Guideline for Proper Usage of Time Temperature Integrator (TTI) Avoiding Underestimation of Food Deterioration in Terms of Temperature Dependency: A Case with a Microbial TTI and Milk

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Abstract Time-temperature integrators (TTIs) can be used to predict food deterioration. However, underestimation of the magnitude of deterioration is not desirable. This study aims to establish guidelines in terms of temperature dependency (Arrhenius activation energy, E_a) to avoid such underestimation by proper use of TTIs. A case study was executed with a microbial TTI and milk. The E_a of the TTI color change was 106 kJ/mol and those of milk deterioration factors aerobic mesophilic bacteria count, lactic acid bacteria count, lactic acid %, and pH were 101, 107, 122, and 145 kJ/mol, respectively. The deterioration factors with values of E_a larger than that of TTI, lactic acid %, LAB, and pH, were found to be underestimated as compared to their actual levels by prediction from TTI color change, leading to potential consumption of deteriorated milk.

Keywords: TTI, underestimation of food deterioration, Arrhenius activation energy, intelligent packaging, food storage

Introduction

Since the quality and safety of chilled and frozen foods are strongly influenced by temperature, maintenance of time-temperature controls in the cold chain is increasingly important owing to more stringent regulations, new market demands, and increased consumer awareness. During the last two decades, there has been growing consumer demand for healthy, safe, and easy-to-prepare convenience food products. These trends have been reflected by researchers as well as by successful food companies by developing and marketing meticulous monitoring systems, such as the time-temperature integrator (TTI). A TTI is attached on the surface of the food package, and its color changes with the time-temperature history (1). The color change results from relevant physical, chemical, or microbial reactions (2-4). The remaining shelf life of food can be predicted from the TTI color.

TTIs have been applied to a number of cases, such as frozen vegetables (5), fish products (6-9), meat products (10-12), mushrooms (13), and dairy products (14,15). TTIs are required to predict the food quality changes accurately. However, some negative scenarios in TTI use can be possible with a practical example as follows. First, some experiments should be performed in which a TTI is stored with food at a certain temperature, during which the color and some food quality factors are measured. Then a comparison sticker including the TTI color grades and the food quality grades, such as “stale”, “less

stale”, and “fresh”, should be prepared. This sticker is used to find the current food quality by comparing the color grades against the observed color of the TTI on the food package. If the food quality grade read from the sticker does not agree with the actual quality, a quality deterioration problem is caused. This agreement between the predicted and actual quality levels is known to depend on TTIs temperature dependency (11). The temperature dependency represents how much the reactions are influenced by temperature change. A reaction with a higher temperature dependency takes place faster than one with a lower temperature dependency. So, food reactions with high temperature dependency would require more careful storage, not experiencing any temperature abuse. If a TTI has a different temperature dependency to that of the food, it would react differently to temperature changes, so that the comparison sticker could not be used at all relevant temperatures to which the product is exposed. On the other hand, if the TTI and the food have the same temperature dependency, the sticker would be universal.

The Arrhenius relationship is a common and convenient method of describing the temperature dependency. The temperature dependency is represented by the Arrhenius activation energy (E_a), which is an important specification of a TTI (16). Practically, the difference in E_a between the TTI and the monitored food should be within ± 25 kJ/mol to assess product quality correctly (1). Strictly, any E_a difference leads to two kinds of errors: under- and over-

estimations. In under- and over- estimations, the quality level is predicted to be lower and higher than the actual quality level, respectively. Overestimation would be acceptable from the food quality deterioration aspect, but underestimation should be avoided. This is because an underestimation of quality would mislead users into thinking the food is still safe to eat. On the contrary, overestimation would lead to fresh food being mistaken as stale.

There are two cases that E_a of TTI is larger and smaller than that of food. However, which case results in the underestimation had not been dealt with rigorously. In this study, the aforementioned errors in the prediction of food quality by TTIs were analyzed in terms of (+) and (-) of the E_a differences. Milk and a microbial TTI were used for a case study. The TTI color and milk properties were measured under isothermal storage conditions and their Arrhenius temperature dependency was investigated. The actual property levels were then compared to those estimated by TTI color to assess under- or over-estimation by the TTI.

Materials and Methods

Materials The materials used to prepare TTI were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) such as glucose, yeast extract, tryptone, monopotassium phosphate, Tween 80, manganese (II) sulfate monohydrate, magnesium sulfate heptahydrate, sodium citrate, sodium acetate, bromocresol purple, methyl red sodium salt, and low melting agarose.

Low Temperature Long Time (LTLT) pasteurized milk, purchased 5 days before the expiration date from L-Mart (Seoul, Korea), was used. A 30-mL aliquot of the sample was placed in a 50-mL conical tube and then stored at 2–4°C until used. It would have been preferable if a milk sample obtained directly after processing (e.g. bottling) was used because the TTI would have been added to the packaging. However, for the study purpose, the samples with uniform qualities were used rather than practically meaningful samples.

Preparation of microbial-based TTI A microbial TTI, which had a similar E_a to milk, was produced according to the method developed by Kim *et al.* (12). Actually, there are more choices of TTI available, but we used a TTI that was developed in our lab because the E_a or shelf life of the TTI could be adjusted by us. A culture solution with 5 log CFU/mL of *Weissella cibaria* CIPF 009 was prepared, 100 μ L of which was added to 20 mL of de Man-Rogosa-Sharpe (MRS; Difco, Detroit, MI, USA) broth. After incubation for 15 h at 37°C, it was centrifuged at 6,860 $\times g$ for 10 min at 4°C. The collected cells were immediately mixed into a sterile microbial TTI base compound (Table 1) and the pH was adjusted to 7.0 as a starting point by using 0.1 N NaOH. A 5.0-mL aliquot of the TTI mixture was used as the TTI prototype in each of six cells of a Cultureware plate (353502; Becton Dickinson, Franklin Lakes, NJ, USA).

Table 1. Formulation of the microbial TTI base

Components	Amounts
Glucose	30.0 g
Yeast extract	7.5 g
Tryptone	5.0 g
Monopotassium phosphate	1.0 g
Tween 80	0.5 g
Manganese(II) sulfate monohydrate	0.05 g
Magnesium sulfate heptahydrate	0.2 g
Sodium citrate	5.0 g
Sodium acetate	5.0 g
Bromocresol purple	3.0 g
Methyl red sodium salt	1.0 g
Low melting agarose	10.0 g
Distilled water	1,000 mL

Storage tests The milk samples were poured into falcon tubes and then both the TTIs and the tubes were stored in the dark in low-temperature incubators (NEX-202S; Nexus Technologies Co., Ltd., Seoul, Korea) under controlled isothermal conditions of 7, 15, and 24°C. The TTI response and milk quality were measured at certain intervals during storage.

Measurement of TTI response The TTI color change is based on lactic acid production by *Weissella cibaria* CIPF 009. For research purposes, the TTI response was measured in lactic acid amount, and then converted into a color index, because the error of color measurements was larger than that of lactic acid measurement. For the conversion, both the color and lactic acid of TTI were rigorously measured during storing at 20°C, the relation of which was used to read the color index value at a measured lactic acid amount.

Although the apparent response of TTIs to time-temperature history is a color, this visible variable usually does not produce reliable data due to inconsistency in photometric or colorimetric measurements of the microorganism solution, i.e., the microbial TTI solution in this study. An alternative variable was chosen to estimate E_a , avoiding such difficulties. The titratable acidity (TA) of TTI with pH change is a variable providing higher reliability in the measurement (17). In order to employ the TA instead of the color index in estimating E_a , a relation curve was rigorously produced between TTI color and TA (Fig. 1), where the color index was obtained from many replications.

The lactic acid amounts of the TTI were expressed in TA. A 5.0-g portion of the TTI mixture was diluted with 20 mL of sterilized distilled water and homogenized for 10 s with 0.5 mL of 1.0% phenolphthalein (w/v). It was finally titrated with 0.1 N NaOH solution to pH 8.2. The TA was estimated by Eq. (1) as % lactic acid (% LA). It was assumed that the titrated acid was almost all lactic acid produced by the lactic acid bacteria in the TTI.

$$\text{TA (\%)} = (M \cdot V \cdot E) / S \cdot 100 \quad (1)$$

where M is the molarity of NaOH, V is the volume of NaOH, E is the equivalence factor, 0.009 for lactic acid (18), and S is the weight of

the sample.

The color of the TTI was measured by using a scanner (CLX-3185WK; Samsung, Suwon, Korea) (12). The digital images of the TTI prototype were taken and the color index was calculated in total color difference (E) as follows.

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \quad (2)$$

where L , a , and b are the differences in Hunter lightness, redness, and yellowness between the samples before and after activation.

To complete the relation curve between the TA and color index of the TTI, a logistic function was employed to fit the data.

$$Y = \frac{1}{1 + \exp(aX + b)} \quad (3)$$

where Y is $E/E_{\max}$, X is TA, and a and b are the parameters. Converting Eq. (3) by taking the logarithm on both sides, Eq. (4) was obtained as follows:

$$\ln\left(\frac{1-Y}{Y}\right) = aX + b \quad (4)$$

Eq. (4) was applied to linear regression to estimate a and b values. Then the original Eq. (2) was used to plot the relation curve (Fig. 1).

Measurements of milk properties Many different milk properties can be measured for quality determination, and some of them were used for this case study (19-21). Actually, there are other quality indices of milk, such as sensory and nutritional parameters (water-soluble and fat-soluble vitamins), protein breakdown, and fat hydrolysis.

The percentage lactic acid of milk samples was measured by titrating 20 mL of samples diluted by adding an equal volume of distilled water with 0.1 N NaOH. The amount of 0.1 N NaOH (mL) necessary for the color of the samples to change the phenolphthalein indicator from white to bright red at pH 8.2 was measured by burette. The % LA (equivalent to titratable acidity) was calculated using Eq. (1).

The pH of the milk samples was measured using a pH meter (S20 SevenEasy™ pH, Mettler-Toledo International Inc., Seoul, Korea).

A microbiological assay was performed for two microorganisms, aerobic mesophilic bacteria (AMB) and lactic acid bacteria (LAB) (22). Decimally diluted milk samples were used with several media, such as Petrifilm™ aerobic count plate (ACP; 3M, St. Paul, MN, USA) and Bromocresol purple count agar (BCP; Eiken Chemical Co., Tokyo, Japan) for AMB and LAB, respectively. The microbial counts are expressed in CFU/mL.

Determination of Arrhenius activation energy For the estimation of Arrhenius activation energy, the lactic acid amount was used as the variable from the TTI rather than the color index. This was because the reaction product in this TTI is lactic acid, not the color index. The product concentration can be used to study reaction

Fig. 1. Conversion graph from titratable acidity (TA) of TTI to color index. The color grades were illustrated according to the previous work (Kim *et al.* (12)). The equation is from logistic function, Eq. (4), obtained by regression analysis. Error bars represent standard deviation ($n=3$).

kinetics and Arrhenius temperature dependency. The color is just a visible reflection of the reaction. The reaction rate constants were estimated by using Eq. (5) and Eq. (6) for zero-order and first-order reactions, respectively.

$$y = k \cdot t + y_0 \quad (5)$$

$$\ln y = \ln y_0 + k \cdot t \quad (6)$$

where y is the lactic acid amount of the TTI or the milk property level, y_0 is the initial level, k is the reaction rate constant (h^{-1}), and t is the storage time. The activation energy was estimated by the Arrhenius equation expressed in logarithmic terms from the calculated rate constant (Eq. (7)).

$$\ln k = \ln A + \left(\frac{-E_a}{R}\right) \cdot \frac{1}{T} \quad (7)$$

where k is the rate constant (h^{-1}), A is the pre-exponential factor ($1/\text{h}$), E_a is the activation energy (kJ/mol), R is the ideal gas constant ($8.314 \times 10 \text{ kJ/K} \cdot \text{mol}$), and T is the storage temperature (K). The slope ($\frac{-E_a}{R}$) and the intercept ($\ln A$) were obtained by linear regression.

Prediction of milk properties from TTI color The prediction was conducted as follows: 1st building calibration curves between TTI color and several milk properties, 2nd predicting milk properties at a given TTI color index. The calibration curves were obtained by establishing the relationships between TTI color and a milk property at 7°C. The curve equations were estimated through regression analysis on the given data, where a logistic function was employed to fit the data.

For the case study, the storage tests at 15 and 24°C, regarded as a temperature abuse, were executed while the color of the TTIs was measured. Then the milk properties were predicted by substituting the color index into the calibration curve equations.

Statistical analysis Milk properties were compared between the prediction from the TTI's color index and the experimental data. To evaluate the prediction error, statistical characteristic indices such as mean square error (MSE), bias factor, and accuracy factor are employed (23). However, in this study, comparison variables are not in the same dimension, such as CFU/mL, pH, and %, and therefore the coefficient of determination (R^2) was used to avoid such differences.

$$R^2 = 1 - \frac{\sum (N_{data} - N_{predicted})^2}{\sum (N_{data} - \bar{N}_{data})^2} \quad (8)$$

where N and $\bar{N}$ represent the milk qualities and their average, respectively, and n is the number of observations.

All experiments were performed in triplicate, and the data are expressed as mean $\pm$ standard deviation. One-way ANOVA was performed to examine significant differences between means. Regression analysis was applied to estimate the kinetic and Arrhenius parameters for the TTI response and the food quality changes. MS Excel 2010 (version 14) was used for the analyses.

Results and Discussion

Ea of TTI response and milk properties As aforementioned, the TA of the TTI was used as the variable for TTI response rather than the color index to estimate the Arrhenius activation energy. The TA of the TTI followed a zero-order reaction, agreeing with a previous report (12). The E_a of the TTI was estimated to be 106 kJ/mol (Table 2).

Milk properties such as percentage lactic acid, pH, aerobic mesophilic bacteria (AMB), and lactic acid bacteria (LAB) were considered as quality indicators of freshness (18). The percentage lactic acid followed a first-order reaction due to its higher R^2 , compared to that of zero-order, and the E_a was estimated to be 122 kJ/mol (Table 2). The pH change turned out to be zero-order, based on a higher R^2 . The estimated E_a value for pH was 145 kJ/mol. Both AMB and LAB are common qualities of milk (18). The kinetics of both of the microorganisms followed a first-order reaction (Table 2). The E_a of the AMB and LAB counts from regression analysis were 101 and 107 kJ/mol, respectively.

Calibration curves for estimation of milk properties from TTI color

In the prediction of food qualities from TTI color, a comparison sticker including the TTI color grades and the food quality grades as "stale", "less stale", "fresh", etc. is usually used. This sticker is compared to a calibration curve. If this relation is universal for the practical range of temperature, it would be ideal. Unfortunately, however, the E_a of a TTI and the labelled food would not be perfectly identical. Therefore, the reality is that the calibration curve should be tested at a representative temperature. In this study, 7°C was considered as the standard temperature for creating the calibration curve. Refrigeration of pasteurized milk at or less than 7.2°C is

Table 2. The coefficients of determination (R^2) for the relationship between the predicted and experimental data of milk qualities in Fig. 3. This indicates a prediction accuracy of TTI

Milk qualities	Temperature (°C)	R^2
Aerobic mesophilic bacteria	15	0.988
	24	0.954
Lactic acid bacteria	15	0.851
	24	0.806
ln lactic acid %	15	0.875
	24	0.852
pH	15	0.754
	24	0.369

regarded as "practical storage conditions", the conditions that can still retain an acceptable quality of pasteurized milk (24).

Figure 2 shows the calibration curves for AMB, LAB, % LA, and pH. Logistic function was employed in the curve fittings, and all the curves had the high coefficients of determination ($R^2 > 0.99$). According to Table 2, the TA of the TTI is linearly correlated to either the milk properties or their logarithmic values. In contrast, the TA of the TTI could be correlated by logistic function to the color of the TTI (Fig. 1). Therefore, the logistic function is reasonable to correlate the color of the TTI with the milk properties.

Determination of under- or over- estimation through comparisons between the predicted and experimental data

The milk properties during storage at 15 and 24°C were estimated by TTI color. To check the accuracy, the actual levels were also experimentally measured and compared with the predicted levels. The measurements were made until the TTI color was saturated around $E = 57.02$ kJ/mol or TA = 0.67, as shown in Fig. 1. The employed storage temperatures were assumed to be plausible temperature abuses.

In Fig. 3, LAB shows very high agreement between the predicted and experimental results, also having the highest coefficient of determination (R^2) (Table 3). However, the other properties, such as % LA, pH, and AMB, had some errors in the predictions, having relatively lower R^2 . The predictions are from the calibration curves established at 7°C. The error should be owing to the difference of the experienced storage temperatures from 7°C. If there were bigger differences of E_a , there would be larger errors. Accordingly, LAB with a smaller E_a difference had less error, whereas the other properties with bigger differences had larger errors.

In addition, there were two kinds of errors in terms of the direction of errors: underestimation and overestimation. In the case of underestimation, the predicted levels of milk properties are lower than the experimentally determined levels. As shown in Fig. 3, LAB, % LA, and the pH were underestimated, whereas AMB was overestimated. In Table 2, the underestimated properties have larger E_a than that of TTI color, whereas the overestimated one has a smaller E_a . This indicates that the direction of errors is related to the E_a differences between the milk properties and the TTI color.

Fig. 2. Prediction graphs from TTI color (ΔE) to milk properties: (A) AMB, (B) LAB, (C) $-\ln$ lactic acid %, and (D) pH. The equations are from logistic function, Eq. 4, obtained by regression analysis. Error bars represent standard deviation ($n=3$).

Table 3. Reaction rate constants (k) of the milk qualities and the microbial TTI response at different temperatures and Arrhenius activation energies (E_a)

		Temperature (°C)	Zero-order reaction		First-order reaction		Reaction order ²⁾	E_a (kJ/mol)	R^2
			k (1/h)	R^2 ¹⁾	k (1/h)	R^2			
Milk qualities	Lactic acid	8	0.0001	0.94	0.0009	0.93	First- order	122	0.95
		15	0.0004	0.95	0.0028	0.99			
		24	0.0023	0.98	0.0150	0.99			
	pH	8	0.0004	0.99	0.0001	0.95	Zero-order	145	0.95
		15	0.0022	0.98	0.0003	0.96			
		24	0.0123	0.95	0.0021	0.93			
	Aerobicmesop hilicbacteria	8	-	-	0.0220	0.95	First- order	101	0.98
		15	-	-	0.0510	0.96			
		24	-	-	0.2210	0.99			
	Lactic acid bacteria	8	-	-	0.0060	0.93	First- order	107	0.99
		15	-	-	0.0188	0.92			
		24	-	-	0.0705	0.98			
Response of microbial TTI	Titratable acidity	8	-	-	0.0024	0.96	First-order	106	0.96
		15	-	-	0.0071	0.99			
		24	-	-	0.0281	0.99			

¹⁾Coefficient of determination for regression analysis.

²⁾Reaction order with higher R^2 was selected.

Heretofore the magnitude of E_a difference (small that 25 kJ/mol) had been employed as a criterion of the prediction accuracy, but in this study whether the E_a of the food was larger or smaller than that of

the TTI was found to be another criterion that should be considered. The case of $E_{a_{food}} > E_{a_{TTI}}$ led to the underestimation, and vice versa. If there is an underestimation, the remaining shelf life estimated

Fig. 3. Differences (errors) between the predicted (empty symbols) and experimental (filled symbols) data of milk qualities (LAB, ln lactic acid %, pH, and AMB) from temperature abuse tests. ○ and ●: 15°C, □ and ■: 24°C. Error bars represent standard deviation (n=3).

from TTI would be longer than the actual one, leading to a dangerous occasion, and vice versa. In the case study, the remaining shelf life of milk, in terms of pH, % LA, and LAB, would be longer than the actual one, whereas it would be shorter in terms of AMB. Meanwhile, the overall shelf life is determined on the basis of the factor reaching the shelf life the fastest. According to KFDA, the threshold values before the shelf life are 4.3 log CFU/mL, 6.6, -1.7 ln % LA, and 4.3 log CFU/mL for AMB, pH, % LA, and LAB, respectively. In Fig. 3, the times reaching those threshold values increase in the order of AMB, pH, LAB, and % LA. AMB is the factor that should be used to determine the overall shelf life. Consequently, AMB turned out to be the factor that the TTI should cover to predict the remaining shelf life.

Based on the Arrhenius relationships, TTI would undergo faster reaction with increased temperature than a factor with lower E_a would. On the contrary, TTI would respond slower than the factors with higher E_a would. In other words, factors with a higher E_a could be of poor quality before the TTI indicated the food to be spoiled. Eventually, TTIs could be formulated so that food qualities with $E_{a\text{ food}} > E_{a\text{ TTI}}$ are underestimated.

Impact of underestimation by TTI on food quality deterioration

The underestimation of food qualities by TTI response should be avoided. Underestimation means that the magnitudes of food quality changes are estimated to be lower by the TTI than those of the actual food ($E_{a\text{ food}} > E_{a\text{ TTI}}$). Eventually, even though the food reaches the end of its shelf life, the TTI may still show a safe indication for product consumption. This will lead to potential food quality deterioration problem. The emphasis of the reliability of the

TTI has been based solely on the similarity of the E_a between the TTI and foods. From this study, however, it has been shown that this conventional requirement is not enough to ensure food quality. To avoid the underestimation, the direction of the difference in the E_a must also be considered. If the quality factors of a food are estimated differently, the factor with the shortest shelf life should be considered to determine whether it is under- or over-estimated. It is known that the threshold values at the end of shelf life are ln 0.18%, 6.6, 4.3 log CFU/mL, and 4.3 log CFU/mL for % LA, pH, LAB, and AMB, respectively (18). In Fig. 3, AMB reached the shelf life the earliest and therefore was used as the determining factor. Accordingly, the milk's deterioration was apparently underestimated by the TTI.

On the contrary, overestimation by the TTI is safe and desirable, even though it would cause early consumption or discarding of food products as the TTI predicts that the food is deteriorated when it may still be fresh.

In conclusion, TTIs are beneficial indicators for predicting food qualities during storage or distribution and their prediction accuracy is crucial. To ensure the accuracy, a similar E_a between the TTI and the food is required. Currently, only this aspect has been of concern for the selection of an appropriate TTI for a target food product. Practically, however, the prediction is not always perfect. In this study, potential errors in the prediction differentiated between under- and over- estimation of food qualities. Underestimation means that the predicted quality levels are lower than the actual ones, resulting in it being dangerous for the consumer to eat the food based on the TTI because the food has already spoiled. Overestimation also produces prediction error, but is nevertheless

safe for consumers. It was found that under- or over-estimation were caused by the E_a of the TTI being smaller or larger than the E_a of the food, respectively.

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