents were coated as prepared without adding PEG. However, for coating the second strip, PEG was added to NED and Griess reagent as a stabilizer before coating.

The first well was left uncoated for both strips, and the subsequent three wells were coated with 1×, 2.5×, and 5× concentration strength of NED. The fifth well was left uncoated, and the subsequent three wells were coated with 1×, 2.5×, and 5× concentration strengths of Griess reagent. These strips with reagent solutions were left to dry at 30 °C for 60 minutes in a vacuum concentrator (Eppendorf, Hamburg, Model No.: 5305). The protocol was chosen to achieve uniform reagent coating on a strip surface while simultaneously reducing drying time without substantially compromising the reagent's activity.[21,24,30] The reagent needed to be coated and, hence, dried on the surface of a microassay strip well. With the limited surface area availability of 0.28 cm^2, the uniform drying of the enzyme solution becomes essential. This ensures maximum interaction with distilled water for activation, reducing the time required for detection of adulteration by quick color change. These imperative pointers help in the production of uniform reagent-covered diagnostic test strips and other similar applications thus avoiding false positive test results. After drying, the reagent-coated wells were covered with cello tape and stored at 4 °C.

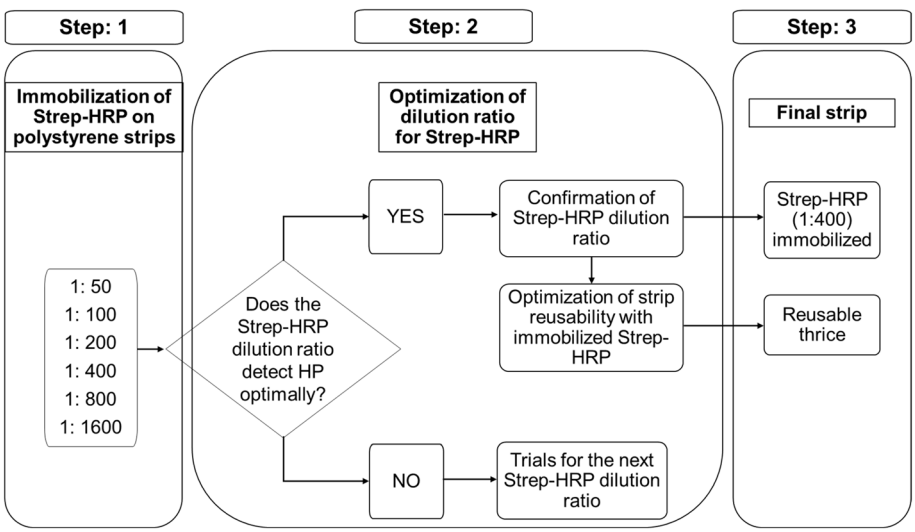

Fig. 2 Protocol for detection of HP in cow milk using immobilized Strep–HRP.

2.5.2 Comparison of NED and Griess reagent-coated strips for nitrite detection. Two of the above-prepared strips were taken. In the strips, 147 μL distilled water was added to wells coated with NED, and 297 μL distilled water was added to wells coated with Griess reagent, and the strips were incubated at room temperature, 26–28 °C, for 15 minutes. After 15 minutes, 150 μL sulfanilamide was added to strips coated with NED. 3 μL of sodium nitrite from 1 mM stock solution was added to each well. The development of pink color was observed, and the absorbance was recorded at 540 nm. The strips were used again to assess reusability and determine the number of days the reagents remained stable with or without PEG.

2.5.3 Detection of nitrite using Griess reagent-coated wells. Griess assay was performed using varying concentrations of sodium nitrite to obtain a standard graph. 1 mL of 1 mM stock solution of sodium nitrite was prepared. For a standard graph, 142 μL of 1× NED and 2–8 μL of sodium nitrite solution were added in each well except the first one, which was left blank. The strip was incubated for 5 minutes, and then 150 μL of 1× sulfanilamide was added to the well. Instantaneous development of pink color was observed. Absorbance was recorded at 540 nm. For performing a standard assay using coated Griess reagent, 292 μL of distilled water was added to the wells with coated reagent, and the strip was incubated for 10 minutes. 2–8 μL of 1 mM sodium nitrite was added to each well, except the first one that was left uncoated, and the strip was incubated at room temperature, 26–28 °C, for 5 minutes for the color to develop. Absorbance readings were taken at 540 nm. The same protocol was followed for detecting nitrite in cow milk samples using strips coated with Griess reagent to which PEG was added for reagent stabilization. The polystyrene strips were rinsed with distilled water, covered with cellophane tape, and stored at 4 °C. The developed method for the nitrite sensor is summarized in Fig. 3:

The following schematic provides a diagrammatic visualization of the working of the adulterant sensing methods (Fig. 4):

3 Results and discussion

The experiments for qualitative and quantitative hydrogen peroxide and nitrite measurements using the developed kit were performed in triplicates. 11 Samples procured from Hyderabad and Pilani were used for the study. Statistical analysis of the results was performed using IBM SPSS 29.0.2.0 software. The variance was calculated using ANOVA for two factors with replication.

3.1 Toxicity prediction using ProTox – III

The toxicity assessment of a compound based on ProTox – III offers insights into its specific effects on organs and organ systems. Based on selections, reports were generated for hydrogen peroxide and sodium nitrite, presented in Fig. 5a and b, illustrating their observed toxicity in human organs and organ systems.

The findings indicated that hydrogen peroxide belongs to class IV with an LD_{50} value of 376 mg kg^{-1}, while sodium nitrite was classified under class II, with an LD_{50} value of 434 mg kg^{-1}. This suggests that significant harm is likely with regular consumption of these compounds relative to body weight. Moreover, the reports were scrutinized to discern potential toxicities depicted in the prediction model as active probabilities. Referring to Fig. 5a and b, it is evident that hydrogen peroxide contributes to ecotoxicity, while sodium nitrite can cause immunotoxicity, mutagenicity, and ecotoxicity. These predictions are based on probability and structural resemblance among toxic components or molecules referred to as analogs, with effects varying depending on human anatomy and genetics.[31]

3.2 Preliminary testing of cow milk samples for hydrogen peroxide and nitrite using NDDB kit

As a preliminary detection using the hydrogen peroxide reagent provided in the NDDB kit, no red color developed for any of the

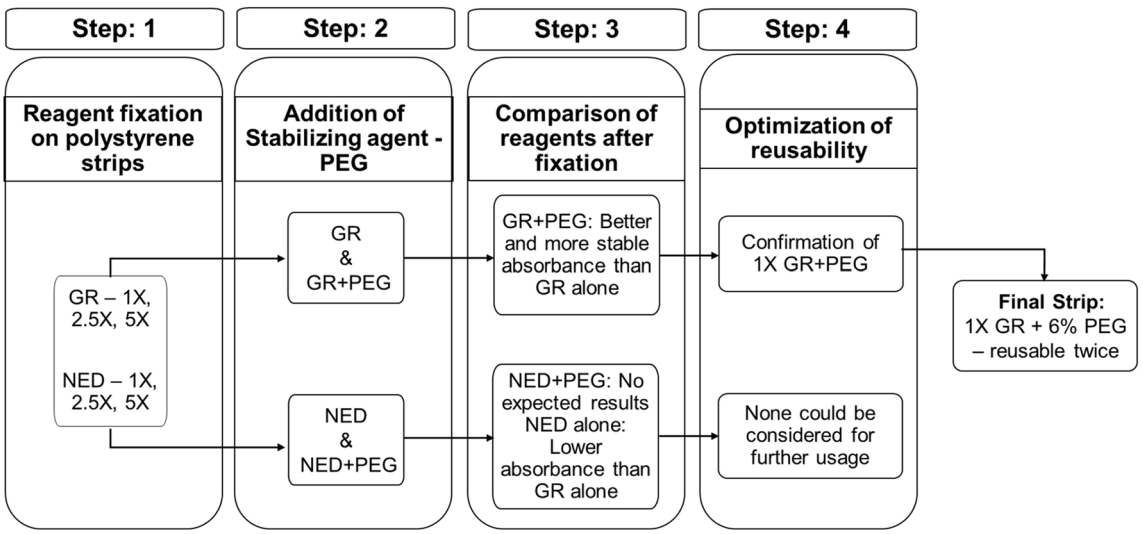

Fig. 3 Protocol summary for detection of nitrite using fixed Griess reagent stabilized with PEG.

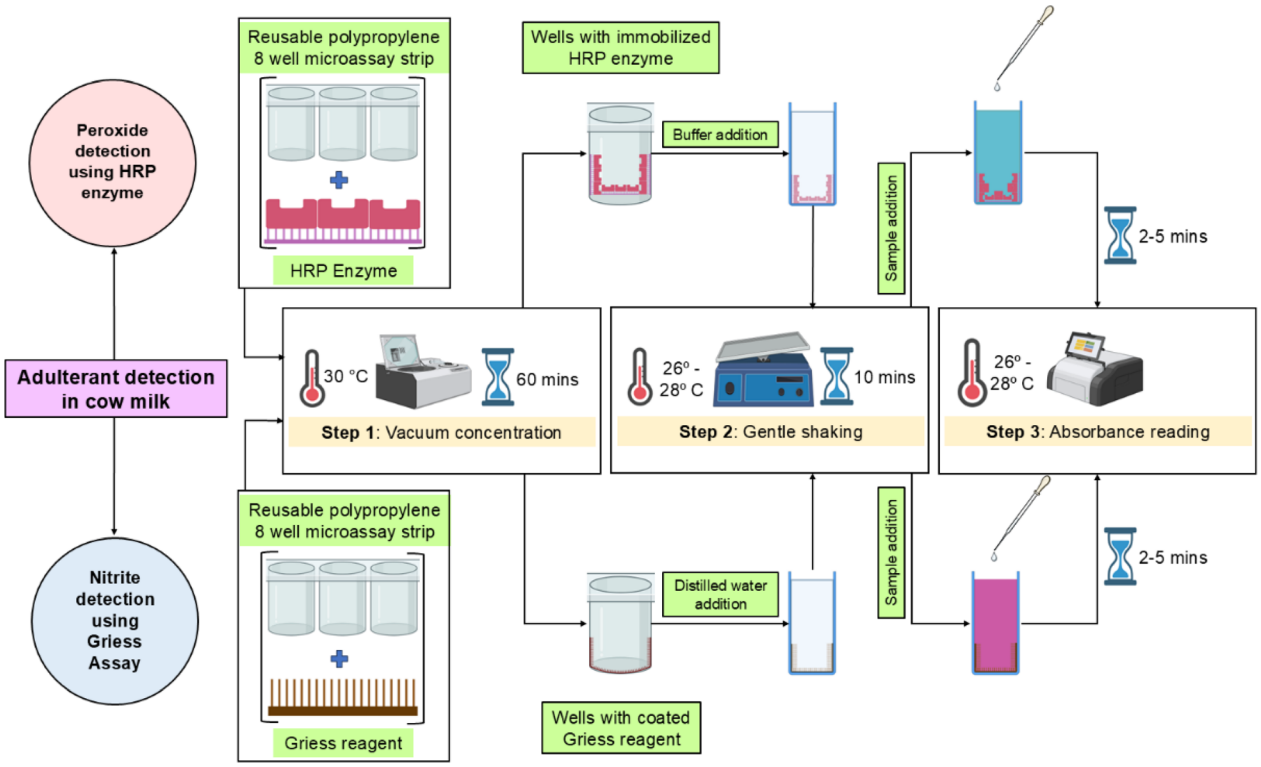

Fig. 4 Schematic for the sensing methods used for hydrogen peroxide and nitrite detection in cow milk.

fresh cow milk samples, confirming that no added hydrogen peroxide was present.[32] Different intensities of red color were observed for spiked samples, confirming the addition of peroxide. Dilution of cow milk with pond or tap water indirectly adds nitrites to cow milk.[33] Upon addition of pond water detection reagent from the NDDB kit, none of the cow milk samples developed a blue color. However, samples spiked with 0.2–1 mM sodium nitrite developed an increasing intensity of blue color, confirming the adulteration with nitrite.

3.3 Qualitative and quantitative detection of hydrogen peroxide using immobilized HRP strips

The HRP is a specific enzyme for detecting hydrogen peroxide in the presence of TMB as its substrate. The tagging of streptavidin with HRP increases its specificity for detection. Hence, a very low enzyme concentration is enough to detect hydrogen peroxide.[34] The reaction can be summarized as follows:

$$\text{Colorless TMB} + \text{hydrogen peroxide} \xrightarrow{\text{horseradish peroxidase}} \text{blue colored TMB}$$

Optimizing the concentration of streptavidin–HRP is critical to efficiently detect hydrogen peroxide in cow milk. Immobilizing a higher concentration of streptavidin–HRP does not provide efficient results as the ratio between substrate and enzyme needs to be optimum for detection. Different dilutions of Strep–HRP in the range of 1 : 50–1 : 1600 were used to understand the best dilution ratio for the development of a reusable hydrogen peroxide detection kit. Two assays were set up in parallel to optimize the following:

(a) The first would confirm the most effective Strep–HRP dilution ratio for optimum color development in 10 minutes, further ensuring the developed color's stability for approximately an hour (60 minutes).

(b) The subsequent assays would help determine the number of times a single well with immobilized Strep–HRP could be used for efficient hydrogen peroxide detection.

Fig. 6a depicts the results of the first assay. Dilutions 1 : 50 and 1 : 100 were highly concentrated for hydrogen peroxide detection, and the results obtained did not give the desired cyan color. Instead, the change was relatively quick from cyan to dark green to colorless, which could not be recorded in real-time. Hence, Fig. 6a does not include the results for these two dilutions. From Fig. 6a, it can be deduced that the color change was quick for 1 : 200 dilution of Strep–HRP, and the highest optimum cyan color absorbance was recorded within four minutes. However, the color intensity later reduced. Among the dilution ratios 1 : 400, 1 : 800, and 1 : 1600, the best development time was observed for the 1 : 400 ratio. Within seven minutes, the highest absorbance for cyan color was obtained, and it remained stable for the next three minutes, and on further incubation, the color continued to remain so. This might be due to the stable nature of Strep–HRP and the optimum substrate-to-enzyme ratio required for best detection.[12]

Repetitive assays were performed with different immobilized Strep–HRP dilutions to optimize the reusability of the detection

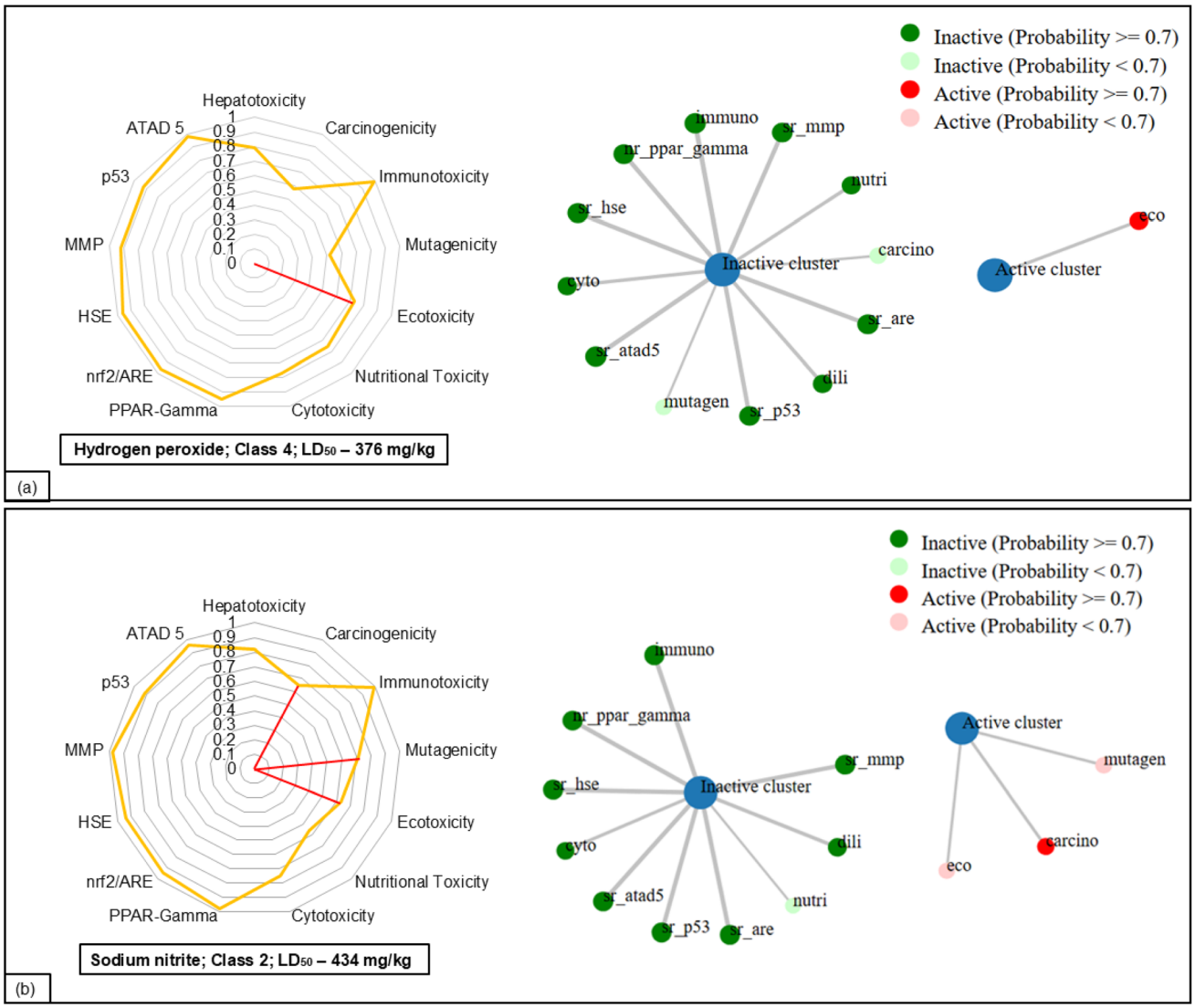

Fig. 5 ProTox – III used for prediction of toxicity in humans for (a) hydrogen peroxide and (b) sodium nitrite.

strip. The results of these assays are depicted in Fig. 6b. Immobilization of 1 : 50 and 1 : 100 dilutions resulted in quick color change followed by rapid loss of enzyme upon washing the well for reuse, whereas for 1 : 200–1 : 1600 dilutions, optimum cyan color was developed that remained stable.[35] Washing the used wells and reusing them for detection helped conclude that optimally, only three uses were possible with the immobilization of a 1 : 400 ratio of Strep–HRP dilution. Rapid enzyme loss and degradation were observed for 1 : 50, 1 : 100, and 1 : 200 Strep–HRP dilutions, whereas the color development was extremely slow for 1 : 800 and 1 : 1600 dilutions after the wash.

From the results in Fig. 6a and b, it can be concluded that the dilution 1 : 1600 did not have an optimum enzyme concentration to detect hydrogen peroxide. In contrast, dilutions 1 : 200, 1 : 400, and 1 : 800 could be compared for their activities and stability of developed color. Two-factor ANOVA analysis was done for the samples using SPSS software, and the results have been summarized in Fig. 6c. For the kit to be considered stable, dilution 1 : 400 seemed appropriate, with the least standard deviations among the results and clear variability. Although for 1 : 800 dilution, the results were optimal, the development of color was slow, and even after 10 minutes, it was not stable. At 1 : 200, the developed color began fading after 5 minutes and hence could not be considered for stability. Thus, 1 : 400 dilution was finalized for the hydrogen peroxide detection strip.

After the dilution of 1 : 400 was finalized for Strep–HRP immobilization, the performance efficacy of the enzyme solution was compared with that of the immobilized enzyme strip, as depicted in Fig. 6d. Based on the standard curves obtained while testing the standard hydrogen peroxide stock solution, the R^2 values were optimal for the Strep–HRP solution and immobilized Strep–HRP 1 : 400 dilution ratio. Immobilized Strep–HRP provided the absorbance results with a better R^2 value ($\approx$0.97) for 1 : 400 dilution, while the other dilutions, 1 : 800 and 1 : 1600, were comparatively inefficient. Lastly, the shelf life and stability of the developed strip are essential for a consumer. Hence, assays were performed every alternate day to check the detection efficiency of the strip with immobilized

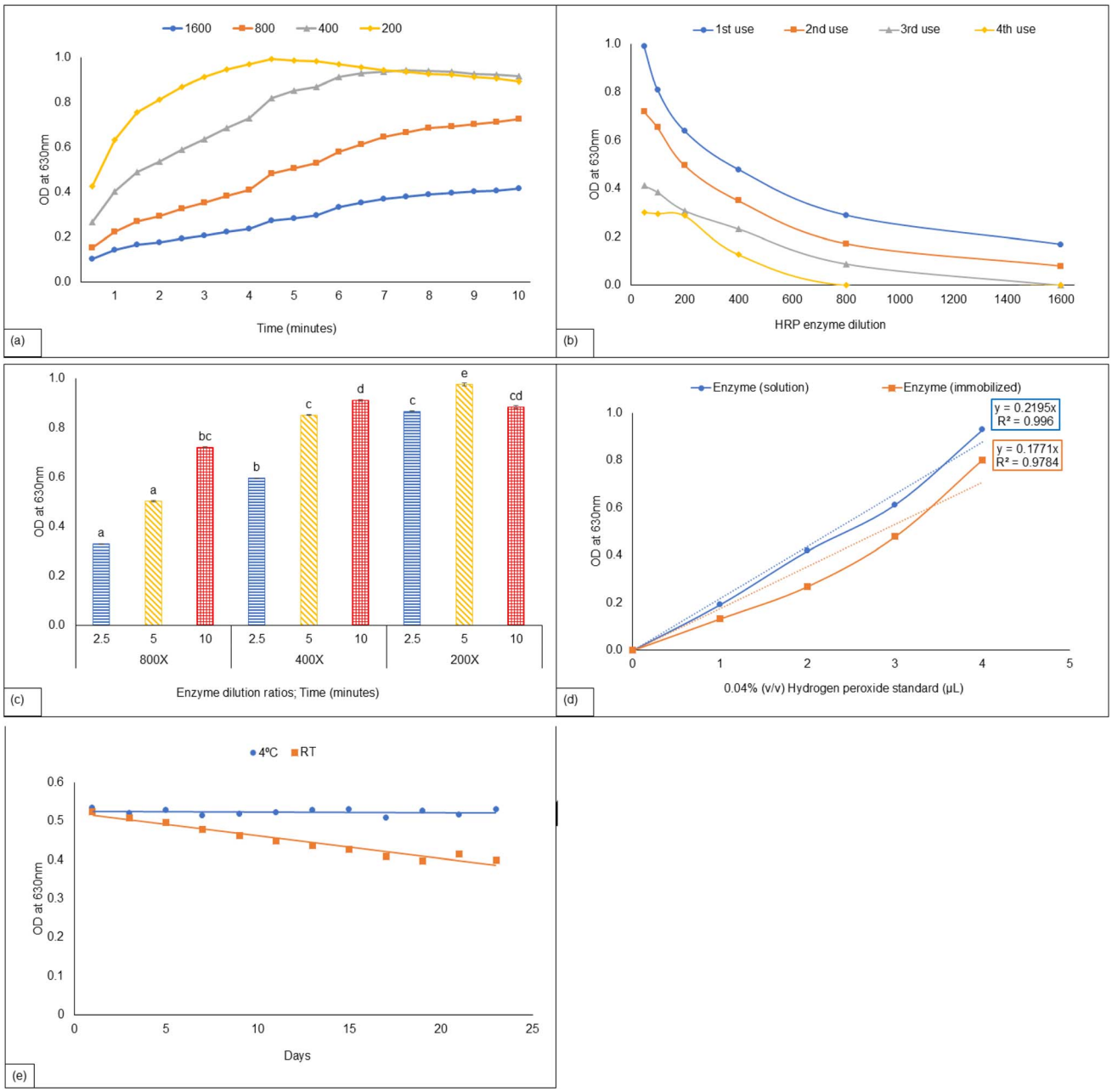

Fig. 6 Results of streptavidin–HRP immobilization for hydrogen peroxide detection – (a) time taken for optimum color development for different Strep–HRP dilutions; (b) optimization of Strep–HRP dilution based on the number of uses/well; (c) ANOVA results for optimum dilutions and time taken for color development; (d) standard curves comparing the efficiency of hydrogen peroxide detection using Strep–HRP solution and immobilized Strep–HRP; (e) shelf life and stability assessment of the immobilized Strep–HRP kit for hydrogen peroxide detection.

Strep–HRP. From Fig. 6e, it is concluded that at 4 °C, the strip is functional at its best. In contrast, detection efficiency was found to slowly reduce over 25 days when stored at room temperature in the range of 24–28 °C.[36]

Based on the results obtained, Fig. 7 indicates the working of the Hydrogen Peroxide Detection Kit. The spiked cow milk samples have been tested using the designed kit.‡[37]

3.4 Qualitative and quantitative detection of nitrite using strips coated with Griess reagent

When a sample containing sodium nitrite is added to the Griess reagent, it forms a magenta-pink-colored azo dye, and the absorbance is recorded at 540 nm.

$$\text{Colorless sample} \xrightarrow[\text{NED+sulfanilamide}]{\text{Griess reagent}} \text{magenta pink colored azo dye}$$

To detect the presence of water in cow milk samples, indirect determination of the presence of nitrite is optimized by using

‡ Indian patents with application no. 202411034469 and 202411043404, incorporating parts of this work, were filed on 30th April and 4th June 2024, respectively.

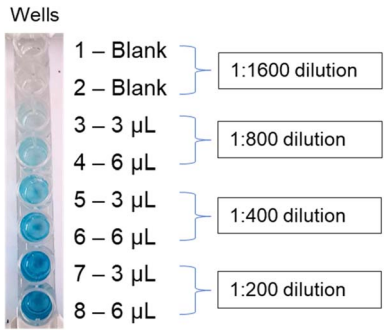

Fig. 7 Testing of spiked cow milk samples for hydrogen peroxide using immobilized Strep–HRP kit.

the Griess assay. The detection consists of the following two aspects:

(a) Determining the concentration of Griess reagent to be coated.

(b) The presence or absence of stabilizing agent to assess reusability of the Griess reagent coated strip.

To detect nitrite in the samples, a comparison was made to assess the detection efficiency of NED-coated strips against those coated with the Griess reagent. However, the efficiency of colour development followed by optimal absorbance was found to be better for Griess reagent-coated strips, and hence, the study was optimized accordingly.

Fig. 8a and b depict the efficiency of detecting sodium nitrite standards using 1× Griess reagent solution against different coated concentrations of Griess reagent coated at concentrations of 1×, 2.5×, and 5×. On comparison, it was observed that the assay performed using 1× solution had a very high detection efficiency with an R^2 value of 0.99, whereas an optimal R^2 value of 0.98 was obtained for 1× coated reagent. R^2 values for other concentrations, 2.5× and 5×, were not comparable and hence disregarded for consideration. Inverse variation between the reagent concentration and absorbance values signifies that the number of molecules of the reagent is far more than the number of molecules of the substance available for interaction, and hence, the absorbance is lower.[38]

Assays were performed to assess the stability and reusability of the wells coated with 1× Griess reagent. Fig. 8c depicts that the best detection was attained when the coated Griess reagent was stabilized with 6% PEG. When the well was coated with Griess reagent without PEG, the absorbance values in the second use dropped considerably. Coating with NED was another option. However, the absorbance values were lower when compared to the coated Griess reagent. The use of PEG as

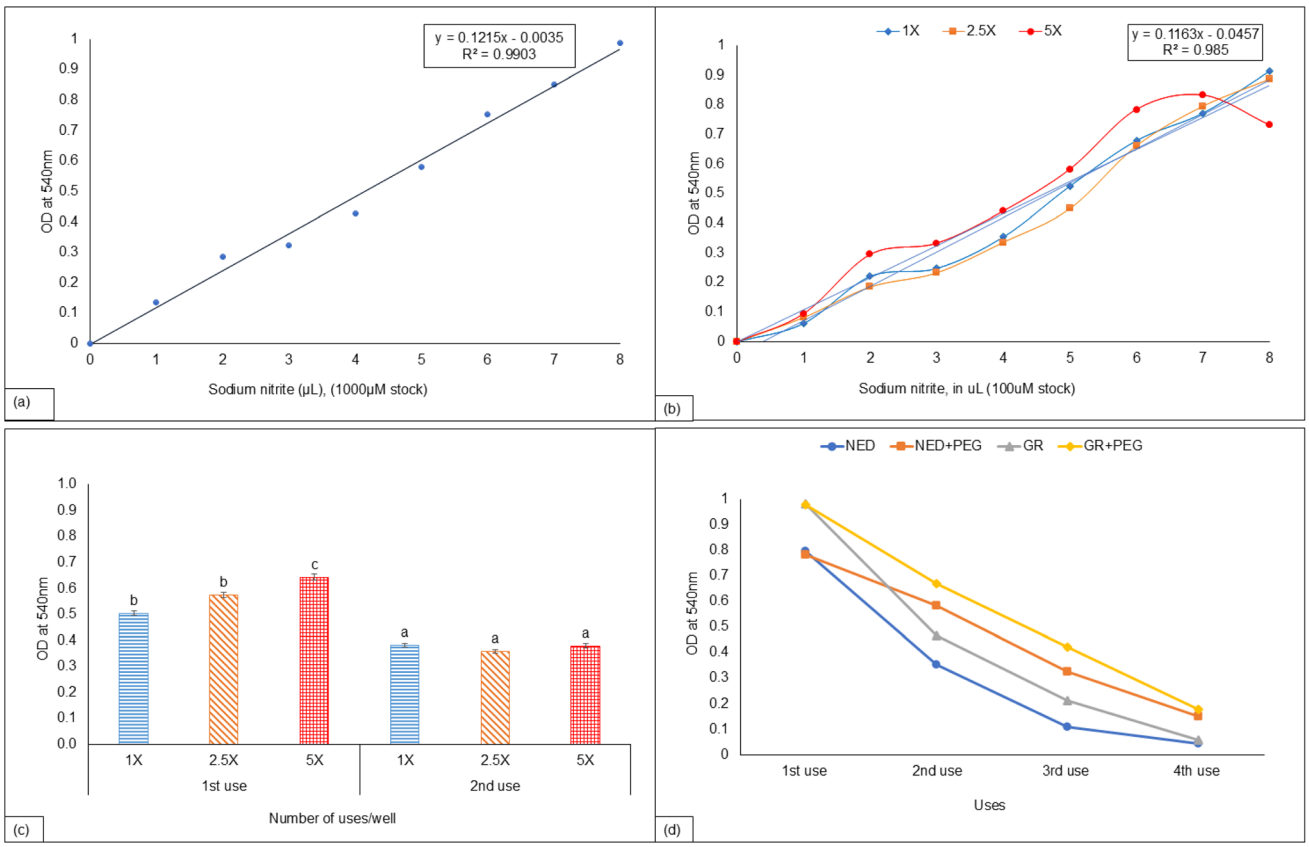

Fig. 8 Results of Griess reagent coating and stabilization for nitrite detection – (a) standard curve for detection of nitrite using 1× Griess reagent solution (b) standard curves for detection of nitrite using coated Griess reagent of different concentrations 1×, 2.5×, 5× (c) assessment of stability of coated Griess reagent for usage in presence and absence of PEG as stabilizing agent (d) ANOVA results for assessment of standard deviation and optimum number of reuses of coated Griess reagent of different concentrations.

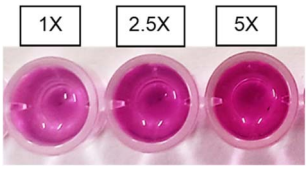

Fig. 9 Testing of spiked cow milk samples for nitrites using PEG stabilized Griess Assay kit.

a stabilizer allowed reusing the well optimally up to 3 times, wherein the third time results were not quantitative but could provide an understanding of the presence or absence of nitrite, and the fourth time results could not be considered for any color change and hence were termed as redundant. These observations, with a reduction of absorbance values and two optimal reuses of the well, were due to the washing of wells after every detection assay and, hence, the loss of coated reagent.[39]

The strip coated with Griess reagent and stabilized with PEG was finalized for nitrite detection. However, ANOVA analysis for samples revealed that the concentration of 1× or 2.5× would be optimal for detection, as depicted in Fig. 8d, and there was no significant difference in detection values. However, 1× concentration would be preferable for cost efficacy and reduced reagent loss. Further, each well could only be used twice to attain recordable results for nitrite detection. The graph shows a 50% retention of Griess reagent activity after the first use. The activity loss was observed to be 24.5%, 37.6%, and 41% for 1×, 2.5×, and 5× concentrations of coated Griess reagent, respectively, after the first use. Hence, 1× proves to be justifiable for coating.

Finalization of strength for Griess reagent coating as 1× with stabilization achieved using 6% PEG resulted in a reusable sensor strip to detect nitrite in cow milk samples. The functioning of the nitrite kit is depicted in Fig. 9 with the strip coated with different concentrations of Griess reagent with cow milk samples spiked with increasing concentrations of sodium nitrite.‡[40] Hence, the evident increase in azo dye formation with a deeper magenta-pink color.

4 Conclusion

Cow milk adulterated with hydrogen peroxide was tested using the HRP–TMB method, while nitrite adulteration in cow milk was tested using the Griess assay. These reactions provide qualitative detection of the adulterants by color change and 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 the presence of hydrogen peroxide and nitrite in the cow milk. The methods were optimized for the following factors: (i) optimization of Strep–HRP concentration immobilized on polystyrene strips for hydrogen peroxide detection, (ii) optimization of concentration of Griess reagent to be coated with a stabilizer for nitrite detection, (iii) time of incubation for quick detection, and stability of color and (iv) days for which the strips can be stored and reused. Although Strep–HRP can be immobilized on a different strip and similarly for coating Griess reagent, these two can also be presented over a single 8-well strip with 4 wells dedicated to each adulterant detection, hence making the kit novel with two biochemical assays being performed on a single platform. The Strep–HRP complex – TMB substrate combination was optimized to identify hydrogen peroxide levels up to 0.04% (v/v), which signifies sensitivity of detecting peroxide concentrations of 13 mM and above. Hence, the limit of detection for the hydrogen peroxide kit was found to range from 1.4–5.9 μg mL^{-1}. In the biosensor setup for nitrite detection, optimization of the Griess reagent followed by stabilization with polyethylene glycol (PEG), enabled the detection of concentrations of nitrite at 32 μg mL^{-1} and above. The assay is sensitive to detect nitrite up to 37 μM concentration; hence, the detection limit for the kit was found to be in the range of 25–185 ng mL^{-1}. The strips were robust enough to support the detection of hydrogen peroxide for over 25 days with three times the reusability of a single well and that for the Griess reagent for around 10 days, with the reusability of each well up to two times. The detection efficiency of both strips was more than 95%. Although the wells were reusable 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 active sites if the strip is used for detecting adulteration of skimmed cow milk, change in the pH of reagents used for detection were some of the most common reasons due to which detection activity might get inhibited. However, developing these strips to detect hydrogen peroxide and nitrite would help many people understand the quality of cow milk they consume. The nitrite detection strip would help solve the significant problem of adding pond or tap water to cow milk in areas where unorganized sectors carry out the distribution. It can be of great assistance to check for cow 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 *RSC Advances Journal*.

Data availability

All data generated or analyzed during this study are included in this published article.

Author contributions

SK and HB conceptualized and developed the methodology for this research work. HB was awarded with the funding, investigated and performed the experimentation, cured the data and formally analyzed it, validated the conclusions, wrote, reviewed, and edited the original manuscript draft. SGP supervised the work, validated the results, reviewed and edited the manuscript draft, supported with the required resources and data curation.

Conflicts of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Acknowledgements

HB thanks the Indian Council of Medical Research (ICMR) for funding this research work vide 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.

References

1 M. N. L. Aishwarya and D. m. badrud, A Review on Adulteration of Milk, *Indo Am. J. Pharm. Res.*, 2017, 7(08), 6.
2 A. Dajsiripun, *Fighting Fraud: Detecting Intentional Adulteration in Milk*, SGS/Food Contaminats, Published online, 2017, pp. 3–5.
3 B. P. Neo, *Milk in India found to be most highly-adulterated food product despite FSSAI assurances of safety*, Food Navigator - Asia, 2019, vol. 8(5), p. 55.
4 M. J. A. Lima, M. K. Sasaki, O. R. Marinho, *et al.*, Spot test for fast determination of hydrogen peroxide as a milk adulterant by smartphone-based digital image colorimetry, *Microchem. J.*, 2020, **157**, 105042, DOI: 10.1016/j.microc.2020.105042.
5 B. Halliwell, M. V. Clement and L. H. Long, Hydrogen peroxide in the human body, *FEBS Lett.*, 2000, **486**(1), 10–13, DOI: 10.1016/S0014-5793(00)02197-9.
6 L. T. Wu, C. C. Chu, J. G. Chung, *et al.*, Effects of tannic acid and its related compounds on food mutagens or hydrogen peroxide-induced DNA strands breaks in human lymphocytes, *Mutat. Res. Fund Mol. Mech. Mutagen*, 2004, **556**(1–2), 75–82, DOI: 10.1016/j.mrfmmm.2004.07.004.
7 G. M. Benhusein, E. Mutch, S. Aburawi and F. M. Williams, Genotoxic effect induced by hydrogen peroxide in human hepatoma cells using comet assay, *Libyan J. Med.*, 2010, **5**(1), 1–6, DOI: 10.3402/ljm.v5i0.4637.
8 G. Razo-Aguilera, R. Baez-Reyes, I. Álvarez-González, R. Paniagua-Pérez and E. Madrigal-Bujaidar, Inhibitory effect of grapefruit juice on the genotoxicity induced by hydrogen peroxide in human lymphocytes, *Food Chem. Toxicol.*, 2011, **49**(11), 2947–2953, DOI: 10.1016/j.fct.2011.07.003.
9 P. Rai, S. Verma, S. Mehrotra and S. K. Sharma, A QR code-integrated chromogenic paper strip for detection of hydrogen peroxide in aqueous samples, *Anal. Methods*, 2023, **15**(40), 5286–5293, DOI: 10.1039/d3ay01584j.
10 N. S. Hill and G. Turner, Calibration of a Fourier transform infrared spectrometer for hydrogen peroxide vapour measurement, *Anal. Methods*, 2011, **3**(8), 1901–1905, DOI: 10.1039/c1ay05170a.
11 N. A. M. Taib, S. N. M. Shazlan, N. N. Adnan and N. E. Emran, Optical sensor response towards hydrogen peroxide in low fat and full cream milk, *J. Phys.:Conf. Ser.*, 2023, **2432**(1), 012005, DOI: 10.1088/1742-6596/2432/1/012005.
12 J. E. Giaretta, H. Duan, F. Oveissi, S. Farajikhah, F. Dehghani and S. Naficy, Flexible Sensors for Hydrogen Peroxide Detection: A Critical Review, *ACS Appl. Mater. Interfaces*, 2022, **14**(18), 20491–20505, DOI: 10.1021/acsami.1c24727.
13 H. Vasconcelos, A. Matias, J. Mendes, *et al.*, Compact biosensor system for the quantification of hydrogen peroxide in milk, *Talanta*, 2023, **253**, 124062, DOI: 10.1016/j.talanta.2022.124062.
14 Z. Xu, C. Zeng, Y. Zhao, *et al.*, Smartphone-based on-site detection of hydrogen peroxide in milk by using a portable ratiometric fluorescent probe, *Food Chem.*, 2023, **410**, 135381, DOI: 10.1016/j.foodchem.2022.135381.
15 M. Tiso and A. N. Schechter, Nitrate reduction to nitrite, nitric oxide and ammonia by gut bacteria under physiological conditions, *PLoS One*, 2015, **10**(3), 1–18, DOI: 10.1371/journal.pone.0119712.
16 M. Moazeni, Z. Heidari, S. Golipour, L. Ghaisari, M. Sillanpää and A. Ebrahimi, Dietary intake and health risk assessment of nitrate, nitrite, and nitrosamines: a Bayesian analysis and Monte Carlo simulation, *Environ. Sci. Pollut. Res.*, 2020, **27**(36), 45568–45580, DOI: 10.1007/s11356-020-10494-9.
17 V. M. Ivanov, The 125th anniversary of the Griess reagent, *J. Anal. Chem.*, 2004, **59**(10), 1002–1005, DOI: 10.1023/B:JANC.0000043920.77446.d7.
18 C. R. Sawicki and F. P. Scaringelli, Colorimetric determination of nitrate after hydrazine reduction to nitrite, *Microchem. J.*, 1971, **16**(4), 657–672, DOI: 10.1016/0026-265X(71)90059-2.
19 Y. Chen, C. Zhao, G. Yue, *et al.*, A highly selective chromogenic probe for the detection of nitrite in food samples, *Food Chem.*, 2020, **317**, 126361, DOI: 10.1016/j.foodchem.2020.126361.
20 M. K. Kirkwood and K. E. Olson, *Enzyme Drying Process*, Published online October 14, 1986, pp. 1–9.
21 S. Maurer, R. Bayer, M. Budde, *et al.*, *Method for Immobilizing and Drying of Enzymes*, Published online June 25, 2015, pp. 1–17.
22 T. A. Costa Silva, C. R. F. Souza, S. Said and W. P. Oliveira, Drying of enzyme immobilized on eco-friendly supports, *Afr. J. Biotechnol.*, 2015, **14**(44), 3019–3026, DOI: 10.5897/ajb2015.14830.
23 S. S. de Jesus and R. M. Filho, Optimizing drying conditions for the microwave vacuum drying of enzymes, *Dry. Technol.*, 2011, **29**(15), 1828–1835, DOI: 10.1080/07373937.2011.605977.
24 G. P. Costa, L. B. Queiroz, V. Manfroi, R. C. Rodrigues and P. F. Hertz, Immobilization of Alpha Acetolactate Decarboxylase in Hybrid Gelatin/Alginate Support for Application to Reduce Diacetyl Off-Flavor in Beer, *Catalysts*, 2023, **13**(3), 601, DOI: 10.3390/catal13030601.
25 S. S. De Jesus and R. Maciel Filho, Drying of α-amylase by spray drying and freeze-drying - A comparative study, *Braz. J. Chem. Eng.*, 2014, **31**(3), 625–631, DOI: 10.1590/0104-6632.20140313s00002642.

26 S. M. Ulven, K. B. Holven, A. Gil and O. D. Rangel-Huerta, Milk and Dairy Product Consumption and Inflammatory Biomarkers: An Updated Systematic Review of Randomized Clinical Trials, *Adv. Nutr.*, 2019, **10**(2), S239–S250, DOI: 10.1093/advances/nmy072.

27 C. D. Arulanandam, J. S. Hwang, A. J. Rathinam and H. U. Dahms, Evaluating different web applications to assess the toxicity of plasticizers, *Sci. Rep.*, 2022, **12**(1), 1–8, DOI: 10.1038/s41598-022-18327-0.

28 S. Ghosh, P. Tripathi, P. Talukdar and S. N. Talaptara, In silico study by using ProTox-II webserver for oral acute toxicity, organ toxicity, immunotoxicity, genetic toxicity endpoints, nuclear receptor signalling and stress response pathways of synthetic pyrethroids, *World Sci. News*, 2019, **132**, 35–51.

29 F. Safety, FSSAI Categorization of Milk Products and their Standards, *Standards of Milk for Indian Markets*, Published online, 2020, pp. 1–4.

30 J. Nam, I. B. Jung, B. Kim, *et al.*, A colorimetric hydrogel biosensor for rapid detection of nitrite ions, *Sens. Actuators, B*, 2018, **270**, 112–118, DOI: 10.1016/j.snb.2018.04.171.

31 P. Banerjee, A. O. Eckert, A. K. Schrey and R. Preissner, ProTox-II: A webserver for the prediction of toxicity of chemicals, *Nucleic Acids Res.*, 2018, **46**(W1), W257–W263, DOI: 10.1093/nar/gky318.

32 Food safety and standards authority of India, *Manual of Methods of Analysis of Foods, Milk and Milk Products*, Fssai, 2012, vol. 204, p. 204.

33 Nitrate and Nitrite in Drinking Water, *Nitrate and Nitrite in Drinking Water*, Ashington WDC, Published online, 1995, DOI: 10.17226/9038.

34 N. Bashir, M. Sood and J. D. Bandral, Enzyme immobilization and its applications in food processing: A review, *Int. J. Chem. Stud.*, 2020, **8**(2), 254–261, DOI: 10.22271/chemi.2020.v8.i2d.8779.

35 J. L. House, E. M. Anderson and W. K. Ward, Immobilization techniques to avoid enzyme loss from oxidase-based biosensors: A one-year study, *J. Diabetes Sci. Technol.*, 2007, **1**(1), 18–27, DOI: 10.1177/193229680700100104.

36 J. Pla-Tolós, Y. Moliner-Martinez, C. Molins-Legua and P. Campins-Falcó, Colorimetic biosensing dispositive based on reagentless hybrid biocomposite: Application to hydrogen peroxide determination, *Sens. Actuators, B*, 2016, **231**, 837–846, DOI: 10.1016/j.snb.2016.03.094.

37 H. Bharwani and S. G. Palani, *Peroxide Testing (PeT) Kit for Adulterant Detection and Method Thereof*, Published online, 2024, vol. 14.

38 L. Hakobyan, B. Monforte-Gómez, Y. Moliner-Martínez, C. Molins-Legua and P. Campíns-Falcó, Improving Sustainability of the Griess Reaction by Reagent Stabilization on PDMS Membranes and ZnNPs as Reductor of Nitrates: Application to Different Water Samples, *Polymers*, 2021, **14**(3), 464, DOI: 10.3390/polym14030464.

39 A. N. Avan, S. Demirci-Çekiç and R. Apak, Colorimetric Nanobiosensor Design for Determining Oxidase Enzyme Substrates in Food and Biological Samples, *ACS Omega*, 2022, **7**(48), 44372–44382, DOI: 10.1021/acsomega.2c06053.

40 H. Bharwani and S. G. Palani, *Nitrite Testing (NiT) Kit for Detection of Adulteration in Cow Milk and the Process Thereof*, Published online, 2024, p. 11.