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Development of Enzymatic - Colorimetric Time - Temperature Integrator for Smart Packaging

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Abstract: A new enzymatic-colorimetric time-temperature integrator (ECTTI) to evaluate temperature abuse in refrigerated products was developed with Tributyrin, pH indicator, and lipase Calb. The kinetic behavior of the ECTTI (0.01 µL.L^{-1} enzyme) was assessed at 5, 10, 15, 20, and 25ºC, evaluating changes in pH (≤7.2) and color (ΔE ≥12). Storage at 5°C provided stability of the enzyme indicator for 16 days, and temperature abuses caused stability reduction to 15, 10, 0.5 0.3 days, with 10, 15, 20, and 25ºC of storage, respectively. The sequential abuse of temperature stability of ECTTI was assessed by exposure at 5.0ºC for 2 h and 25ºC for 1 or 5 min, returning to 5.0ºC for another 2 h, until the change in pH and color. The ECTTI subjected to temperature abuses for 5 and 1 min/25°C, remained stable for 5 and 9 cycles, respectively. The results of ECTTI stability before application, obtained with separate buffer/enzyme/water + substrate/indicator solutions, stored at 5 or 25ºC, before the reactive mixing of solutions, kept their stability, demonstrating the possibility of use as a tool against the temperature abuse conditions.

Keywords: Refrigeration; Microorganisms; Temperature Abuse; Food preservation; Chicken.

1. Introduction

The quality and safety of chilled foods are strongly influenced by temperature, requiring monitoring throughout the distribution chain [1-3]. Unexpected changes or abuses in temperature in the cold food chain can compromise food safety and quality, which can result in loss of consumer confidence and increased levels of food waste [4]. Temperature abuse occurs when food is stored at temperatures that exceed the safe storage limit (5°C) [5]. This type of temperature abuse usually occurs during product distribution, retail display, or domestic storage, when products in transit or storage are refrigerated under sub-optimal conditions [6-8].

The development of smart packaging with specific devices that allow the monitoring of the conditions of packaged products is becoming increasingly popular in response to the demand for high quality and food safety [9-12]. Different indicator systems such as temperature and time [13], gas sensors [14], pH [10], biosensors [15-17], and miniaturized biosensors [18]

can provide qualitative information and quantitative in real-time, through color changes, incorporating them into the packaging material [11,19].

The time and temperature indicators (TTI) are based on irreversible changes that can be physical, chemical, or biological [19-21]. The rate of this change depends on time and temperature, providing a cumulative indication of the storage conditions to which the product has been exposed [22]. The performance of the TTI system in monitoring food spoilage at both temperatures used in storage distribution has been studied for kimchi [23], frozen vegetables [24,25], fish products [26-28], meat products [26-31], mushrooms [32], dairy products [33,34], fresh food [35].

There are different types of TTIs on the market, enzymatic, microbiological, diffusion, polymeric, and photochemical [36]. Enzymatic TTIs are based on enzyme chemistry and have many benefits over other types of TTIs due to unwavering performance, low production cost, and easy control. An enzymatic TTI enables the development of a TTI with various activation energy values, as it can be used by conjoining different enzymes and substrates. Thus, it has the advantage of being applied as a tailored type of TTI for various food products undergoing a lot of quality changes during the supply chain. Its working principle is to produce an acid/base by enzymatic hydrolysis to increase or decrease pH value and then to dynamically display the cumulative effect of time and temperature by color changes of an acid-base indicator. The system can be calibrated to match the rate of spoilage of a designate product by altering several parameters: enzyme type and concentration, substrate type and concentration, pH, and presence of a buffer [9]. Lipase based TTI shows an irreversible color change induced by a pH decline resulting from the controlled enzymatic hydrolysis of a lipid substrate, for example, tributyrin.

However, the literature still lacks results showing TTIs with similar durability to refrigerated food products when stored correctly, that allows handling of the packaging with removal from the refrigeration gondola for short periods of time, and with stability before being placed in contact with the product. In this context, the objective of this work was to develop a colorimetric enzyme system of time and temperature, with stability similar to that of chilled raw meat products, that allow product manipulations in market shelves and with stability prior to its application in the product packaging, in view of food security due to the negligence of some establishments regarding the lack of temperature control in refrigerated food gondolas.

2. Materials and Methods

2.1. Development of the enzymatic indicator.

The preparation of the colorimetric enzyme indicator was carried out with a commercially available enzyme, lipase Calb L Novozym (batch LCN 02115), with the declared activity of 5000 $LU.g^{-1}$. The reactional system of the time and temperature indicator was composed of 4 mM Tributiryn, 0.1 % Triton X-100, 2.5 % buffer (Na_2HPO_4 0.2 mM, citric acid 0.1 mM, pH 7.5), 8.5 % pH indicator (mixture of 0.1 % bromothymol blue, methyl red, neutral red, 12:4:1) [37] varying the enzyme concentration (20000; 15000; 10000; 5000; 1000; 500; 100; 250; 1; 0.1; 0.05; 0.01 e 0.005 $\mu L.L^{-1}$). The pH indicator led to an irreversible color change from green to orange and finally to red (the endpoint), indicating a progressive decline from pH 8.0 to 6.0. The experiments were carried out, exposing the reaction system in controlled temperature conditions to 5 and 25°C (temperature abuse), determining the pH and ΔE of each sample every 6 h.

In carrying out experiments to define the concentration of enzyme to be used in the colorimetric system, it was observed that the color rapidly altered when exposed to room temperature (around 25ºC) at the time of the analyzes. Thus, was evaluated the influence of temperature fluctuations when reading the pH of the reaction system, exposing the colorimetric system to room temperature (25.0°C ± 2.0) with and without bath ice for up to 5 min, for the reading of pH.

pH values were determined using a digital pH meter (Digimed DMPH-2). The chromaticity change (ΔE value) was measured using CIE Lab color space coordinates to objectively describe the irreversible TTI color change per unit time at different constant temperatures, and the ΔE value was expressed by the following Equation 1.

$$〖\Delta E〗^{\wedge} = \sqrt{((〖\Delta L〗^{\wedge}(*))^{\wedge}2 + (〖\Delta a〗^{\wedge}(*))^{\wedge}2 + (〖\Delta b〗^{\wedge}(*))^{\wedge}2)} \quad (1)$$

Where L* is the difference in brightness (white-black) change between t=0 and measured unit time; a* is the difference in redness-greenness, and b* is the difference in yellowness-blueness [38].

2.2. Effect of temperature on the kinetic behavior of the indicator.

The kinetic behavior of colorimetric enzymatic indicator of time and temperature was evaluated, keeping the enzyme concentration of 0.01 $\mu L.L^{-1}$ and exposing the reaction system to different temperatures (5, 10, 15, 20, and 25°C). For each temperature tested, the final samples' follow-up time was determined by change in pH and color. During the period of exposure to 25°C, the reactive system was kept in contact with a bath ice base.

2.3. Evaluation of the stability of the reaction system.

To evaluate the stability of the reactive system to small temperature fluctuations, a condition of handling the food packaging by the consumers was simulated in the choose the product during the day and also without manipulation, during the night. For that, a test was carried out, submitting the reactive system to cooling (5.0ºC ± 1.0) for 2 h and then exposed to 25°C for 1 and 5 min and then returned to 5.0ºC for another 2 h (simulating manipulation during the day). During the exposure to 25°C, the reactive system was kept in contact with a cold base (gel ice -Techgel), to simulate a refrigerated product in contact with the reactive system package. During the night, this system was kept under constant refrigeration (5.0ºC ± 1.0). The reactive system was monitored to changes pH and color, evaluating stability at different cycles of 1 and 5 min at 25°C.

2.4. Evaluation of the reactional stability of the solutions that make up the indicator.

Aiming at the practical applicability of the developed reaction system, the conservation of the in separated solutions was studied, since, when all the relational components are mixed, the color and pH change process can already be started. This study is important because, during the filling of the products, the packaging can go through the process of thermo shrinkage and vacuum sealing, which causes an increase in temperature and thus could trigger the reaction system. For this, the conservation at different temperatures (5 and 25°C) of two different solutions was studied, aim to evaluate the need to preserve the reaction components under refrigeration or ambient temperature. The reaction systems I (buffer/enzyme/water + substrate/indicator) and II (buffer/enzyme/indicator/water + substrate) with the groups of

compounds still separated, were maintained at 5 and 25°C for 24 h, with subsequent mixing between the groups of reaction components of each system. All the reaction systems were evaluated by determining the pH and color, with temperature abuse at 25°C.

2.5. Statistical analysis.

All analyzes were performed in triplicate, and graphs and linear regression were performed using the Excel software.

3. Results and Discussion

3.1. Development of the enzymatic indicator.

A time-temperature indicator must provide an answer that is understandable, perceptible, and easy to read by consumers [39]. In this sense, a reactive system of easy responses was developed, where after the addition of the enzyme in the indicator, a color change can be observed in the pH variation functions by hydrolysis of the taxed substrate in case of temperature abuse (Fig. 1).

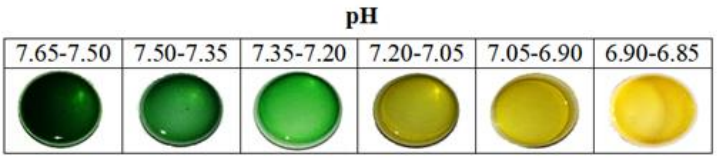

Figure 1. The color variation of the enzymatic indicator of time-temperature in relation to the reduction of pH.

The results related to the colorimetric enzymatic system developed with different concentrations of enzyme and exposed to 5 and 25°C, showed that the enzymatic indicator with the same concentration of enzyme, when exposed to different temperature conditions, results in different stability times. It is worth noting that the higher the exposure temperature of the enzymatic indicator, the faster the pH reduction and the consequent color change occurs in any of the enzyme concentrations evaluated. This phenomenon was also observed by Wu *et al.* [40], who developed an enzymatic time-temperature indicator using lipase from *Aspergillus niger*.

When analyzing or behavior of the enzyme indicator in relation to the enzyme concentration, it was observed that the system containing 20000 $\mu L.L^{-1}$ of enzymes changed the pH from 7.80 to 5.27 and the visual aspect (green to medium yellow) in 3 h. While with 0.01 $\mu L.L^{-1}$ it remains stable for 9 days, with no color change and without significant reduction in pH (7.75 - 7.21), when maintained at 5°C. The lowest concentration of the enzyme studied (0.005 $\mu L.L^{-1}$) was insufficient to promote the tributyrin hydrolysis reaction and consequent reduction in pH and color change. Thus, the enzyme concentration defined for the subsequent steps was 0.01 $\mu L.L^{-1}$.

According to Jaiswal *et al.* [41], the use of lipases for the development of time-temperature indicators is economically viable and can be considered of great commercial value, as these enzymes catalyze various reactions in liquefied and non-liquefied states. In addition to presenting high catalytic activity, such as the Calb lipase used in the present work, which allowed the efficiency of the reaction system in small quantities.

Regarding the stability of the reactive system to small temperature fluctuations, maintaining the enzymatic reaction system in an ice bath during pH determination, it was possible to minimize the influence of temperature fluctuation, keeping it stable for 18 days with

a pH above 7.20. The samples kept without an ice bath presented a pH of 5.9 at 18 days. This result demonstrates that the exposure of the reaction system to temperature variations, even for a short time, causes a cumulative and irreversible effect on the reaction result (color change from green to yellow).

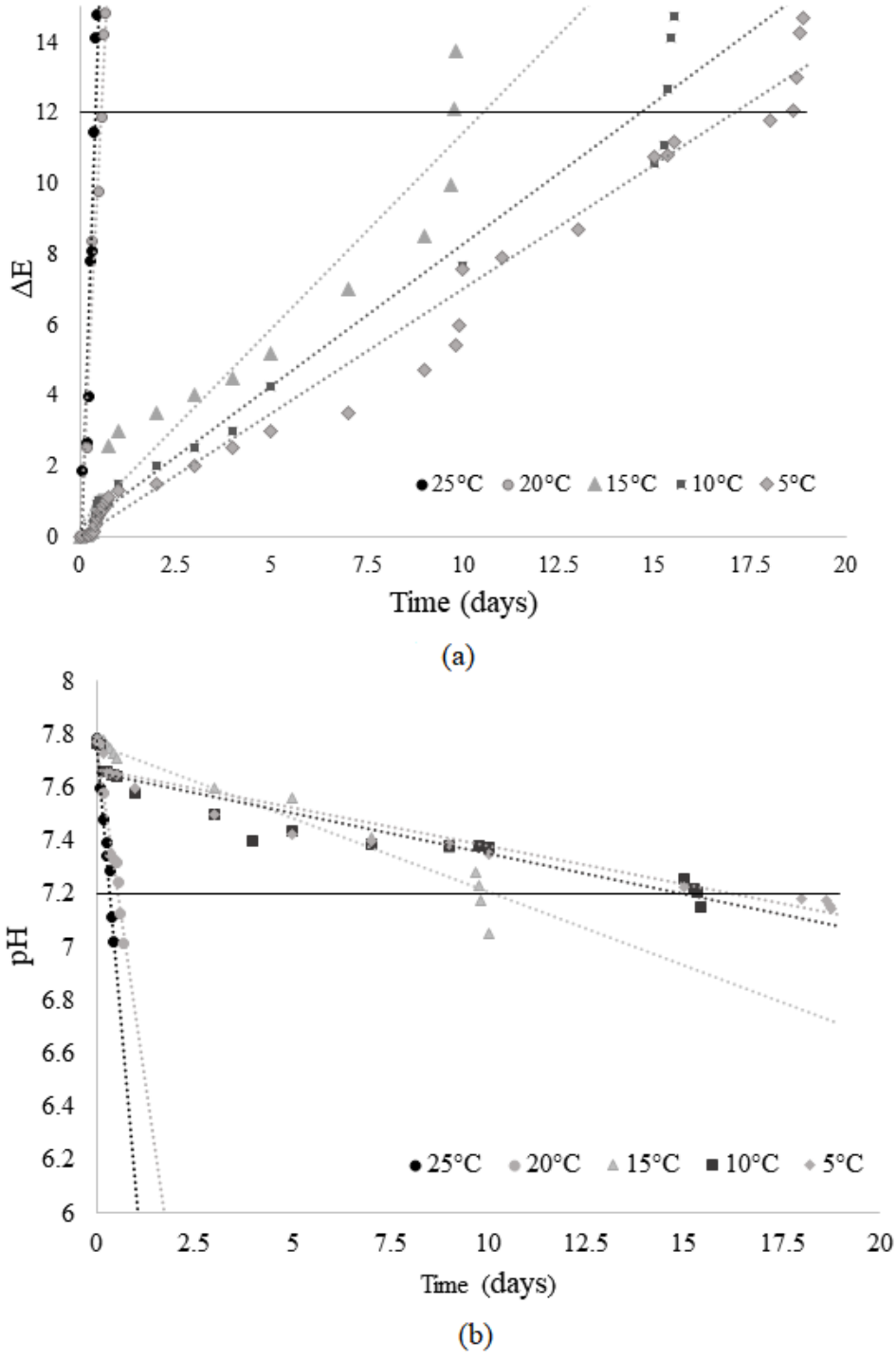

Figure 2. Kinetic behavior of the time-temperature indicator as a function of ΔE (a) e pH (b) subjected to different temperatures. Where ΔE = 12.0 and pH de 7.2 refer to the perceived color change by consumers.

3.2. Influence of temperature on the color of the enzymatic indicator.

After defining the enzyme concentration (0.01 μL.L^{-1}) and conditions for evaluating the enzymatic indicator (ice bath), it was stored at different temperatures, 5, 10, 15, 20, and

25°C, to determine the behavior kinetic through changes in the ΔE value and pH as a function of temperature (Fig. 2). The temperature values studied represent the conditions used in the chilled product distribution chain, from the producer to the final consumer [42-44].

In Fig. 2a, ΔE = 12.0 refers to the perceived color change by consumers. A progressive reduction in the time of color change can be observed with the increase of the storage temperature and a drastic reduction of this time in storage with temperature abuse (20 and 25°C). In Fig. 2b, pH 7.2 indicates the limit of pH variation for maintaining the green color. Slow pH reduction at lower storage temperatures and rapid reduction when stored in 20 and 25°C are identified.

From the kinetics of ΔE and pH presented in Fig. 2a and 2b, the equations for each temperature were obtained, and these are used to determine the validity time of the indicator at each temperature when the ΔE reaches 12.0 and pH 7.2 (Table 1).

There is a linear behavior of pH reduction and an increase in color variation ΔE (green to yellow) in relation to the storage time for all evaluated temperatures. Storage at 5°C caused stability of the enzyme indicator for 16.33 days in relation to the minimum pH (7.2) and 17.02 days in relation to the noticeable color variation (ΔE = 12.0). These results indicate that it could be used for products with a shelf-life of up to 16 days and that temperature abuses would reduce this shelf-life to up to 0.3 days, demonstrated by the enzymatic indicator when submitted to 25°C.

Kim *et al*. [37], evaluating the development of the enzyme time-temperature indicator with lipase from *Burkholderia cepacia* observed lower reaction stability when evaluating different exposure temperatures. These authors obtained times from 0.4 days to 26ºC, 0.67 days to 20ºC, 1.1 days to 15°C, 2.0 days to 10ºC, and 3.8 days to 5ºC to reach the final color change point, stability times lower than obtained in the present work.

Table 1. Validity time of the temperature indicator when ΔE = 12.0 and pH = 7.2

Temperature (ºC)	pH		ΔE	
	Equation	Time (days)	Equation	Time (days)
5	y = -0.0287x + 7.6687 R² = 0.9348	16.33	y = 0.7067x – 0.0317 R² = 0.9794	17.02
10	y = -0.0304x + 7,6555 R² = 0.9057	14.98	y = 0.8054x + 0.2375 R² = 0.977	14.60
15	y = -0.0554x + 7,7645 R² = 0.9312	10.19	y = 1.1115x + 0.3207 R² = 0.9463	10.50
20	y = -1.0138x + 7.7605 R² = 0.9523	0.55	y = 21.7x – 0.1836 R² = 0.9781	0.56
25	y = -1.6676x + 7.7756 R² = 0.9709	0.34	y = 32.749x – 1.4901 R² = 0.9429	0.41

The ΔE values (Table 1) obtained showed that there was a significant color change in the reaction systems with a gradual increase in temperature. This result must corroborate with the microbiological changes when exposing the samples of refrigerated products in the condition of temperature abuse [39], with different times of product's useful life at a different temperature of exposition.

3.3. Evaluation of the stability of the reaction system to sequential abuse of temperature.

Fig. 3 present the number of cycles obtained from exposing the reaction system to sequential temperature abuses, kept at room temperature (25°C) for 1 or 5 min and subsequent return to refrigeration (5°C) for 2 h to stabilize the temperature, simulating withdrawals and returns of the product to the gondola, until a change in pH (<7.2). The indicator subject to

temperature abuse for 5 min remained stable for 5 cycles, whereas the indicator exposes to temperature abuse for 1 min remained stable for 9 cycles.

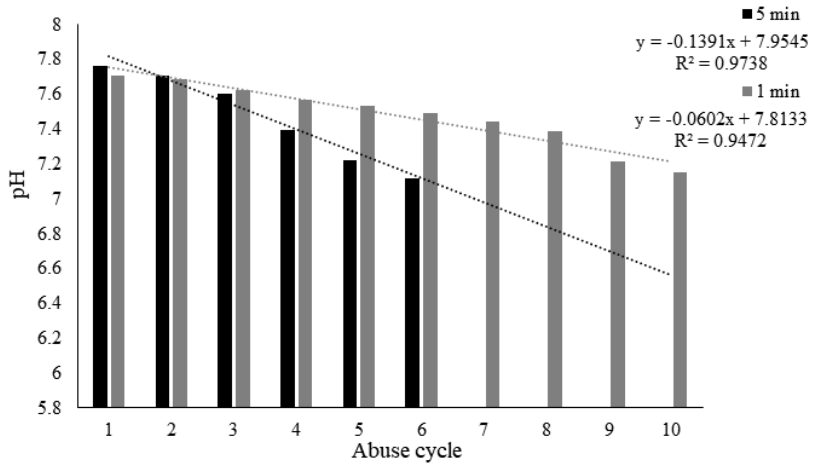

Figure 3. Sequential abuse cycles of the reaction system exposed to 25 °C for 1 and 5 min and then returned to refrigeration for 2 h.

3.4. Evaluation of the reaction stability of the solutions that form the indicator.

Time-temperature indicators are devices used on the surface of a package that undergoes color changes, for example, showing the accumulated history of temperature abuse of a food [20,45,50]. According to Mataragas *et al*. [5], TTI can be applicable to a wide range of methods, only promoting small adjustments in its parameters, taking into account the deterioration kinetics of the respective product, thus improving the management of the cold chain and reducing food waste.

Thus, it is interesting that the enzyme indicator associated with a package can be activated only after the products are stored (<5.0°C) and can be maintained without starting the reaction before manipulation and temperature variation. In this sense, combinations of the reaction system I (buffer/enzyme/water + substrate/indicator) and II (buffer/enzyme/indicator/water + substrate) were monitored at 5 and 25°C in order to apply them to the packaging surfaces, with the homogenization of the groups of compounds after refrigeration.

From the kinetic monitoring of the 2 systems, it was possible to obtain the equations for each of these, which are used to determine the validity time of each indicator when it reaches the value of ΔE 12.0 and pH 7.2 (Table 2).

Table 2. Validity time of systems I, II, III, and IV time-temperature indicators when ΔE = 12 and pH = 7.2.

Temperature (ºC)	pH		ΔE	
	Equation	Time (days)	Equation	Time (days)
System I (5°C)	y = -0.0768x + 7.8735 R² = 0.9765	0.41	y = 1.2868x – 0.6747 R² = 0.9848	0.41
System II (5°C)	y = -0.011x + 7.5671 R² = 0.6833	33.37	y = 0.1415x + 2.268 R² = 0.6312	68.78
System I (25°C)	y = -0.0563x + 7.753 R² = 0.9765	0.41	y = 1.2749x – 0.3926 R² = 0.9788	0.40
System II (25°C)	y = -0.0096x + 7.6087 R² = 0.8023	42.57	y = 0.1243x + 2.1304 R² = 0.6132	79.40

The test results show the inefficiency of the enzymatic indicators for the systems where there was no homogenization of the substrate with the other components of the reaction (system II at both temperatures of storage), which caused the pH and color not to change.

The results obtained for the system I had a reduction in pH and color change after 0.4 h at 25°C. This result is due to the ease of mixing between the reagents (buffer/enzyme/water + substrate/indicator) stored at 5 or 25°C for 24 h, mixed and submitted to temperature abuse (25°C).

Thus, the enzymatic indicator developed (a system I) has the potential to be applied in the monitoring of temperature abuses in foods that receive refrigeration. In addition, this enzymatic indicator can be used as a tool to identify possible negligence of some establishment's regarding the lack of temperature control in refrigerated food expositors. Considering the microbiological risk associated with temperature abuses, this tool is of great importance throughout the distribution and storage chain of chilled products, aiming at food security.

4. Conclusions

The concentration of enzymes, temperatures influence, and the stability of the reaction system was studied to obtain an easy-to-read and stable time-temperature indicator for small temperature fluctuations after commercial application.

The colorimetric enzyme time-temperature indicator was developed with 0.01 $\mu L.L^{-1}$ of the enzyme Calb L, remained stable for approximately 16 days, stored at 5ºC. The enzyme indicator allowed 9 and 5 cycles of product removal from the refrigerator and exposure to 25°C, for 1 and 5 min, respectively. The colorimetric enzymatic system divided into 2 groups of solutions (buffer/enzyme/water) and (substrate/indicator) can be maintained without starting the reaction, under refrigeration, or at 25°C. After homogenization of the solution and 25ºC, pH and color changes in 9h (0.4 days).

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Conflicts of Interest

The authors declare no conflict of interest.

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