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# Developing a novel colorimetric indicator for monitoring rancidity reaction and estimating the accelerated shelf life of oxygen-sensitive dairy products

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**Abstract**

A colorimetric mixed pH dye-based indicator to real-time monitoring rancidity reaction of infant milk powder formula is described. Milk powder stored in 30, 40, and 50°C was analyzed in terms of peroxide value (PV), moisture content, volatile compounds and sensory score. In addition, moisture sorption isotherms of infant milk powder formula were assessed at various temperatures including 30, 40, and 50°C. It was found that milk powder kept at higher temperature had a shorter shelf life, whereas milk powder with higher moisture content yielded a higher PV, and vice versa. A rancid sensory score revealed a similar fashion to PV. Both hexanal and acetic acid concentrations increased with increasing moisture content of milk powder. An indicator label consisting of mixed pH-sensitive dyes of both bromothymol blue and methyl red responds through visible color change to volatile compounds e.g. hexanal and acetic acid released from oxidation and hydrolytic reaction during storage period. Indicator labels placed in headspace of milk powder bottles that stored in 30°C. Indicator label characteristics were studied, as well as their response to volatile compounds by changing color from light green to orange. An orange was a sign of either warning or rejecting. An accelerated shelf life of milk powder was estimated at 26 days.



## Introduction

Off-flavors in infant milk powder formula during storage are one of the most critical problems of this product. Usually off-flavors in infant milk powder formula are produced by lipid oxidation and hydrolytic. Lipid oxidation by a free radical chain reaction process involving three stages: initiation, propagation and termination. Unsaturated fatty acid was involved by an initiator to formed free radicals in the initiation stage. Common initiators include ultraviolet light, photo sensitizers and visible light, radicals from other sources, ionizing radiation, metals, home compounds and heat. Free radical would be reacting with oxygen to form proxy radical. Proxy radical is very selectively abstract hydrogen from adjacent lipid molecules forming hydro peroxides and generating new free radicals, hydroperoxides as the primary oxidation products in the propagation stage (Schaich, 2009). In the termination stage, lipid free radicals terminate to form non radical products, which are the secondary lipid oxidation products (alkenes, alkenes, aldehydes, ketones, etc.), that are responsible for a change in taste and odor of food products (Woo and Lindsay, 1983). In this case, hexanal may be serve as a useful index of lipid oxidation but off-flavors in rancid dairy products may be caused by the release of free fatty acids (FFA) from milk through the action of lipases are known as hydrolytic rancidity or lipolyzed flavors. Hydrolytic rancidity results from the hydrolytic degradation of milk lipid, triglyceride, the hydrolysis is catalyzed by lipase, that involved are two sources as endogenous milk enzyme and enzymes of microbial origin, and

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produces free fatty acids (Deeth and Fitz-Gerald, 2006). The most methods to detect rancidity reaction but its take time in laboratories do not have easy to understand. Freshness indicator is good alternative to monitor rancidity in milk powder formulas. This technique has shown potential for this application in many products. The amount of published works on freshness indicators is still limited. However, they have constructed indicators for the volatile compounds produced in microbial spoilage e.g. myoglobin-based indicators for modified-atmosphere-packed poultry meat on the basis of the presence of hydrogen sulphide (HS), a colorimetric dye-based sensor and indicator for monitoring fish spoilage on the basis of the presence of total volatile basic nitrogen (TVB-N), a novel colorimetric mixed-dye-based indicator for monitoring freshness of intermediate-moisture dessert spoilage (Smolander *et al*., 2002; Pacquit *et al*., 2006; Pacquit *et al*., 2007; Nopwinyuwong *et al*., 2010). There exist many known indicators of freshness of food products that indicate whether a certain food product may be spoiled from microbial growth. There are indicators of microbial growth that change color only after the microbe grows. The pH dye is chemicals that change color when pH changes have been used to mark the presence of bacterial growth. For rancidity reaction that is a main deteriorate reaction for oxygen sensitive foods, there is no published work related to rancidity indicator available in public domain. However, there is only one study of Vo *et al*. (2007), which revealed an aldehyde indicator pad for the rapid detection of aldehyde using in chemical industrial application. It was shown that this indicator pad responded to glutaraldehyde with a visible color change from yellow to red. The color change of aldehyde indicator was occurred with pH decreased when adding glutaraldhyde. Therefore, a freshness colorimetric indicator for monitoring rancidity reaction of oxygen-sensitive dairy products is supposed to be a novel rancidity indicator for monitoring hexanal and acetic acid producing from oxidation and hydrolysis, respectively. The objectives of this present work aimed at determining the quality of infant milk powder formula kept at ambient and elevated conditions, at developing a novel colorimetric indicator for monitoring rancidity reaction and at estimating the shelf life of infant milk powder formula.

## Materials and Methods

### *Materials*

Bromothymol blue (Ajax Finechem, Australia) and methyl red (Panreac Quimica, Spain) were used to prepare a dye mixture. Food-grade methylcellulose (MC) and hydroxypropyl methylcellulose (HPMC) (Methocel, Dow Chemical, USA) were used as the carbohydrate biopolymer for coating formulations. Polyethylene glycol-400 (Fluka, Sigma-Aldrich, Singapore) was added as a plasticizer. Optically clear linear low-density polyethylene films (50 micrometer) were obtained from Alcan Packaging Strongpack, Thailand.

### *Oxygen-sensitive dairy product study: Infant milk powder formula case*

### *Experimental setup*

Enfalac (Mead Johnson Nutrition, Thailand), infant formula contains omega 3, 6, 9, choline and 2 fibers mixed, is brought from supermarket in Thailand was studied. Main ingredient of infant formula includes that lactose (33.35%), whey protein (15.6%), palm olein oil (13.21%) skim milk powder (13.116%), Soybean oil (5.87%), coconut oil (5.87%), safflower oil (4.403%), galacto- oligosaccharides 28% (2.9%), inulin (2.7%), lecithin (1.222%), minerals (1.19%), corn syrup (0.205%), vitamins (0.16%) choline chloride (0.134%), taurine (0.05%) and nucleotide (0.02%). The approximate shelf life was not exceed 3 months. About 35 g infant milk powder formulas were placed into glass bottles (100 ml contained). Milk powder bottles were separated in 2 part, first part (sealed), closed by rubber with aluminum cap and put indicator labels and a moisture absorber sachet (1g silica gel contained) and second part (non-sealed), not closed. Infant milk powder formula samples were kept under an isothermal controlled condition of 30°C, 40°C and 50°C and exposed to daylight fluorescent light tube, 14 watt and 1,150 lm (Lamptan Lighting Technology co. Ltd) for accelerated shelf life estimation. The light tubes were suspended 20 cm above the samples. The powder samples were stirred every day with checker for maintaining a uniform exposure to light and temperature. Three samples were withdrawn for analyses. Indicators labels were compared with control that bottle not have milk powder.

### *Moisture content*

Moisture content of infant milk power was according to AOAC International that developed by Lane (1998).

### *Peroxide value*

Fat of infant milk powder was isolated using the technique developed by Pont (1955) and subsequently improved by Newstead and Headifen (1981). Fat was evaluated peroxide value, expressed as meq.$O_2$/kg oil, was determined in the fat extract according to method cd 8-53 of the American Oil Chemists' Society (AOCS, 1998).

*Volatile compounds*

Headspace vial contained 3 g milk powder formula as received were matrix-spiked with 10 µg of 0.001 ppm benzene to serve as internal standards and the vials were incubated at 60°C for 45 min. Following incubation, 1.0 mL of head space vapor were withdrawn and injected into the GC-MS for analysis using a pre-heated gas-tight syringe equipped with an on/off valve. The analysis of volatile compounds was carried out using a Shimadzu TQ8030 Gas chromatography (Scientific Equipment Source, Japan) equipped with an HP-5MS capillary column (30m x 0.25 mm I.D., 0.25 µm film thickness). The oven temperature was maintained at 40°C for 1 min, increased to 240°C for 2 min at a rate of 3°C/min. The injection and interface line temperature are 220°C and 230°C, respectively. The injection port was in split less mode. The ion source temperatures were 220°C. Electron impact ionization was used at a voltage of 70 eV.

*Sensory evaluation*

Infant milk powder formula, sampling from accelerated condition, were presented to six trained panelists to measure intensity rancid odor. The panelists practiced rating the intensity using line scale (0 = no rancid to 3= extremely rancid).

*Moisture sorption isotherm*

The moisture sorption isotherms for milk powder were measured using the static gravimetric method. Dried Infant milk formulas were placed in air tight bottle, which contained saturated salt solutions (Sigma-Aldrich, Singapore) of known %RH: 11 (LiCl); 22 ($CH_3COOK$); 33 ($MgCl_2$); 43 ($K_2CO_3$); 54 ($Mg(NO_3)_2$); 63 ($SrCl_2$); 75 (NaCl); 85 (KCl); and 94 ($KNO_3$)) placed inside temperature controlled rooms (30°C, 40°C and 50°C). After equilibrium, milk powder formula were measured moisture content and water activity ($A_w$) was determined using a water activity instrument (Testo 650, Testo, Inc., Germany).

*Colorimetric indicator development*

*Indicator solution preparation*

Three types of indicator solution, such as methyl red solution, bromothymol blue solution and mixed dyes solution, were prepared. Methyl red solution and bromothymol blue solution were prepared with 125 µl methyl red indicator solution (or bromothymol blue indicator solution) in 4.875 ml ethanol and 5 ml distilled water. Mixed dyes solution was prepared by mixing bromothymol blue (75µl v/v) and methyl red (50 µl, v/v) in 4.875 ml ethanol and 5 ml distilled water in a ratio of 3:2.

*Indicator label fabrication*

Cellulose-based coating was prepared by mixing 7 g methylcellulose, 3 g hydroxypropyl methylcellulose in 300 ml distilled water, heat at 70°C and adding with 1% plasticizer such as polyethylene glycol-400 and then kept in refrigerator about 12 hr. Indicator label was obtained by casting a cellulose-based coating incorporated with indicator solution onto 50 µ LLDPE film to obtain a thickness of indicator 140 µ, label was then dried at 25°C and 30 %RH for 24 hr.

*Color changes of indicator solution caused by free fatty acid*

Color changes of an indicator solution due to contact with free fatty acid as butyric acid were studied by enclosing 10 mL of dye solutions in gas-tight vials (20 mL). For sensitivity test in vary volume, 0.1 N butyric acid was injected into the vials, obtaining final butyric acid volume of 0-26 µl depending on type of dye solutions to change color of indicator. Another way, vary concentration of butyric acid are used to test sensitivity of changes color of indicator. Fixed volume in maximum and vary concentration of butyric acid from 0 to 0.1 N and then injected into indicator solutions. Indicator solution samples were incubated at 30°C for 30 min, and their optical spectrum recorded with a T60 UV spectrophotometer (PG Instruments, UK).

*Color changes of indicator labels*

The color change of the colorimetric mixed-dye-based indicator was evaluated visually and measured instrumentally with a Minolta CM-400 spectrophotometer, using L, a, b values to describe the color of the indicator. The index describing the total color difference (TCD) was suggested by Hunt (1991). Color change (ΔE) was calculated with formula (1):

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2} \quad (1)$$

*Statistical Analysis*

The experiments were conducted in three replication of each treatment. Statistical analysis was performed using Student's a one-way ANOVA test followed by LSD's test comparison of multiple

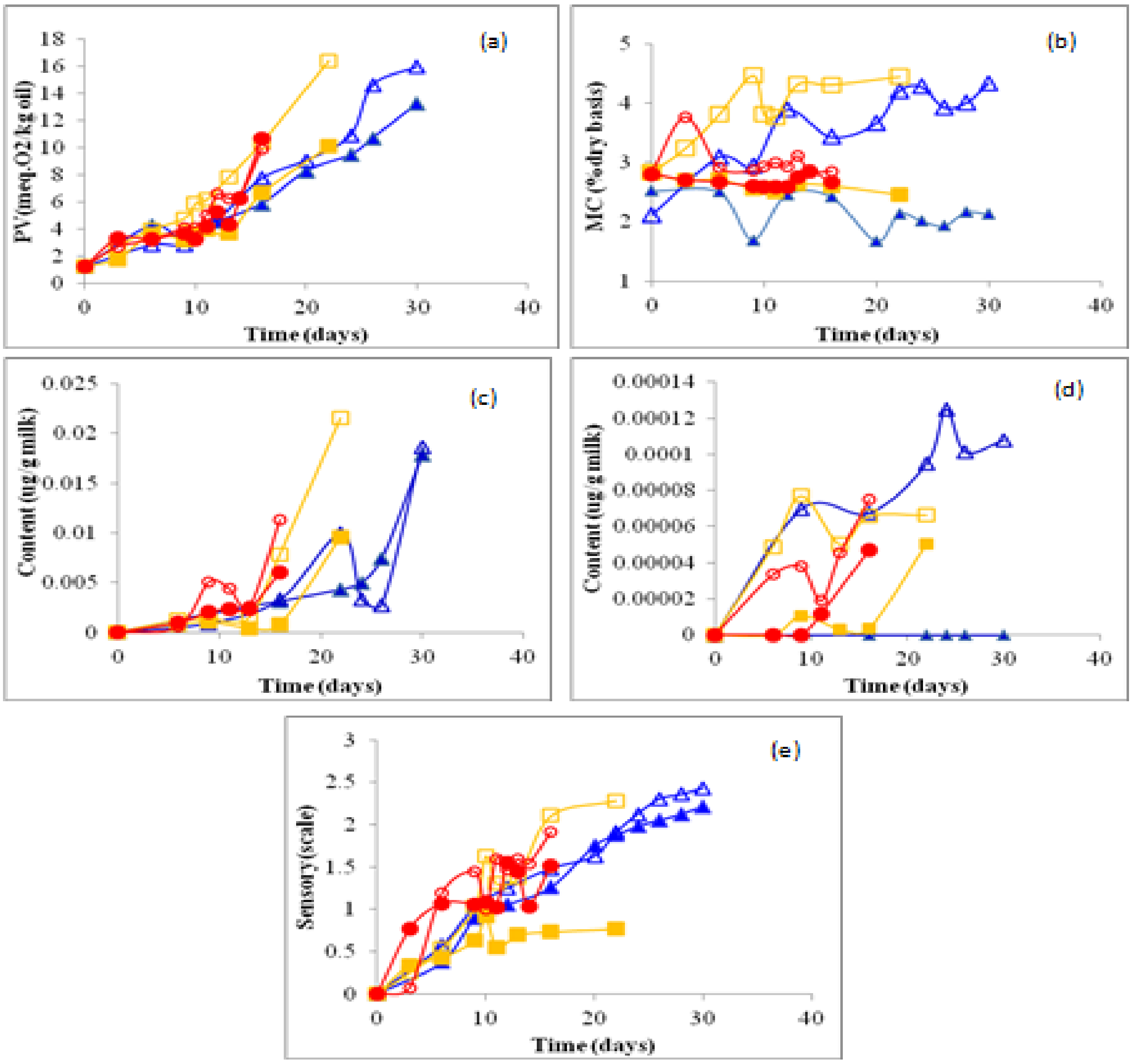


Figure 1. Effect of moisture content and temperature on rancidity milk powder formula, (a) peroxide value, (b) moisture content (dry basis), (c) hexanal, (d) acetic acid and (e) sensory evaluation (▲) sealed 30°C, (△) non-sealed 30°C, (■) sealed 40°C, (□) non-sealed 40°C, (●) sealed 50°C and (○) non-sealed 50°C

means. Statistical analysis was performed using the SPSS for Windows version 15.

## Results and Discussion

*Oxygen-sensitive dairy product study: Infant milk powder formula case*

*Rancidity reaction of milk powder formula*

Peroxide value is a quality index which is used at the early stage of the lipid oxidation (Xingjian *et al*., 2009). In Figure 1(a), peroxide value of milk powder in each temperature shows increasing trend. Milk powder in non-sealed bottles had higher PV than those of milk in sealed bottles. Especially, milk powder in non-sealed bottles which kept at 40°C presented the highest PV value. PV value of milk powder in sealed bottles were 3.59, 3.19, 3.64 and 6.63 meq. $O_2$/kg oil at 6, 9, 12, and 16 days, respectively, whereas PV value of milk powder in non-sealed bottles were 3.93, 4.75, 7.81 and 10.40 meq.$O_2$/kg oil at 6, 9, 12, and 16 days, respectively. Generally, temperature is one of major factors which accelerated the deterioration of oxygen sensitive food products, resulting in oxidative rancidity of unsaturated fatty acid. (Xiangjin and Yali, 2011; Rufian-Henares *et al.* 2005) In turn, the higher temperature, accelerated the faster lipid oxidation, and vice versa. In this experiment, there were influences of both storage temperature and moisture content of milk powder on rancidity reaction. The results showed PV value of milk powder in non-sealed bottles which kept at 40°C presented higher PV value than those of milk powders in both sealed and non-sealed bottles which kept at 50°C ($p<0.05$). The presence of either water or free radicals combined with higher temperature may increase the rate of hydrolysis, leading to undesirable odors and tastes. Stewart and Bewley (1980) reported that high temperature and high relative humidity aged soybean axes contained malondialdehyde derived from the peroxidation of unsaturated fatty acid. In addition, the rate of lipid oxidation depends on water activity. With an $A_w$ of 0.3, the product is most stable with respect to lipid oxidation, whereas an $A_w$ of approximately 0.8 contributed to the rate of lipid oxidation reaching to the maximum and following by a declination (Labuza, 1982). As shown in Figure 1a and Figure 1b, milk powder in non-sealed bottles stored at 40°C had an increase in moisture content from 2.83 to 4.31 (% dry basis) along 16 days which had the highest moisture content ($p<0.05$). It was followed by milk powder in non-sealed stored at 30 and 50°C, which had increases in moisture content from 2.12 to 3.43 and from 2.83 to 2.85 (% dry basis), respectively. In addition, milk powder in non-sealed stored at 30 and 50°C substantially yielded the highest PV value. This is because temperature around 37-44°C, significantly enhanced not exclusively the growth of lipase-producing microorganisms, but also lipase activity. As catalyzed, lipase lead to hydrolysis rancidity (Onilude *et al*., 2010). From these findings, however, moisture content plays a major role on lipid oxidation as presented in PV, in comparison with temperature

In Figure 1 (c) and 1(d), at storage time of 16 days, milk powder in ether non-sealed or sealed bottles stored at 50°C exhibited a significant increase in hexanal, an oxidation state index, compared to acetic acid, a hydrolytic state index, due to more pronounced temperature on hexanal production. Conversely, milk powder, especially in non-sealed bottles stored at either 30 or 40°C, to higher moisture content, gave a higher level of acetic acid via hydrolysis. Milk powder in non-sealed bottles at 30 and 40°C at 22 days had an increase in both hexanal and acetic acid via oxidation and hydrolysis, respectively.

In Figure 1(e), sensory score of high moisture milk powder could fluctuate during 8-10 days, and then increase significantly in the longer term. An unacceptable sensory score for non-sealed milk powder presented high score at high moisture content and high temperature. Sealed bottles milk powder was considered unacceptable (score ≥ 1.5) for 20 and 16 days when kept at 30 and 50°C, respectively, and non-sealed milk powder for 16, 14 and 13 days when kept at 30, 40 and 50°C, respectively. Normally, the flavor of fresh milk powder has a light, almost bland taste, but with a characteristic sweet and salty flavor from lactose and milk salts. A large number of volatile compounds in fresh milk powder is more difficult to detect. Ogden (1993) reported that some common off flavors are acidic, astringent, unclean, cowy, bitter, cooked, feedy, weedy, fruity, foreign, lacking freshness, malty, rancid, salty and oxidized.

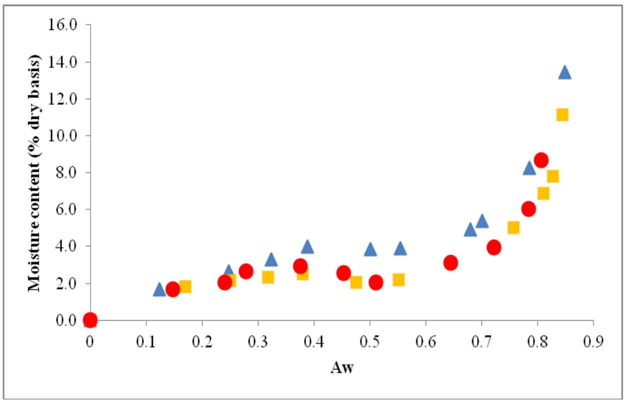


Figure 2. MSI of milk powder formula in different temperature condition (▲) 30°C, (■) 40°C and (●) 50°C

High moisture content in milk powder had affected to microbial growth, thus many volatile compounds produced by microorganism were interfered with oxidative rancid odors.

*Moisture sorption isotherm*

The moisture sorption isotherm curve of milk powder, represented in Figure 2, can be classified as a type II sigmoidal isotherm, which is obtained for soluble materials and shows an asymptotic trend as water activity tends toward 1 (Bell and Labuza, 2000). Moisture sorption was more rapid in the initial stages, and a lesser amount of moisture was adsorbed as adsorption time increased. The higher the relative humidity used, the more pronounced the effect. The equilibrium moisture content of the product dramatically soared above $A_w = 0.8$.

The results is in accord with hypothesis, An increase in temperature caused an increase in $A_w$ for the same moisture content and, if $A_w$ was kept constant, an increase in temperature caused a decrease in the amount of absorbed water (Stencl *et al*. 2010). Adsorption of milk powder between $A_w = 0.12$ and 0.38 were slightly increased and decreased at $A_w$ above 0.40 that showed this trend in each storage condition. This result showed according to the results of many researchers (Bronlund and Paterson 2004; Shrestha *et al*. 2007; Murrieta-Pazos 2011).

This phenomenon is characteristic of amorphous lactose crystallization which occurs at temperature above the glass transition temperature (Roos and Karel, 1990). McCarthy *et al.* (2013) reported moisture content during the initial period increased as amorphous lactose and proteins absorbed water. Milk powders with lower protein levels absorbed less water prior to lactose crystallization (which was indicated by a sudden drop in water content). Additional, Shrestha *et al.* (2007) reported higher lactose level in powder absorbed more water and lower point adsorption at which crystallization starts.

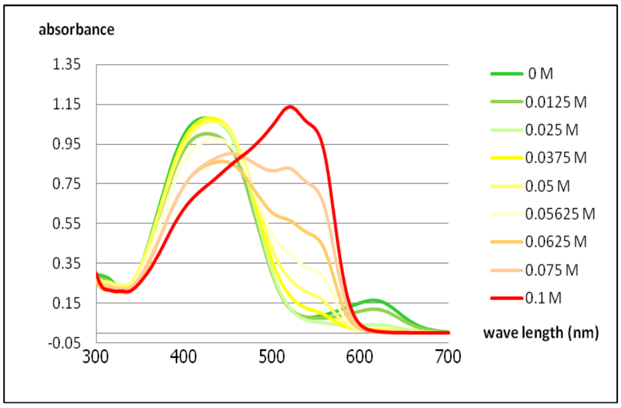


Figure 3. Absorption spectra of mixed-dye-based indicator solution, bromothymol blue solution and methyl red solution reacted with concentration of butyric acid

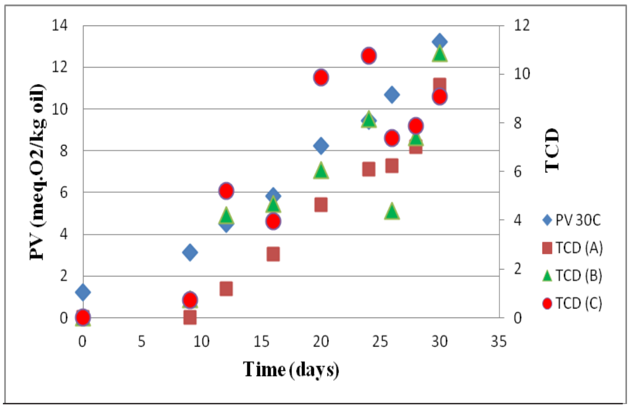


Figure 4. TCD of indicator labels correlate PV level of milk powder after kept at 30°C

MSI determine optimal conditions for storage from the point of view microorganisms grow, $A_w < 0.6$.

*Color changes of indicators solution*

When butyric acid were added to vials containing colorimetric mixed-dye-based indicator solution, a visual color change of the solution from bright green to bright red was detected. The most remarkable change in the absorption spectra after 30 min of reaction time took place at high absorption peaks in the wavelength range of 430-526 nm (Figure 3). Bromothymol blue, which shifts from basic form (blue, pH 7.6) to acidic form (yellow, pH 5.8), results in maximum lambda ($\lambda_{max}$) shifting from 615-618 to 430-435 nm. Methy red, which shifts from basic form (yellow, pH 6.2) to acidic form (red, pH 4.5), results in maximum lambda ($\lambda_{max}$) shifting from 430-435 to 523-526 nm. As a mixed-dye-based indicator, bright green changed to bright red in relation to a shift in the absorption peak from 430-435 to 523-526 nm when exposed to level of butyric acid (0.0125–1.0 M).

A visual color change of the solution from bright green to bright red was detected according to Nopwinyuwong *et al.* (2010). This vivid color spectrum of mixed-dye indicator solution is in agreement with Wallach (1996), who reported that a mixed indicator could enhance an expansion of the range of color change, as compared with a single indicator.

*Color changes of indicator labels during infant milk powder spoilage trial*

Due to limitation of utility of indicator labels, high moisture and high temperature had affected on color changes of indicator. This experiment would presented color changes of indicator labels in sealed bottles milk powder which only kept at 30°C.

Changes in PV of milk powder could directly associate with changes in total color difference (TCD) of colorimetric indicator labels (A-, B- and C-types) when kept at 30°C (Figure 4). The PV levels of milk powder slowly increased at the beginning of period, and then rapidly increased. Rosell (1999) reported that hydroperoxide level of freshly refined fats should be less than 1 meq. $O_2$/kg oil, and the limit of PV level specified by Joint FAO/WHO Standards (1989) was 10 meq.$O_2$/kg oil. This limited value was assumed to a minimal deterioration level of oxygen sensitive food product and indicated shelf life of this product as well. An accelerated shelf life of milk powder in sealed bottles was estimated at 26 days when stored at 30°C with light exposure. TCD values of each type gradually increased with time as well as PV levels. The TCD value also changed continuously and consistently with the response of the indicator label. TCD values at 26 days were 7.280, 5.108 and 7.367 for A-, B- and C-types, respectively. Francis (1983) reported that TCD more than 5.0 could be easily detected by unaided eyes and TCD more than 12 presented a clearly different shade of color. Figure 5 presents the colorimetric indicator labels were gradually change from bright green to yellow, and orange, respectively, when exposed to volatile compounds such as hexanal, acetic acid and other compounds. Color change of indicator labels correlated with increasing the concentration of PV during storage time. The color of B- and C-types could change so quickly as compared with these of A-type. Both types could not well relate with the change of PV. Therefore, the indicator response of A-type could monitor rancidity of milk powder at 30°C. Mixed-dye-based food freshness indicator could be accurately used to monitor the freshness of oxygen sensitive food products. Milk powder in sealed bottles with food freshness indicator labels (A-, B- and C-types) are shown in Figure 6.

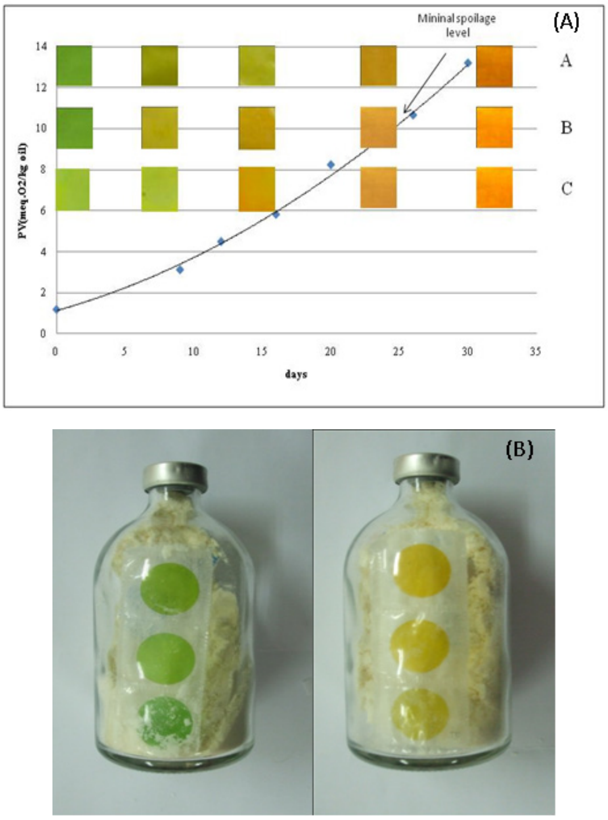


Figure 5. PV value of milk powder in sealed bottles at 30°C (A) attached with indicator labels changing in color from bright green (fresh) to orange (warning) (B)

## Conclusion

For rancidity reaction, moisture content and temperature affected to the rancidity in milk powder. It can be concluded that milk powder kept at higher temperature had a shorter shelf life, whereas milk powder with higher moisture content yielded a higher PV, and vice versa. A change in color of A-type indicator label could correlate well with PV levels in milk powder, therefore enabling the non-invasive and real-time monitoring of product deterioration. An accelerated shelf life of milk powder in sealed bottles kept at 30°C was uppestimated at 26 days. Hence, this study demonstrated that a novel colorimetric indicator could be accurately used for monitoring rancidity reaction of milk powder formula.

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