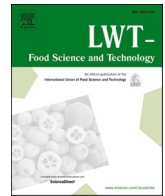




Contents lists available at ScienceDirect

LWT

journal homepage: [www.elsevier.com/locate/lwt](http://www.elsevier.com/locate/lwt)

## Use of natural plant extracts as a novel microbiological quality indicator in raw milk: An alternative for resazurin dye reduction method

Ranga Madushan<sup>a</sup>, Janak K. Vidanarachchi<sup>a,\*\*</sup>, P.H.P. Prasanna<sup>a</sup>, Shanika Werellagama<sup>a</sup>, Hasitha Priyashantha<sup>b,\*</sup>

<sup>a</sup> Department of Animal Science, Faculty of Agriculture, University of Peradeniya, Peradeniya, 20400, Sri Lanka

<sup>b</sup> Department of Molecular Sciences, Swedish University of Agricultural Sciences, Box 7015, SE-750 07, Uppsala, Sweden

### ARTICLE INFO

#### Keywords:

Anthocyanin  
Plant aqueous extracts  
Microbiological safety  
Milk keeping quality  
Platform test

### ABSTRACT

In the present study, we propose a novel field-scale analytical tool for rapid screening of microbiological quality in raw milk using aqueous extracts of plant materials, i.e. flowers (e.g. *Hibiscus rosa-sinensis* L. and *Clitoria ternatea*), taproots (e.g. *Beta vulgaris*) and pricklypears (e.g. *Opuntia dillenii*). For each plant extract, the colour changes in raw milk were evaluated between 4.5 and 6.5 pH against the resazurin dye as a control. The plant anthocyanin content in each extract was analyzed by the differential pH method using a spectrophotometer. The *Hibiscus rosa-sinensis* flower extract was opted to further test since it had a pH-sensitive colour change (6.5; maroon to 6.2; light-pink) compared to other plant extracts, which did not indicate a noticeable colour variation with pH. Anthocyanin content of the Hibiscus extract was 0.59 g/mL. The novel method showed high linearity ( $R^2 = 0.95$ ), 100% accuracy and greater repeatability with an intermediate level of precision. The limit of quantification and detection was 0.46 and 0.15 g/mL, respectively. In conclusion, we demonstrated the potential in using water extract of *Hibiscus rosa-sinensis* L. flowers as an alternative to the resazurin dye reduction method for rapid and accurate microbiological quality control for raw milk procurement in remote areas.

### 1. Introduction

Many countries have adopted numerous milk quality regulations and standards that must be used in raw milk procurement (More, 2009). In most cases, among the small-scale dairy farmers, on-farm chilling of the raw milk is practically limited and thus, milk is kept at ambient temperature until it reached the regional chilling centres or pickup by the tank driver or milk collector. The time from milking to chilling may vary due to several practical reasons and because of this, microbial quality of the raw milk might be affected, since milk is perishable and often subjected to microbial quality deterioration, if not chilled immediately after milking (Muhammad et al., 2018). A study by De Silva, Kanugala, and Weerakkody (2016) reported, 20% of raw milk is discarded at chilling centres due to spoilage in every month in Sri Lanka, alarming the need for improved quality control in the dairy supply chain. Dairy processors, however, demand high-quality raw milk, often characterized by lower counts in somatic cells and viable microbes (More, 2009; Murphy, Martin, Barbano, & Wiedmann, 2016). Besides these quality control

requirements imposed by the producers, dairy farmers are receiving incentives for supplying high-quality raw milk, determined based on the limits of somatic cells and the bacterial count in the raw milk (Murphy et al., 2016). Hence, the microbiological content of raw milk is an important economical aspect for subsistent dairy farms, which often lacks the control or required knowledge for preliminary evaluation. Processing plants regularly monitor the quality and safety of incoming raw milk using platform tests to secure the safety and processability of raw milk (Murphy et al., 2016). One of the main platform tests used to identify the microbial quality of milk is resazurin dye reduction method and it has been long used to identify the microbial content of milk (Hudman & Sargentini, 2013; Muliro, Shalo, & Kutima, 2013). Resazurin dye is purple, non-toxic, oxidation-reduction indicators that convert into pink (resorufin) when subjected to oxidative-reduction reaction (Hudman & Sargentini, 2013). However, several problems are associated with this method and mainly the high-cost which limits the access of this technique at field-level and milk chilling centres. Thus, creating a necessity for seeking alternative and cost-effective control

\* Corresponding author.

\*\* Corresponding author.

E-mail addresses: [janakvid@pdn.ac.lk](mailto:janakvid@pdn.ac.lk) (J.K. Vidanarachchi), [Hasi.tvp@slu.se](mailto:Hasi.tvp@slu.se) (H. Priyashantha).

<https://doi.org/10.1016/j.lwt.2021.111221>

Received 30 December 2020; Received in revised form 17 February 2021; Accepted 27 February 2021

Available online 2 March 2021

0023-6438/© 2021 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

techniques that can be easily accessible in raw milk collection centres or small-scale dairy farmers.

Raw milk is an excellent medium for various microbial communities and provides a favourable physicochemical environment for harbouring a wide range of microbial consortiums (Garnier, Valence, & Mounier, 2017; Oliveira, Favarin, Luchese, & McIntosh, 2015; Perera, Munasinghe, & Marapana, 2019; Sowmya, 2017). The growth of microbiota in raw milk has great concerns regarding the safety and quality of the milk intended for consumption or processing (Li et al., 2018). This also leads to deterioration of the raw milk quality (e.g. reduction of pH, production of extracellular lipases and proteases) and eventually impacting the final processed product quality (Huck, Hammond, Murphy, Woodcock, & Boor, 2007; Schornsteiner, Mann, Bereuter, Wagner, & Schmitz-Esser, 2014; Lu et al., 2013; Knight-Jones, Hang'ombe, Songe, Sinkala, & Grace, 2016). The growth of microbiota in raw milk eventually leads to limit the shelf-life of milk, if not handled properly. It has shown if milk is not chilled immediately after milking (e.g. delayed up to 72 h) will reduce the milk pH and lactose content (Muhammad et al., 2018), which could have a serious impact on milk processing characteristics. These changes in physicochemical properties are suggested to associated with the growth of bacteria in milk.

Anthocyanins are natural compounds that belong to the group of natural pigments (flavonoids) possessed predominantly by higher plants (e.g. fruits, vegetables and flowers) although some are identified in lower plants (e.g. mosses and ferns) (Martín, Navas, Jiménez-Moreno, & Asuero, 2017). The diversity of anthocyanins are mainly influenced by its number and position of hydroxyl and methoxy groups on the basic skeleton (Miguel, 2011). The colour of the anthocyanins is pH-dependent and (Cisowska, Wojnicz, & Hendrich, 2011; Dangles & Fenger, 2018; Ibrahim, Muhammad, & Salleh, 2011; Miguel, 2011) it also influences the chemistry and stability of anthocyanins (Martín et al., 2017). Thus, changes in the colour of anthocyanins could serve as a complementary pH indicator to identify different pH levels in different aqueous media. Anthocyanins are water-soluble, mainly because of its sugar molecules (Gallik, 2011) and thus provide the potentials for using in aqueous media such as milk. Currently, anthocyanins are used in the food, pharmaceutical, cosmetic and textile industries due to their anti-oxidant activity, detoxifying, anti-proliferation, induction of apoptosis, anti-inflammatory activity and its anti-angiogenic activity (Cisowska et al., 2011; Dangles & Fenger, 2018; Hayat & Jacob, 2019; Miguel, 2011).

However, the use of plant anthocyanin to assess the microbial quality of milk has not received attention. Therefore, this study hypothesised the anthocyanin content of the natural plant extract can be used to determine the microbial quality of raw milk instead of the commonly practised resazurin dye reduction method. Thus, we aimed to identify a suitable, reliable, easy and commonly available natural plant source (in Sri Lanka) to obtain anthocyanin and develop it as an indicator for variation in pH to replace the resazurin dye reduction method in field-level microbiological quality determination of raw milk.

## 2. Materials and methods

### 2.1. Sample preparation

#### 2.1.1. Milk samples

The experiment was conducted using a series of bovine raw milk samples which were collected from the University Dairy Farm, University of Peradeniya, Sri Lanka. After milking, samples were kept chilled (4–5 °C) until used for the analysis (within 3 h milk samples were used for the analysis). Milk samples (pH 6.5) were adjusted to defined pH values (6.5, 6.2, 6.1, 6.0, 5.9, 5.8, 5.7, 5.6, 5.5, and 4.5) using acetic acid (0.05 M).

#### 2.1.2. Natural plant extracts

Four different natural plant extracts were collected to determine the

anthocyanins content in respective plant materials (Fig. 1). The plant extracts used in this experiment were; (a) bloomed and fresh hibiscus flower petals (*Hibiscus rosa-sinensis*) without any visible quality defects; (b) bloomed and fresh butterfly pea flower (*Clitoria ternatea*) petals without any visible quality defects; (c) washed and peeled taproot of beetroot (*Beta vulgaris*); (d) ripened, cleaned and peeled cactus pricklypears (*Opuntia dillenii*). All the plant materials were thoroughly cleaned using cold distilled water and cut into small pieces and then mixed with 50 mL of distilled water. The weight of plant materials were changed (1–8 g) depending on the required final concentrations (e.g. 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16 g/mL). The mixture was homogenized using a polytron homogenizer at 0.044×g (50 rpm) for 5 min and filtered using a cheesecloth to obtain the plant extract without coarse particles. The pH of the fresh aqueous extracts was; 6.0 (*Hibiscus rosa-sinensis* flowers), 7.6 (*Clitoria ternatea* flowers), 7.5 (*Beta vulgaris* taproots) and 4.0 (*Opuntia dillenii* pricklypears).

### 2.2. Preliminary method development

An equivalent amount (1 mL) of each plant extract was added into 10 mL of milk in a sterilized test tube, for all the milk samples with varying pH levels as described in section 2.1.1. Subsequently, the milk samples were heated in a water bath at 37 °C and colour changes were observed after 10 min and 1-h intervals. The same procedure was performed for the four natural extracts at each pH level to identify the most suitable natural plant extract and the appropriate concentration to distinguish a noticeable colour change.

### 2.3. The control colourant test vs. novel plant colourant test

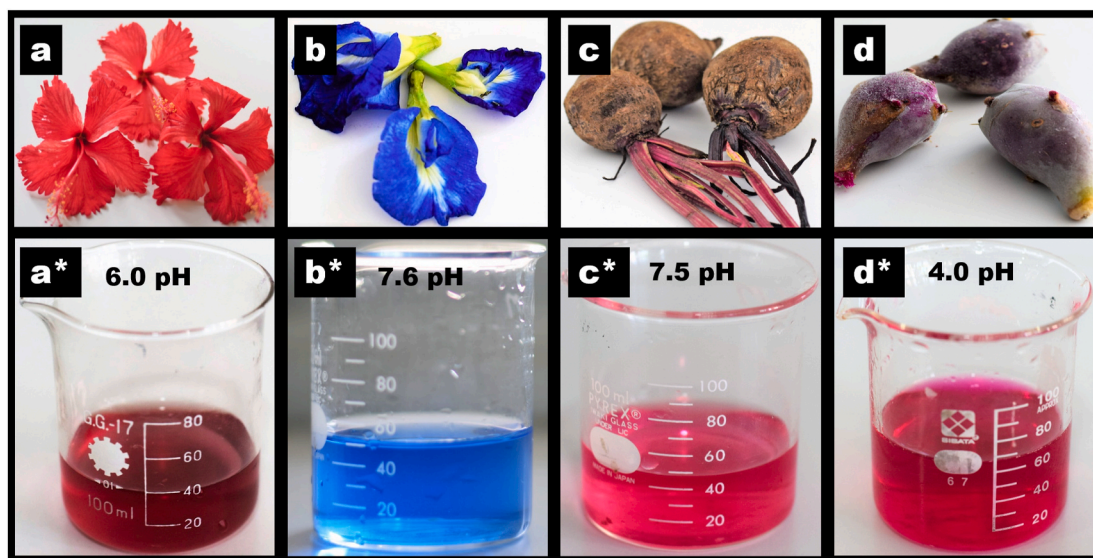
The resazurin dye reduction method was performed as a positive control to compare with the plant colourants. A standard resazurin solution was prepared by dissolving a resazurin tablet (Chemical Formula- C<sub>12</sub>H<sub>6</sub>NNaO<sub>4</sub>; Molecular Weight- 251.17 g/mol) (VWR CHEMICALS®, UK) in 50 mL of sterilized distilled water. Then, 1 mL of resazurin solution was added quickly to the test tube pre-filled with 10 mL of milk. Subsequently, milk samples were heated in a water bath at 37 °C and colour changes were observed after 10 min and 1 h. From the results of preliminary studies (section 2.2), *Hibiscus rosa-sinensis* was opted as the best natural plant extract due to visible colour changes with varying pH levels. Hence, it was used as the potential candidate for developing a novel indicative plant extract in subsequent studies.

### 2.4. pH value and microbiological analysis

The pH of the milk samples was determined using a Hanna micro-processor pH meter (HannaNorden AB, Kungsbacka, Sweden). The microbial count of the same milk samples was analyzed using the Total Viable Plate Count (TVPC) method. A dilution series was developed from each milk sample using aqueous peptone solution (HIMEDIA, India) and nutrient agar was used for enumeration of viable counts using the spread plate technique (Jayarathna et al., 2020). The total viable microbial count was expressed as log colony formation unit per millilitre of the sample (log CFU/mL) (Hudman & Sargentini, 2013).

### 2.5. Determination of anthocyanin concentration

The total anthocyanin concentration of the natural extract of *Hibiscus rosa-sinensis* was calculated using equation (1) according to the pH differential method using a spectrophotometer (GENESYS®, G10s UV-Vis, Thermo Fisher Scientific, United States) at pH 1.0 and pH 4.5 (Gallik, 2011). In brief, the anthocyanin concentration of *Hibiscus rosa-sinensis* was measured using the anthocyanin standard curve [X-axis: concentration (g/mL); Y-axis: absorbance (520 nm)], which was developed using a commercial anthocyanin standard (Biopurify Phytochemicals Ltd., Chengdu, China).



**Fig. 1.** Plant materials (a) *Hibiscus rosa-sinensis* flowers (b) *Clitoria ternatea* flowers (c) *Beta vulgaris* taproots and (d) *Opuntia dillenii* pricklypears. Their aqueous extractions are marked with an asterisk with the pH of the extraction, respectively.

The absorbance of the Anthocyanin = Absorbance at pH 1.0 – Absorbance at pH 4.5 (1)

## 2.6. Preparation of the colour chart

A colour chart was prepared for rapid determination of milk pH and corresponding microbiological quality for outdoor uses. First, the colour of the natural plant extract was identified by observing the absorbance of the extract using a spectrophotometer (GENESYS®, G10s UV-Vis, Thermo Fisher Scientific, United State). Then, the colour chart for the proposed novel method was developed using a Lovibond Comparator (Lovibond®, 2000 MK.11, United Kingdom). Afterwards, a range of colours (from light to dark colours) was prepared into a colour chart by comparing colour containing test tubes (i.e. test tubes filled with milk in different pH levels and natural plant extract) with different colour ranges using Lovibond Comparator.

## 2.7. Method validation

The analytical method was validated for linearity, precision, repeatability, accuracy, the limit of quantification (LOQ) and limit of detection (LOD) in accordance with International Conference on Harmonisation (ICH) guidelines and Eurachem guide (Magnusson & Örnemark, 2014; ICH Harmonised Tripartite Guideline, 2005).

### 2.7.1. Linearity

The calibration curve developed by plotting the absorbance versus concentration of *Hibiscus rosa-sinensis* flower water extract was used to evaluate the linearity. The linearity of the proposed model was judged by examining the correlation coefficient ( $R^2$ ) of the linear regression (Matos et al., 2015; Shabir, 2003).

### 2.7.2. Precision

The precision of the method was evaluated using both repeatability and intermediate precision (Ravisankar, Naga Navya, Pravalika, & Navya, 2015).

**2.7.2.1. Repeatability.** The repeatability of the novel method was determined using the Relative Standard Deviation (RSD) (Peris-Vicente,

Esteve-Romero, & Carda-Broch, 2015). The RSD was calculated using both pH and absorbance using three individual samples of *Hibiscus rosa-sinensis* flower water extract within a day (intraday). As described in Ravisankar et al. (2015), repeatability was analyzed using the same laboratory conditions and the same analyst over a short period.

**2.7.2.2. Intermediate precision.** The intermediate precision of the proposed method was calculated as RSD with triplicates over five consecutive days (interday). RSD was computed using pH and absorbance of the *Hibiscus rosa-sinensis* flower water extract that performed during different days, different analysts and using different instruments (e.g. different spectrophotometers and pH meters in different laboratories) (Belouafa et al., 2017; Ravisankar et al., 2015).

### 2.7.3. Accuracy

Accuracy was considered as the closeness between the calculated values from the treatment and the true values of the experiment (Ravisankar et al., 2015). As previously described by Chauhan, Mittu, and Chauhan (2015), the accuracy of the proposed method was evaluated by obtaining the difference between the true values of pH and absorbance versus the calculated means of the pH and absorbance of the *Hibiscus rosa-sinensis* flower water extract.

### 2.7.4. Limit of quantification (LoQ)

LoQ was computed using the Standard Deviation (SD) of the absorbance of the *Hibiscus rosa-sinensis* flower water extract and the slope (S) of the calibration curve using equation (2) (Ravisankar et al., 2015).

$$\text{LoQ} = 10 (\text{SD}/\text{S}) \quad (2)$$

SD= Standard Deviation  
S= Slope of the calibration curve

### 2.7.5. Limit of detection (LoD)

LoD was calculated using the Standard Deviation (SD) of the absorbance of the *Hibiscus rosa-sinensis* flower water extract and the slope of the calibration curve using equation (3), as previously described by Chauhan et al. (2015).

$$\text{LoD} = 3.3 (\text{SD}/\text{S}) \quad (3)$$

SD= Standard Deviation

S= Slope of the calibration curve

## 2.8. Statistical analysis

Statistical analysis was carried out using one-way ANOVA using PROC ANOVA with Complete Randomized Design (CRD) using three replicates. Mean separation was accomplished through the Least Significant Difference (LSD) method ( $p < 0.05$ ). Data were analyzed using SAS® 9.1 statistical computer package (SAS Institute Inc., Cary NC, USA).

## 3. Results and discussions

### 3.1. Relationship between total viable plate counts (TVPC) and the pH of the raw milk

We evaluated the relationship between the pH and viable microbial counts in milk during the spoilage to assess the possibility of using pH as an indicator for the microbiological quality in milk (Fig. 2). The TVPC of raw milk increased when the pH of raw milk decreased. This is mainly due to bacterial growth resulting in spoilage of raw milk, which will eventually reduce the pH of the milk as a result of lactic acid production by lactic acid bacteria (Lu et al., 2013; Perera, Munasinghe, & Marapana, 2019). Thus, as expected, the results revealed that the pH of the milk is inversely proportional to the TVPC of the milk. Therefore, understanding the pH of the sample would enable us to predict or speculate the total viable microbial counts of the milk, which will be used in the proposed model.

### 3.2. Determination of suitable plant extract and concentration

Unrevealing the relationship of microbial counts and pH of milk samples, we further investigated the use of plant extract as an indicator to detect changes in the pH of aqueous media. During the preliminary study, we studied the variation in colours with varying pH levels using aqueous extracts of plant materials (Fig. 3). At around 6.5 pH, varying plant aqueous extracts observed to contain a range of colours (e.g. dark pink colour for *Hibiscus rosa-sinensis* flower, light blue colour for *Clitoria ternatea* flower, light pink colour for *Beta vulgaris* tap roots and dark pink colour for *Opuntia dillenii* pricklypears). Studying their varying concentrations, it was found that *Hibiscus rosa-sinensis* flower water extract (0.16 g/mL) had a dark pink colour. Depending on the pH of the

medium, the aqueous extract of *Hibiscus rosa-sinensis* flowers showed a marked variation in colour. At extremely low pH ( $pH < 2$ ), *Hibiscus rosa-sinensis* flower water extract appeared orange, while at moderately low pH ( $pH = 4.5$ ) it was pink. At neutral pH, it was dark pink and exhibited a dark green colour at extremely alkaline pH ( $pH > 11$ ). This variation in colours is mainly due to pH dependency of anthocyanin's charge since anthocyanin pigments are predominantly positively charged (flavylium cation) on the C-ring of the molecule (Gallik, 2011). Further, if C-ring carries the positive charge, the molecule is pigmented. On the other hand, if the C-ring is hydrated, the positive charge is neutralized and then pigmentation is recessive (Gallik, 2011).

Having observed the pH dependency of the colours in anthocyanin pigment in aqueous media, we further investigated their possible colour changes in raw milk (Fig. 4). An interesting colour change was observed for *Hibiscus rosa-sinensis* flower water extract, when pH changed from 6.5 to 6.2, demonstrating the possibility of further improving this to detect pH changes in the milk. However, we observed, no noticeable colour changes in *Clitoria ternatea* flower water extract, *Beta vulgaris* tap roots water extract and *Opuntia dillenii* pears water extract in milk varying with pH values. Thus, potentially disqualifying those extracts to use as an indicator for pH changes. Ruiz-Gutiérrez, Amaya-Guerra, Quintero-Ramos, Pérez-Carrillo, & Meléndez-Pizarro, 2017 revealed that cactus and beetroot mostly contain betacyanins as natural plant pigment. Betacyanins are more stable to pH and temperature as described by Singh & Singh (2014). Thus, low pH sensitivity in cactus and beetroot may due to the betacyanin which consists naturally in those plants. Due to the betacyanin, both cactus and beetroot have a good potential to be used as a natural food colourant in the food processing industry, but not as an indicator for detecting changes in pH. Furthermore, butterfly pea and *Hibiscus* extract contains a high amount of anthocyanins (Pachpore & Punjabi, 2017) and thus we expected both colours to be sensitive to changes in pH. These changes, however, are visible at aqueous media for both flower extracts (Fig. 3), but no visible changes were observed in milk for butterfly pea (Fig. 4). Due to the sensitivity in colour with changing pH in aqueous media, butterfly pea extract has been used as a natural dye in the food industry as well as an indicator of acid-base titrations (Saptarini, Suryasaputra, & Nurmalia, 2015). Despite *Hibiscus rosa-sinensis* flower water extract, other three plant extracts did not show a clear colour variation in different pH conditions when milk was used as a medium. Hence, it could be argued that *Hibiscus rosa-sinensis* flower water extract is more sensitive to small pH changes in milk when compared to the other three plant extracts, demonstrating the possibilities of utilizing it for detecting pH changes.

Because of *Hibiscus rosa-sinensis* flowers' water extract's ability to distinguish the differences between pH 6.2 and pH 6.5 in milk, we compared it with the current standardized test for detecting the milk with varying pH, i.e. resazurin dye reduction test. The aqueous solution of *Hibiscus rosa-sinensis* flowers was dark maroon colour (Fig. 5d) and then, once it introduced to fresh milk, the colour changed to maroon colour at 6.5 pH (Fig. 5e) and light pink colour at pH 6.2 (Fig. 5f). Resazurin dye [dark blue colour (Fig. 5a) at aqueous media] also change its colour in the same pH range, where it changed from bluish colour (Fig. 5b) to pinkish colour (Fig. 5c), when the milk pH changed from 6.5 to 6.2, respectively. Thus, verifying the potential use of the aqueous extract of *Hibiscus rosa-sinensis* flowers. Furthermore, the colour of hibiscus has reported depending on the medium of extraction, e.g. in methanol a dark brown colour, in ethanol a light red colour and 5% acetic acid a dark red colour was obtained (Hayat & Jacob, 2019).

As we demonstrated earlier, pH of the milk samples is inversely correlated with viable microbial counts (section 3.1), we used pH dependency of anthocyanins to indirectly determine the viable microbial count of the milk samples using aqueous extract of *Hibiscus rosa-sinensis* flowers. Similar to the present study, Ibrahim et al. (2011) also have reported, anthocyanin molecules change their colour depending on the pH of the medium. When pH increases, thermodynamic and kinetic competition occurs between the hydration reaction of the flavylium

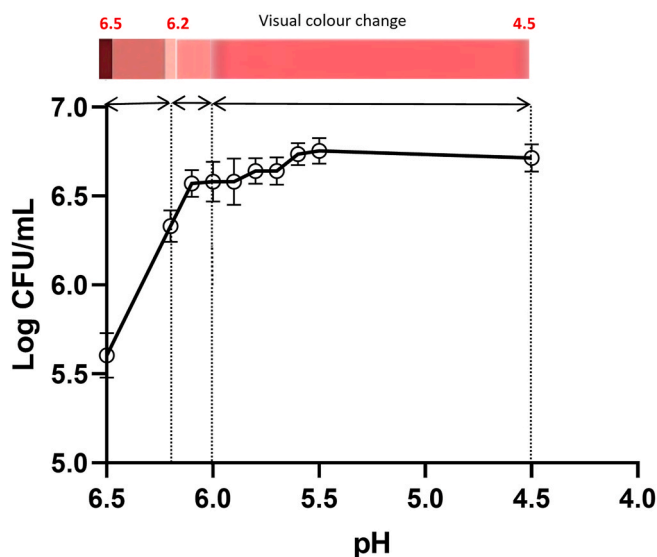


Fig. 2. Relationship between total viable plate counts (colony forming units: CFU/mL) and the pH of the raw milk.

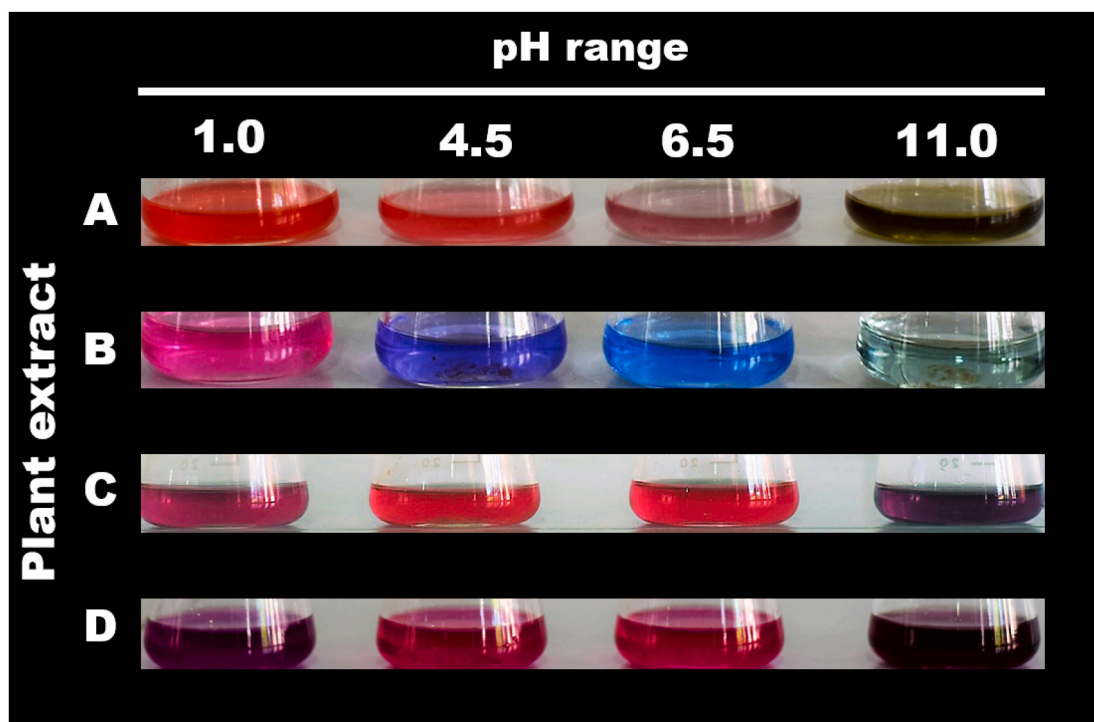


Fig. 3. Colour changes of the plant water extracts with respect to the pH change from acidic (1.0 pH) to alkaline (11 pH) conditions, (A) *Hibiscus rosa-sinensis* flower water extract, (B) *Clitoria ternatea* flower water extract, (C) *Beta vulgaris* tap roots water extract and (D) *Opuntia dillenii* pricklypears water extract. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

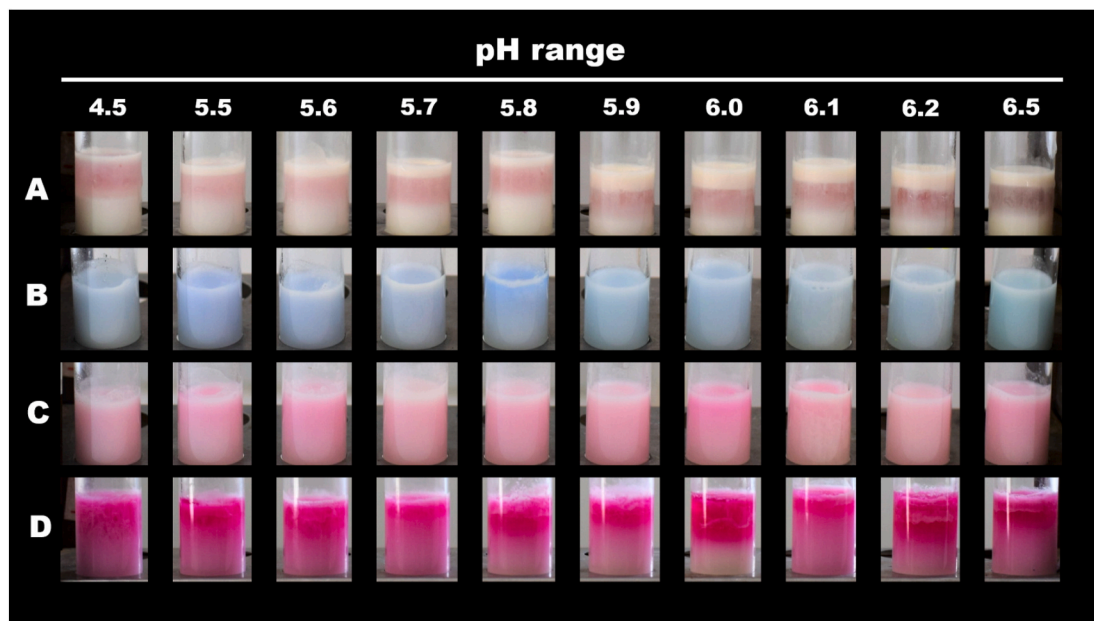
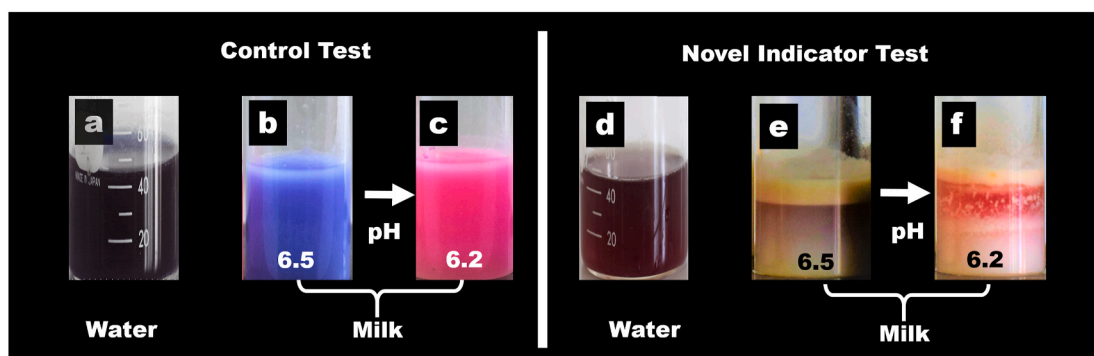


Fig. 4. Colour changes in water extracts of plant materials, when added into milk with varying pH levels. (A) *Hibiscus rosa-sinensis* flower water extract, (B) *Clitoria ternatea* flower water extract, (C) *Beta vulgaris* tap roots water extract and (D) *Opuntia dillenii* pricklypears water extract. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cation. In low pH, yellow retro-chalcones produce and when pH increases and converts to the quinonoidal bases by further deprotonation which is more stable quinonid anion (Ibrahim et al., 2011). According to Draaiyer, Dugdill, Bennett, and Mounsey (2009), the same principle is applied by the resazurin dye reduction method. As the microbial load in milk increases, the amount of oxygen present in the milk decreases and an oxidation-reduction reaction occurs. Also, the colour of the resazurin

dye changes with the oxidation-reduction reactions that occur in milk due to microbial activities. Overall, it can be argued that the aqueous extract of *Hibiscus rosa-sinensis* flowers has the potential to replace resazurin dye to visually examine the pH difference and thereby predict the corresponding microbial quality of raw milk.



**Fig. 5.** Comparison of colour changes in milk when resazurin dye (Left) or *Hibiscus rosa-sinensis* flower water extract (Right) is added into milk. (a) Resazurin dye aqueous solution of dark blue colour, change into (b) blue colour at pH 6.5 in fresh milk and (c) pink colour at pH 6.2 in spoiled milk. In the novel indicator method using (d) dark maroon colour *Hibiscus rosa-sinensis* flower water extract change into, (e) maroon colour at pH 6.5 in fresh milk and (f) light pink at pH 6.2 in spoiled milk. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

### 3.3. Development of a novel colour chart for rapid colour identification

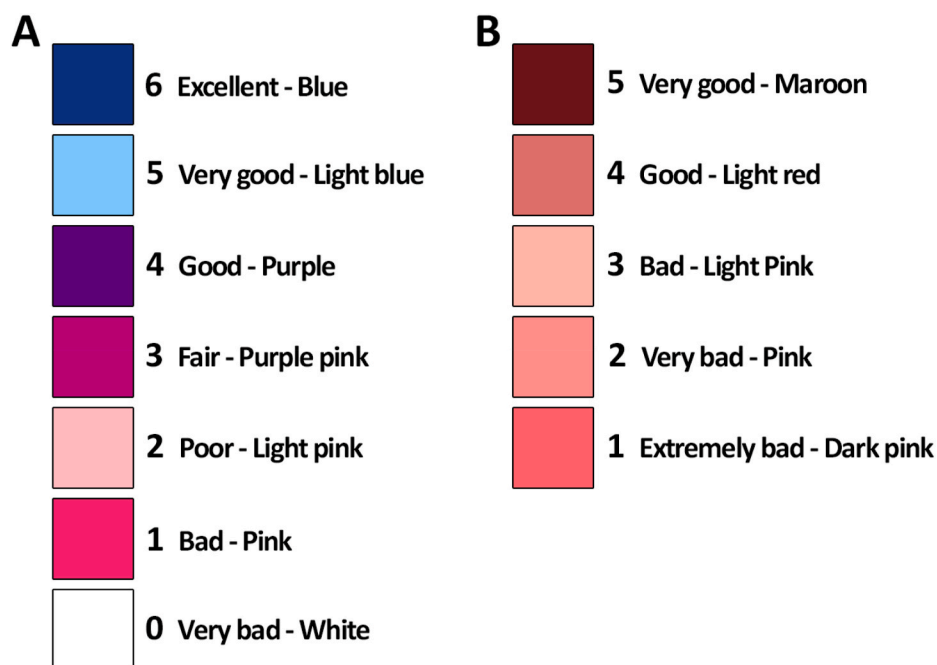
Since, novel plant indicator also can be used to evaluate the pH and indirectly determine the microbiological quality of the milk, in parallel to the resazurin dye reduction test, we aimed to develop a colour chart for rapid identification of changes in pH. This will facilitate convenient usability and user-friendliness in outstation milk quality determinations. In the new method, the colour change from maroon to light pink was observed within 15 min whereas, in resazurin dye reduction method blue colour changed into pink within 10 min. According to the resazurin colour chart, at pH 6.2 milk has to be rejected (Draaiyer et al., 2009).

The minimum required viable microbial counts for identification of the colour change in milk for both resazurin and the new method (water extract from hibiscus flowers) was 6.3 log CFU/mL (Fig. 2). According to the novel colour chart (Fig. 6B), milk has to be rejected at number three (3) position when the colour of the milk change into light pink.

Similarly, milk has to be rejected at number one (1) position when the colour of the milk change into pink by comparing the colour of the milk samples (after adding resazurin dye) and the resazurin colour chart (Draaiyer et al., 2009). The same principle is followed when using the novel colour chart to determine the microbiological quality of raw milk.

### 3.4. Analysis of anthocyanin content of the *Hibiscus rosa-sinensis* flower water extract

The concentration of anthocyanin in the extracts was determined using a spectrophotometer according to a pH differential method. This was preferred due to its simplicity and low cost, providing very accurate data which was analyzed in the specific absorbance band (520 nm–560 nm) (Martín et al., 2017; Viskelis, Anisimoviene, Rubinskiene, Jankovska, & Sasnauska, 2010). In the current study, the anthocyanin concentration of the *Hibiscus* extract was 0.59 g/mL which was calculated



**Fig. 6.** Colour charts for identification of microbial quality of milk, (A) Color chart of the resazurin dye reduction method (milk reject at number 1- Bad condition when milk turn in to pink colour), (B) Novel colour chart using *Hibiscus rosa-sinensis* flower water extract (milk reject at number 3- Bad condition when colour change into light pink). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

using the pH differential method using equation (4).

coefficient of greater than 0.9 (>0.9) is generally considered as evidence of acceptable fit of the data to the regression line (Peris-Vicente et al., 2015; Shabir, 2003). Moreover, a high value of the correlation coefficient

The absorbance of the anthocyanin = Absorbance at pH 1.0 – Absorbance at pH 4.5

$$= 2.97 - 0.75$$

$$= 2.22$$

According to the anthocyanin standard curve (Fig. 6):

$$Y = 3.765 * X - 0.004118$$

$$X = \frac{2.22 + 0.004118}{3.765}$$

$$X = 0.59 \text{ g/ mL}$$

(4)

### 3.5. Method validation

The validation of an analytical method demonstrates the scientific soundness of the measurement or characterization (Chauhan et al., 2015). Furthermore, the key objective of method validation is to demonstrate the suitability for its intended use and therefore, the proposed method was validated to use in further applications (i.e. field level or as a rapid and low-cost platform test in small-scale milk chilling centres). Upon validation, this can be used as a reliable analytical method in routine analysis of raw milk procurement.

#### 3.5.1. Linearity

As shown in Fig. 7, the proposed predictive method showed a very high correlation coefficient ( $R^2 = 0.95$ ) between absorbance and concentration. The linearity of the regression line can be evaluated using correlation coefficient (Boqué, Maroto, Riu, & Rius, 2002), of which 0.95 for the present study. According to the Ravisankar et al. (2015), the linearity of a method demonstrates the accuracy of the calibration curve of response vs. concentration, e.g. if  $R^2$  value close to one (1) indicates adequate linearity, whereas a  $R^2$  close to zero (0) indicates the total absence of proportionality (Peris-Vicente et al., 2015). A correlation

coefficient is normally sufficient to evaluate the linearity of the method (Boqué et al., 2002).

#### 3.5.2. Precision

As reported in Matos et al. (2015), precision was evaluated to identify variability due to random errors which occurred unavoidably during the analysis, e.g. analysis time, reagents, glassware, and during sample preparation etc. Hence, precision was examined in terms of repeatability and intermediate precision in the present study.

**3.5.2.1. Repeatability.** The RSD of both pH and absorbance was, 0.27% and 0.15%, respectively in the present study and 0.38% and 0.75% in resazurin dye reduction method, respectively (Table 1). As stipulated by Paithankar (2013), repeatability is the precision under the same operating conditions over a short interval of time. It involves the repeated determination of the same sample and also named intra-assay precision (Belouafa et al., 2017). The repeatability is expressed in terms of standard deviation and is generally dependent on analyte concentration (Belouafa et al., 2017). Moreover, in method validation, repeatability is evaluated by calculating the variability of the results which was obtained during the analytical method (Belouafa et al., 2017). The

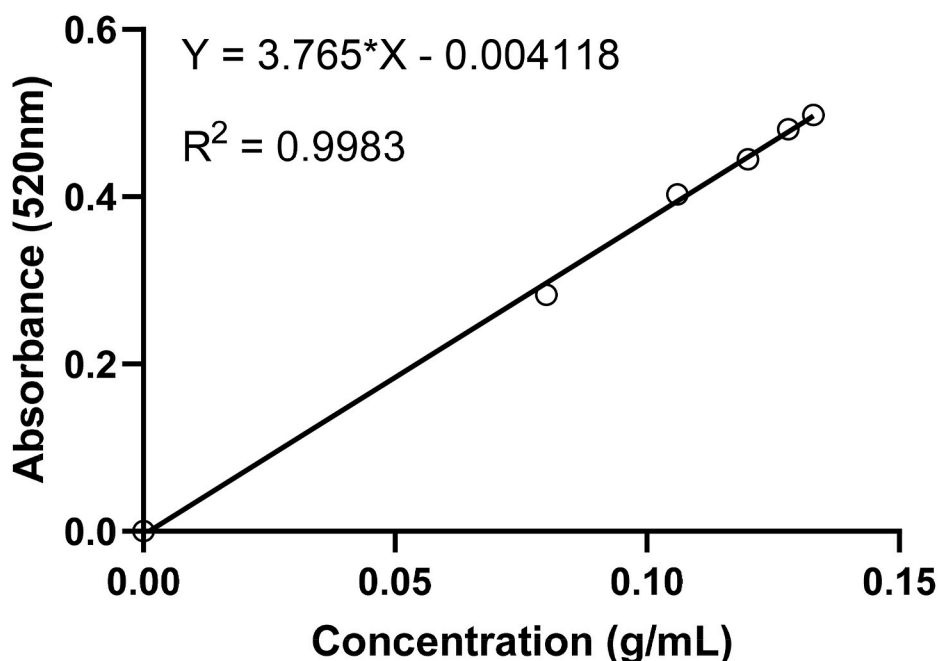


Fig. 7. Anthocyanin standard curve used for determination of anthocyanin concentration in the Hibiscus rosa-sinensis flower water extract.

**Table 1**

Repeatability (intraday) and intermediate precision (interday) analysis of the novel method validation and control method.

Variables	Novel method ( <i>Hibiscus rosa-sinensis</i> flower water extract)				Control method (Resazurin Dye Reduction method)			
	pH	RSD <sup>a</sup>	Absorbance (520 nm)	RSD <sup>a</sup>	pH	RSD <sup>a</sup>	Absorbance (520 nm)	RSD <sup>a</sup>
<b>Intraday (n = 3)</b>								
Sample 1	5.88 ± 0.02		0.65 ± 0.00		7.74 ± 0.03		1.34 ± 0.01	
Sample 2	5.90 ± 0.01	0.27	0.65 ± 0.00	0.15	7.73 ± 0.03	0.38	1.36 ± 0.01	0.75
Sample 3	5.90 ± 0.01		0.65 ± 0.00		7.72 ± 0.03		1.36 ± 0.00	
<b>Interday (n = 3)</b>								
Day 1	5.88 ± 0.03		0.65 ± 0.00		7.74 ± 0.03		1.34 ± 0.01	
Day 2	5.89 ± 0.02		0.65 ± 0.00		7.73 ± 0.03		1.36 ± 0.01	
Day 3	5.89 ± 0.02	0.28	0.65 ± 0.00	0.18	7.72 ± 0.03	0.36	1.36 ± 0.00	0.76
Day 4	5.90 ± 0.01		0.65 ± 0.00		7.74 ± 0.03		1.36 ± 0.01	
Day 5	5.88 ± 0.02		0.65 ± 0.00		7.73 ± 0.03		1.35 ± 0.01	

<sup>a</sup> Relative Standard Deviation (RSD) = (Standard Deviation/Mean) × 100 (samples are in triplicates).

observed data in the present study have also confirmed the findings of [Shabir \(2003\)](#) who delineated that, in precision criteria for an assay method is that the RSD will be ≤ 2%. Therefore, the novel method shows a high level of repeatability.

**3.5.2.2. Intermediate precision and accuracy.** In the current study, the intermediate precision was 0.28% for pH and 0.18% for absorbance and 0.36% for pH and 0.76% for absorbance in the control method ([Table 1](#)). Intermediate precision is expressed within laboratory variations: different days, different analysts and different equipment ([Belouafa et al., 2017](#); [Paithankar, 2013](#)). Literature suggested that the most useful and essential method to identify precision for a laboratory was intermediate precision because it gives an overall idea of the variability that the laboratory can expect as well as it is an essential component of the uncertainty of the results ([Boqué et al., 2002](#)). According to [Shabir \(2003\)](#), precision criteria for intermediate precision would be ≤ 2%. Similarly, intermediate precision of this method shows acceptable values which are less than two per cent (2%). Therefore, this method shows very high precision according to the repeatability and intermediate precision.

According to the computed data, the accuracy showed 100% recovery in both pH and absorbance in the current study and control method (data not shown). The accuracy of an analytical method describes the closeness of the determined value obtained by the method to the nominal concentration of the analyte ([Belouafa et al., 2017](#); [EMEA, 2006](#)). Accuracy can be determined using recovery studies and measured by comparison to a reference standard, recovery of the analyte spiked into a blank spike and standard addition of the analyte ([Ravisankar et al., 2015](#)). In the current study, the reference standard was used to analyze the accuracy. As specified by [Shabir \(2003\)](#), the accuracy criteria for an assay method could be considered as the mean recovery of 100 ± 2% at each concentration. Therefore, results revealed that the novel method shows the maximum level of accuracy.

### 3.5.3. Limit of quantification

In the proposed novel method, LoQ value was 0.46 g/mL. LoQ can be defined as the lowest concentration of an analyte in a sample which can be quantified reliably, with acceptable accuracy and precision ([Boqué et al., 2002](#); [EMEA, 2006](#)). Further, LoQ can be used as a performance characteristic which shows the ability of an analytical method to quantify and analyze easily ([Boqué et al., 2002](#)). LoQ was calculated using the standard deviation (0.10) and the slope (2.26) of the plotted linearity curve using equation (2). Thus, the lowest quantifiable *Hibiscus rosa-sinensis* flower water extract concentration was 0.46 g/mL to analyze raw milk microbial quality.

### 3.5.4. Limit of detection

LoD is the lowest concentration of a substance in a sample that can be detected reliably according to a given analytical method ([Boqué et al., 2002](#); [Shabir, 2003](#)). In the present study, LoD value for the novel

method was 0.15 g/mL, which implied that lowest detectable concentration of the *Hibiscus rosa-sinensis* flower water extract was 0.15 g/mL, to determine the microbiological quality of raw milk. Furthermore, [Boqué et al. \(2002\)](#) described that the concentration of the analyte in the material which used to analyze is higher than that in the blank sample. Literature suggested that LoD can be determined by visual evaluation, signal to noise ratio and standard deviation and the slope of the linearity graph ([Chauhan et al., 2015](#); [Ravisankar et al., 2015](#)).

## 4. Conclusions

The water extract of *Hibiscus rosa-sinensis* L. flowers contains anthocyanin (0.59 g/mL), which is very sensitive to minute changes in pH compared to other plant sources of anthocyanin used in the current study. Due to the linear and inverse relationship between pH and viable microbial counts, the pH-sensitive colour changes of *Hibiscus rosa-sinensis* L. can be used to visually distinguish milk with varying pH and correlate with respective microbial counts to rapidly determine the milk quality. The proposed method resulted in acceptable model performances for testing the microbial quality of milk using water extract of *Hibiscus rosa-sinensis* L. flowers. Accordingly, the minimum viable microbial counts in milk for observing a colour change was 6.3 log CFU/mL. Overall results of the present study revealed the potential for using aqueous extract of *Hibiscus rosa-sinensis* L. flowers as an alternative to resazurin dye reduction method in microbial quality determination of raw milk at field-level for rapid screening of milk at milk collection centres. This because, proposed novel analytical tool is a valuable time- and cost-saving strategy that can be used in milk quality determination in rural areas, if properly conducted and intelligently interpreted. However, this should be supplemented with further laboratory quality analyses to determine the results of the filed test. Future studies are recommended to develop a user-friendly application of the water extract of *Hibiscus rosa-sinensis* L. flowers, e.g. water-soluble tablet (similar to resazurin tablets), after freeze-drying of the anthocyanin extract. This will enable the transferring of knowledge generated from this study into a rapid and convenient field-level routine to assess the routine microbiological quality of milk.

## Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## CRedit authorship contribution statement

**Ranga Madushan:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualization. **Janak K. Vidanarachchi:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing



– review & editing, Visualization, Supervision, Project administration. **P.H.P. Prasanna:** Validation, Data curation, Writing – original draft, Writing – review & editing, Supervision. **Shanika Werellagama:** Software, Data curation, Writing – original draft. **Hasitha Priyashantha:** Software, Validation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision.

### Declaration of competing interest

The authors declare no conflict of interest.

### Acknowledgements

Technical assistances at Dairy Science Laboratory, Departement of Animal Science, Faculty of Agriculture, University of Peradeniya, Peradeniya, Sri Lanka are acknowledged for the material support and support given during the practical work.

### References

- Belouafa, S., Habti, F., Benhar, S., Belafkih, B., Tayane, S., Hamdouch, S., et al. (2017). Statistical tools and approaches to validate analytical methods: Methodology and practical examples. *International Journal of Metrology and Quality Engineering*, 8(9).
- Boqué, R., Maroto, A., Riu, J., & Rius, F. X. (2002). Validation of analytical methods. *Grasas Y Aceites*, 53(1), 128–143.
- Chauhan, A., Mittu, B., & Chauhan, P. (2015). Analytical method development and validation: A concise review. *Journal of Analytical & Bioanalytical Techniques*, (1), 06.
- Cisowska, A., Wojnicz, D., & Hendrich, A. (2011). Anthocyanins as antimicrobial agents of natural plant origin. *Natural Product Communications*, 6(1), 149–156.
- Dangles, O., & Fenger, J. (2018). The chemical reactivity of anthocyanins and its consequences in food science and nutrition. *Molecules*, 23(8).
- De Silva, S. A. S. D., Kanugala, K. A. N. P., & Weerakkody, N. S. (2016). Microbiological quality of raw milk and effect on quality by implementing good management practices. *Procedia Food Science*, 6, 92–96.
- Draaiyer, J., Dugdill, B., Bennett, A., & Mounsey, J. (2009). *Milk Testing and Payment Systems Resource Book a practical guide to assist milk producer groups*.
- EMA. (2006). *Note for guidance on validation of analytical procedures: Text and methodology*. European Medicines Agency.
- Gallik, S. (2011). *Determination of the anthocyanin concentration in table wines and fruit juices using visible light spectrophotometry cell biology OLM*.
- Garnier, L., Valence, F., & Mounier, J. (2017). Diversity and control of spoilage fungi in dairy products: An update. *Microorganisms*, 5(3), 42.
- Hayat, L., & Jacob, D. (2019). Dyeing wool and cotton fibres with acidic extract of *Hibiscus rosa sinensis* flower. *Natural Product Research*, 33(7), 980–986.
- Huck, J. R., Hammond, B. H., Murphy, S. C., Woodcock, N. H., & Boor, K. J. (2007). Tracking spore-forming bacterial contaminants in fluid milk-processing systems. *Journal of Dairy Science*, 90, 4872–4883.
- Hudman, D., & Sargentini, N. (2013). Resazurin-based assay for screening bacteria for radiation sensitivity. *SpringerPlus*, 2, 55.
- Ibrahim, U.k., Muhammad, I. I., & Salleh, R. M. (2011). The effect of the pH on color behavior of Brassica oleracea anthocyanin. *Journal of Applied Sciences*, 11(13), 2406–2410.
- ICH Harmonised Tripartite Guideline. (2005). Validation of analytical procedures: Text and methodology. In *International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. Q2(R1)*.
- Jayarathna, S., Priyashantha, H., Johansson, M., Vidanaratchi, J. K., Jayawardana, B. C., & Liyanage, R. (2020). Probiotic enriched fermented soy-gel as a vegan substitute for dairy yoghurt. *Journal of Food Processing and Preservation*, Article e15092, 00.
- Knight-Jones, T., Hang'ombe, M., Songe, M., Sinkala, Y., & Grace, D. (2016). Microbial contamination and hygiene of fresh cow's milk produced by smallholders in Western Zambia. *International Journal of Environmental Research and Public Health*, 13(7).
- Li, N., Wang, Y., You, C., Ren, J., Chen, W., Zheng, H., et al. (2018). Variation in raw milk microbiota throughout 12 Months and the impact of weather conditions. *Scientific Reports*, 8–2371.
- Lu, M., Shiau, Y., Wong, J., Lin, R., Kravis, H., Blackmon, T., et al. (2013). Milk spoilage: Methods and practices of detecting milk quality. *Food and Nutrition Sciences*, (7), 113–123, 04.
- Magnusson, B., & Örnemark, U. (2014). *Eurachem guide: The fitness for purpose of analytical methods – a laboratory guide to method validation and related topics* (2nd ed.).
- Martin, J., Navas, M., Jiménez-Moreno, A., & Asuero, A. (2017). Anthocyanin pigments: Importance, sample preparation and extraction. In M. Soto-Hernandez, M. Palma-Tenango, & R. M. Garcia-Mateos (Eds.), *Phenolic compounds - natural sources, importance and applications*. InTech.
- Matos, B., Oliveira, P., Reis, T., Gratieri, T., Cunha-Filho, M., & Gelfuso, G. (2015). Development and validation of a simple and selective analytical HPLC method for the quantification of oxaloplatin. *Journal of Chemistry*, 2015. Article ID- 812701.
- Miguel, M. (2011). Anthocyanins: Antioxidant and/or anti-inflammatory activities. *Journal of Applied Pharmaceutical Science*, 1(6), 7–15.
- More, S. J. (2009). Global trends in milk quality: Implications for the Irish dairy industry. *Irish Veterinary Journal*, 62, 5–14.
- Muhammad, A., Muhammad, N., Muhammad, I., Muhammad, A., Maryam, B., Imran, T. K., et al. (2018). Impact of immediate and delayed chilling of raw milk on chemical changes in lipid fraction of pasteurized milk. *Lipids in Health and Disease*, 17, 190.
- Muliro, P., Shalo, P., & Kutima, P. (2013). Quality assessment of raw camel milk using dye reduction tests. *African Journal of Food Science and Technology*, 4(5), 116–121.
- Murphy, S., Martin, N., Barbano, D., & Wiedmann, M. (2016). Influence of raw milk quality on processed dairy products: How do raw milk quality test results relate to product quality and yield. *Journal of Dairy Science*, 99(12), 10128–10149.
- Oliveira, G. B. D., Favarin, L., Luchese, R. H., & McIntosh, D. (2015). Psychrotrophic bacteria in milk: How much do we really know? *Brazilian Journal of Microbiology*, 46(2), 313–321.
- Pachpore, S., & Punjabi, K. (2017). Study on chemical constituents, antioxidant properties and toxicity of *Hibiscus rosasinensis* flower extract. *Journal of Pharmacognosy and Phytochemistry*, 6(3), 116–119.
- Paithankar, H. (2013). HPLC method validation for pharmaceuticals: A review. *International Journal of Universal Pharmacy and Bio Sciences*, 2(4), 229–240.
- Perera, P., Munasinghe, H., & Marapana, R. (2019). Quality assessment of selected dairy products in Sri Lankan market. *Journal of Food Quality*, 2019. Article ID- 697227.
- Peris-Vicente, J., Esteve-Romero, J., & Carda-Broch, S. (2015). In J. L. Anderson, A. Berthod, V. P. Estévez, & A. M. Stalcup (Eds.) (1st ed.) *Analytical separation science/Validation of analytical methods based on chromatographic techniques: An overview*. Wiley-VCH Verlag GmbH & Co. KGaA.
- Ravisankar, P., Naga Navya, C., Pravallika, D., & Navya, D. (2015). A review on step-by-step analytical method validation. *IOSR Journal of Pharmacy*, 5(10), 7–19.
- Ruiz-Gutiérrez, M., Amaya-Guerra, C., Quintero-Ramos, A., Pérez-Carrillo, E., & Meléndez-Pizarro, C. (2017). Use of red cactus pear (*Opuntia ficus-indica*) encapsulated powder to pigment extruded cereal. *Journal of Food Quality*, 2017. Article ID- 7262464.
- Saptarini, M. N., Suryasaputra, D., & Nurmalia, H. (2015). Application of Butterfly Pea (*Clitoria ternatea* Linn) extract as an indicator of acid-base titration. *Journal of Chemical and Pharmaceutical Research*, 7(2), 275–280.
- Schorsteiner, E., Mann, E., Bereuter, O., Wagner, M., & Schmitz-Esser, S. (2014). Cultivation-independent analysis of microbial communities on Austrian raw milk hard cheese rinds. *International Journal of Food Microbiology*, 180, 88–97.
- Shabir, G. A. (2003). Validation of high-performance liquid chromatography methods for pharmaceutical analysis Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization. *Journal of Chromatography A*, 987, 57–66.
- Singh, B., & Singh, H. B. (2014). Chemical composition, functional properties and processing of Beetroot-a review. *International Journal of Scientific Engineering and Research*, 5(1).
- Sowmya, Y. (2017). A short review on milk spoilage. *Journal of Food and Dairy Technology*, 5(3).
- Viskalis, P., Anisimovienė, N., Rubinskienė, M., Jankovska, E., & Sasnauska, A. (2010). Physical properties, anthocyanins and antioxidant activity of blackcurrant berries of different maturities. *Journal of Food Agriculture and Environment*, 8(2), 159–162.