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Application of pH Indicator Label Based on Beetroot Color for Determination of Milk Freshness

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ABSTRACT

Introduction: Applying of a new indicator in food packaging can be effective to inform consumers about the freshness and quality of the products.
Materials and Methods: In the current study, a new milk freshness label was investigated containing beetroot color and multi layers of polystyrene. The label characteristics were investigated by estimating color number, release test, and scanning electron microscopy (SEM). The total bacterial count, pH, lactic acid concentration in milk, and label color number were measured using standard plate count, pH meter, titration of acidity, color analysis software, and UV spectrophotometry on days 0, 3, 4, 5, 6, and 7 at refrigerator temperature (4 ± 0.2°C).
Results: The label reacted to total bacterial count and pH changes with a visible color during milk spoilage. A positive correlation was found between the label color changes, total bacterial count, and pH. The color of label turned from dark red to light brown, which was related to the chemical changes and bacterial count of milk.
Conclusion: According to this simple, visible, and affordable label, the shelf life of pasteurized milk was estimated as five days.

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Introduction

During the last decades, the consumers' concern have increased about the quality and safety of food. So, application of new sensors have increased in food packaging, which informs consumers about the freshness and quality of products [1-2]. The traditional and time-consuming methods can be easily replaced by new smart packaging [3]. Therefore, the food spoilage can be detected easily using intelligent (smart) packaging. The application of intelligent packaging revolutionized the food packaging industry potentially. According to European Union (EU), intelligent packaging refers to a system that provides the opportunity to observe the condition of packaged food or its surrounding environment during the transportation and storage [4]. Dairy products are an important part of people's daily diet. Among these foodstuffs, milk is a highly perishable food with nutritious components for people, especially children [5]. Microbial contaminations of food products limit their shelf life and increase the risk of food borne diseases in consumers [6]. Milk pasteurization was introduced as a healthy way to eliminate the pathogens and reduce the spoilage microorganisms [7]. During storage of the pasteurized milk in refrigerator, its shelf life decreases due to the gradual growth of microorganisms [3]. Generally, milk quality is assessed in factories and laboratories using common methods such as standard plate count (SPC), which provides a quantitative measure of the total bacterial count by culturing, counting, and biochemical analysis [8]. Although these methods are valuable, they take a lot of time and must be accomplished in microbiology laboratories by skilled staff. Therefore, different studies were conducted on different kinds of food quality indicators [9-10]. The pH indicators that undergo color changes are applicable in milk and dairy products [11]. Natural pigments in some herbal sources such as anthocyanin have a great potential to be applied as a color index in smart packaging [12]. The time/temperature indicator was also prepared as a smart packaging using polyvinyl alcohol/ chitosan polymers and anthocyanin to show the changes in pasteurized milk quality [13]. Beetroot (Beta vulgaris L.) is composed of both red (betacyanins) and yellow pigments (betaxanthins), which are known as betalains [14]. Betalains are more resistant to temperature and pH; so, the pH stability of betalains makes it appropriate for using in the low acid foods [15]. Betanin, as a betacyanin, is one of the main colors of beetroot, [16] which is stable at pH rates of 4 to 7 [17]. The use of beetroot as an additive color to foodstuffs was approved by the European Union E-162 [15]. Although the beneficial effects of smart packaging have been emphasized for controlling the food spoilage by many researchers over years, little attention has been paid to the selection of an appropriate natural color ingredient.

During inadequate transportation and storage of milk, the bacteria can grow and cause some problems in consumers. So, the presence of a simple monitoring system that warns consumers about the milk safety is necessary. In the present study, a new milk freshness label was presented containing beetroot color ingredients and multi layers of polystyrene. The designer label was attached to the inner side of a milk package and different microbial and chemical analysis was done. Moreover, the correlation between color changes of the label and milk spoilage was assessed.

Materials and Methods

Fresh cow milk was obtained from Yazd city, Iran. Several materials were also purchased: beetroot powder (Freer Flavor and Color Company, Iran), phenolphthalein (Merck, Germany), ethanol 96% (Parsteb, Iran), NaOH (Sumchun, Korea), alum powder (Merck, Germany), cellulose paper (Whatman, USA), sterile polypropylene bottles (Iran), polystyrene powder (Petrochemical company, Tabriz, Iran), xylene (Merck, Germany), distilled water, and plate count agar (Merck, Germany) for microbiological tests of the samples.

Milk preparation

Fresh cow milk was gathered and transferred to the sterile glass tubes. The tubes were then placed in water bath at 63.5 °C and kept for 30 minutes. The water level was kept several inches above the milk level in tubes. In order to control the temperature, a thermometer was placed in one milk tube. After pasteurization, the tubes were placed in ice containers for rapid cooling followed by refrigeration at 4 ± 0.2 °C [8].

Label preparation

To prepare the beetroot solution, 10 g of the beetroot powder was dissolved in 30 ml of distilled water and heated at 100 °C until it was dissolved completely. The alum solution was made by solving 10 g of alum powder in 25 ml of distilled water and heating at 100 °C. The cellulose paper strips were washed with distilled water three times, added to the alum solution, and heated for 30 min. The cellulose paper strips were immersed in the beetroot color solution and heated for half an hour. To remove the extra colors, the strips were washed by distilled water and left to dry. In order to prepare the polystyrene solution (PS), 1.8 g powder was dissolved in 15 ml of xylene under a hood. The labels were cut into square pieces (1×1 cm), immersed in PS solution, and dried near heat. This process was repeated 10 times and the thickness of PS layers was finally checked by scanning electron microscopy. The pieces of label were immersed in melted paraffin and dried near heat. The outer layer of the package was made of polyethylene (Figure 1). In order to investigate the proper function of produced label, release tests and label scanning electron microscopy were performed.

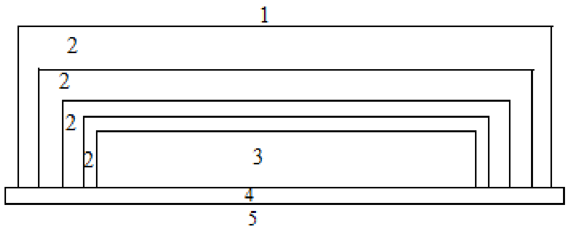

Figure 1: Schematic shape of designed label with different layers, (1: inner side (milk), 2: polystyrene layers, 3: beetroot color and cellulose, 4: polyethylene layer, 5: outer side)

Label release test

The release of color was detected by a UV spectrophotometer at the end of the seventh day. In order to isolate the proteins and fat of milk, 30 µl of milk sample was mixed with 3 ml of ethanol. The mixture was centrifuged at 4500 g for 15 minutes, the transparent liquid was directly transferred into cuvette in the spectrophotometer and the absorbance was measured at 450 nm [18]. The final value of dye release into the milk sample containing complete label was obtained by comparing it with the absorption values for the negative control (milk sample without freshness label on the package, i.e., 0% dye release into milk) and positive control (milk sample with freshness label lacking polystyrene layers and white alum, i.e., 100% dye release) [19].

Scanning electron microscopy (SEM) of label

Microstructure of the cross-sections of dried label was observed by scanning electron microscopy (EM-3200, KYKY, China). The labels were mounted on the specimen holder with aluminum tape and then sputtered with gold in BAL-TEC SCD 005 sputter coater (BAL-TEC AG, Balzers, Liechtenstein). Samples were photographed at tilt angles of 60–90 °C to the electron beam in order to show the cross-section view [20].

Milk freshness analysis

Milk freshness analysis was measured by pH, titratable acidity, and total bacterial count (TC) in days 0, 3, 4, 5, 6, and 7 at refrigerator temperature (4 ± 2C°).

Measurement of milk pH

A digital pH meter (digital pH meter, Taiwan) was applied to measure the pH, which was calibrated in standard buffer solutions of pH 7 and pH 4 prior to each measuring. In each measurement, the electrodes were completely immersed in the milk samples [21].

Measurement of milk acidity

The concentration of lactic acid (LA) was calculated by titration with NaOH 0.1 M. Each one ml of 0.1 N NaOH was equal to 0.009 gram of lactic acid. The lactic acid concentration was calculated using the equation 1 [8].

Equation 1: Acidity (%) = 0.009 × NaOH (0.1 N) used (g) × 100 / sample weight (g)

Quantification of TC

One ml of the milk sample was diluted with 9 ml of 0.1 % peptone in sterile conditions. A series of dilutions were prepared from 10^{-1} to 10^{-9} and cultured on the Plate Count Agar (PCA) to observe the countable (30-300) range of colonies. Moreover, 1 ml of the diluted milk was cultured on a PCA plate in triplicates. The plates were incubated at 30 ℃ for 72 h until visible colonies appeared on agar plates [8]. The colonies were counted using a colony counter.

Label color number

In order to determine the label color number, a 13-megapixel digital camera was used to take photos at a distance of 10 cm for 7 days. Later, the color number was calculated with the graphics software Adobe Photoshop CS3 (Adobe Inc., USA). The final color number was calculated.

Statistical analysis

Statistical analysis was performed using Statistics Package for Social Sciences (SPSS) software, version 17 (SPSS Inc., IBM Corp, Chicago, USA). The results were analyzed using ANOVA, regression analysis and Pearson's correlation coefficient at the significance level of P < 0.05. This procedure was repeated three times and all results were reported in mean ± SD. The relationship between color change and other parameters (TC, acidity and pH) were evaluated by Pearson's correlation.

Ethical issues

This study was conducted with approval of Shahid Sadoughi University of Medical Sciences. Research Ethics Code was IR.SSU. SPH. REC.1395.122.

Results

Label response

There was not seen any changes in the color of freshness label with the naked eye during four days. From the fifth day, the dark red color began to fade slowly. After seven days, along with the increase in TC and acidity, the pH decreased due to the spoilage and the label color changed obviously from dark red to light brown and the warning sign indicating milk spoilage appeared. Figure 2 shows the color change of milk freshness label at 4 °C.

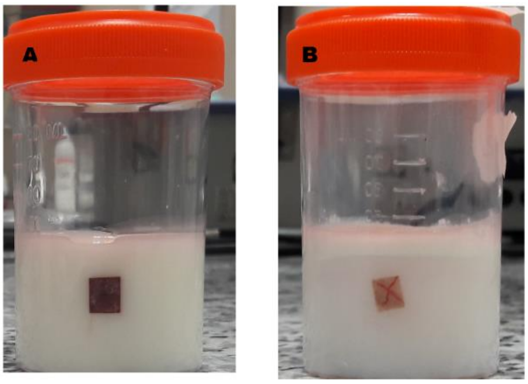

Figure 2: Change in the color of milk freshness label at 4 °C. A: Fresh milk; B: Spoiled milk.

Label color number

As figure 3 shows, the color number of labels increased regularly during seven days. The label color number on the first day was calculated as 91 ± 3, which had a rising trend over time and reached 167.09 ± 1 on the fifth day and 213 ± 4 on the last day. A significant difference was observed in color number during all days of the study.

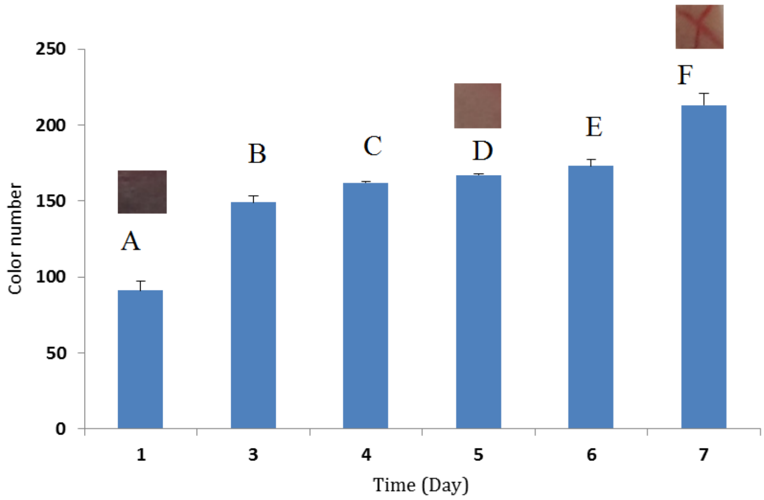

Figure 3: Range of color number of milk freshness label in 7 days
Different letters for each column shows significant differences at $p < 0.05$

Label release test

In order to estimate function of the designer label, possible release of beetroot color into the milk was investigated. The release of beetroot dye into the milk after 7 days was estimated as 0.2 by spectrophotometry. According to the absorption values calculated for the zero percent, 100 percent, and unknown samples, the amount of dye release into the milk was calculated as 20 percent after seven days of storage at refrigerated temperature.

Cross-section SEM of label

As figure 4 (a) depicts, the beetroot particles on the cellulose layer are amorphous with the size of about 50 µm. Figure 4 (b) illustrates the polystyrene layers.

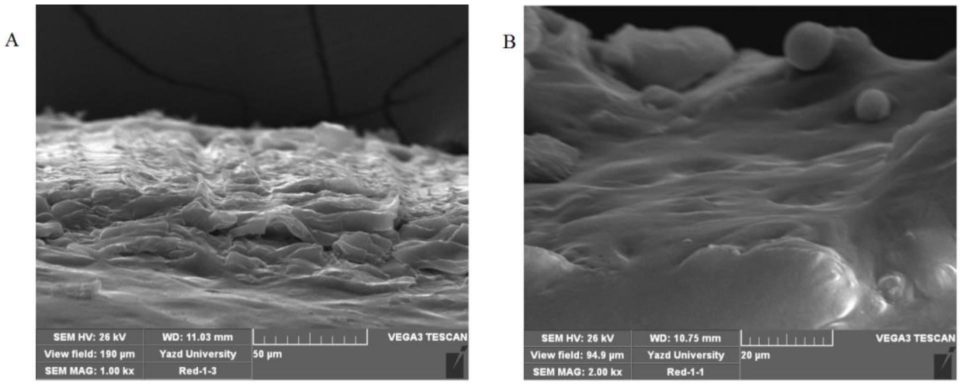

Figure 4: SEM pictures of milk freshness label. The size of the beetroot particles (a) and polystyrene (b)

Milk pH

The pH values of pasteurized milk samples are shown in figure 5. The pH values decreased steadily during seven days of storage at refrigerator temperature. The pH value of milk was 6.78 ± 0.02 at the first day, which decreased to 6.45 ± 0.01 after five days and eventually reached to 6.5 ± 0.03 at the last day. The results showed no significant difference ($p > 0.05$) up to the third day. However, after the third day, pH of milk had a significant decrease ($p < 0.05$).

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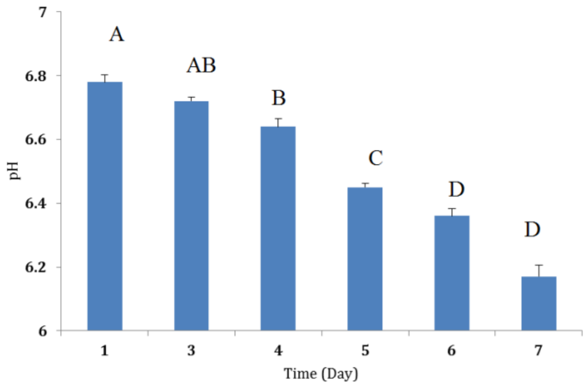

Figure 5: Values of milk pH at 4 °C
Each different letter means that the column has significant differences with other columns at $P < 0.05$.

Milk acidity

As indicated in figure 6, the initial acidity of the milk was 0.14 ± 0.007 in the first day, which increased gradually as the milk storage time increased at the refrigerator. The acidity increased to 0.18± 0.003 on the fifth day and reached to 0.21 ± 0.015 on the seventh day. No significant difference ($p > 0.05$) was observed up to the fourth day. After the fourth day, the acidity value of milk had a significant increase ($p < 0.05$).

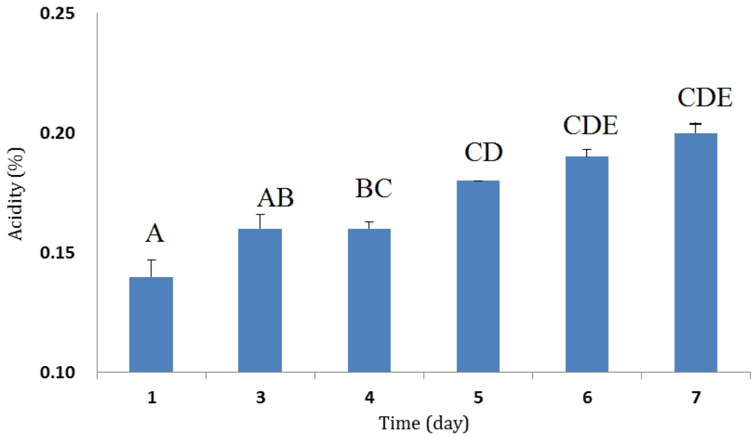

Figure 6: The acidity values of pasteurized milk acidity at 4 °C
Different letters mean that the column has significant differences with other columns at $P < 0.05$.

Quantification of the TC values in pasteurized milk at 4 °C

On the first day of experiment, the TC was 4.86 ± 0.04 Log CFU/mL. However, on the fifth day, it reached to 5.20 ± 0.009 Log CFU/mL and on the seventh day it increased to 5.74 ± 0.007 (Figure 7). No significant difference ($p > 0.05$) was observed between the first and third days. However, after the third day, milk samples showed a statistically significant increase ($p < 0.05$) in TC.

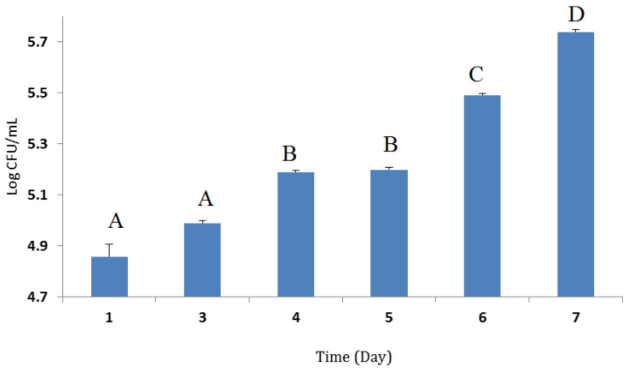

Figure 7: The value of TC of pasteurized milk at 4 °C.

Different letters mean that the column has significant differences with other columns at P < 0.05.

Analysis of the relationship between label color changes, acidity, pH, and TC in pasteurized milk

Based on the Pearson correlation coefficients, the label color changes had a strongly positive correlation with TC, acidity, and storage time. Furthermore, a strong negative correlation was found between the label color changes and pH (range from 0 to 1).

Discussion

Smart packaging has important applications in monitoring freshness of food, especially with regard to the highly perishable food stuffs such as pasteurized milk due to rapid spoilage and limited shelf life. The aim of current study was to evaluate the applicability of a novel color label for detecting the spoilage of pasteurized milk and determining the correlation of its color change with milk pH, acidity, and total bacterial count during the refrigerator storage. In recent years, studies on color-based indicators, which could warn consumers about the chemical and microbial changes of foods, received great attention. Synthetic reagents, such as methylene blue, methyl red, bromothymol blue, and phenol red were frequently used as pH indicators in different studies. For example, bromothymol blue and methyl bromide were utilized as the milk spoilage indicator by Guggilla et al. [24]. Tassanawat et al. applied bromothymol blue in nano-composite indicator film for beverage smart packaging [25]. However, some concerns exist about application of synthetic reagents in food. Application of natural pigments extracted from vegetable sources in smart packaging was also investigated in some studies. For instance, grape anthocyanins was examined as an indicator of chilled pork deterioration [12]; natural dyes extracted from the flower of rose and red cabbage were used as a sensor in intelligent packaging to determine decay in buffalo meat [4]. Furthermore the natural dye curcumin was used for real-time monitoring of the shrimp spoilage [26]. In the present study, beetroot color ingredients were used, since betalains in beetroot are resistant to temperature and can display the range of pH changes, which are suited to low-acid foods like milk [15]. The beetroot color ingredients reacted to the bacterial activity and production of lactic acid in milk, changing from dark red to light brown, which indicated a warning sign.

In current study, multi layered polystyrene label was used for milk freshness determination because it is commonly used to make packages in food industries and studies [6, 27]. The multi layered polystyrene is cheap, lightweight, recyclable, and adhesive with high level of humidity resistance. It also enhanced the adhesion of beetroot color ingredients to the label. To achieve higher levels of absorption and better stability of beetroot ingredients on the cellulose substrate, the white alum that created some scratches on the surface of cellulosic paper was utilized. Kavosi et al. used Alum to treat the recurrent aphthous stomatitis for its adhesion property [28]. The changes in color of label is attributed to production of H^+ ions in the spoiled milk; transition of H^+ ions throughout the polystyrene layers and active beetroot color

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changes the color. Adhesion of beetroot color to cellulose paper makes the release of the color to the milk impossible.

In the present study, a positive correlation was found between the label color changes and TC. The bacteria consume milk compounds such as lactose, lipid, and protein during the milk spoilage. A similar study on milk spoilage detection [9] supports our result. The researchers found that the dye reagent on a modified polypropylene film faded with the growth of microorganisms and decomposition of milk ingredients. Although accurate determination of TC was not aimed in this study, another study [29] demonstrated that color change of the reagent was directly related to the growth of bacteria stated as Log CFU/mL.

Our findings showed a significant correlation between the label color and pH. Generally, bacteria adapt themselves to growth conditions in the lag phase. Afterwards, they started to increase in the log phase, which is a period characterized by cell doubling. Later, LA was produced and milk pH was decreased. A similar study used an indicator based on pH to show the milk spoilage and the color change of the reagents (from light green to red) caused by the gradual reduction of pH in milk [24]. The correlation between TC and milk pH was investigated in another research, where bromothymol blue changed from yellow to blue [11]. According to Iran's National Standard, the acidity range and pH of fresh bovine milk is 0.14-0.16 % and 6.6-6.8, respectively [30]. In the current study, the acidity and pH measurements were within those recommended limits up to the fourth day. From the fifth day, the acidity value (0.18%) was higher than the national standards and the pH (6.45) was lower than the standard.

The TC was in accordance with the Iran's National Standard limits from the first to the fourth day of the experiment. However, in the fifth day, these measures reached 4.87 Log CFU/mL that was higher than the standard [31]. The psychrophilic bacteria can grow in pasteurized milk kept in refrigerator. As the bacteria grow in milk, their products also pile up. Label color change reflects different factors including bacterial activity, production of LA, and pH changes during spoilage.

Beetroot color was chosen in this milk freshness label since the natural dyes in packaging do not cause harmful effects on health and enjoy high acceptability among the consumers. Similar studies employing natural products show this trend[12-13, 26]. To the best of our knowledge, this was the first study that applied natural beetroot ingredient as a freshness label in pasteurized milk packaging. Since milk spoilage can cause poisoning and is dangerous for health, such warning labels should be applied to help the consumers. In this regard, the milk should not be consumed when the color of label turns from dark red to light brown.

Conclusion

In the current study, the natural dye of beetroot was used as a freshness indicator to display the changes of pasteurized milk stored at refrigerator. Application of polystyrene layers, white alum and beetroot as a natural reagent on cellulose substrate looks affordable and simple. Since the reagent was natural and edible, it is appropriate for food packaging. We recommend other researchers to study the freshness labels with various pigments from natural sources such as carotene in carrots or anthocyanin in tomato. Furthermore, studies can be conducted on stabilizers such as carboxymethyl cellulose or gelatin to improve the stability of pigments on substrates.

Authors' Contributions

Akrami, jebali, Hekmatimoghaddam, and khalili designed the experimental protocols. Eshaghi carried out the experiments. Eshaghi, Akrami, and khalili wrote the manuscript. All authors contributed to the final version of the manuscript.

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Conflict of interest

There is no conflict of interest.

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