Patient-Centered Application of α-Amylase-Based Enzymatic Time-Temperature Indicator for Real-Time Insulin Monitoring in Koronadal City

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ABSTRACT

Insulin stability remains a major concern in tropical regions where elevated ambient temperatures may compromise its therapeutic efficacy. This study evaluated the potential application of an α-amylase-based enzymatic time–temperature indicator (TTI) for real-time insulin monitoring in Koronadal City, Philippines. The study investigated the physicochemical properties of α-amylase relevant to TTI development, examined the relationship between TTI color change and insulin degradation above 25°C, assessed patient usability, and compared the prototype with a commercially available vaccine vial monitor (VVM). A mixed-methods approach combining laboratory experimentation and descriptive survey design was employed. Enzymatic activity was evaluated under varying temperature and pH conditions, while insulin and TTI samples were monitored for 28 days using UV-Visible spectrophotometry and Turbidimetric analysis. Patient-centered usability was assessed through structured questionnaires among diabetic participants. Results showed that α-amylase exhibited optimal activity at 45°C and pH 7, with progressive decline in activity at higher temperatures. The enzymatic TTI demonstrated a strong negative correlation with insulin degradation ($r = -0.84$, $p < 0.001$), indicating that visible color changes reliably reflected cumulative thermal exposure. Participants also reported high usability, clarity of interpretation, and increased confidence in insulin safety monitoring. Comparative analysis revealed that the α-amylase-based TTI performed comparably with commercial VVMs under controlled conditions. These findings suggest that the proposed enzymatic TTI is a practical tool for monitoring insulin stability in hot-climate settings and may support safer insulin storage practices in resource-limited communities.

Keywords: α-amylase, time-temperature indicator, insulin stability, patient-centered monitoring, Koronadal City

INTRODUCTION

Insulin is an essential medication for the management of diabetes mellitus, and maintaining its stability during storage is critical to ensuring its therapeutic effectiveness. According to the Food and Drug Administration (FDA, 2024), unopened insulin should be stored at 2–8 °C, while opened insulin may be kept at room temperature (20–25 °C) for up to 28 days under controlled conditions. However, exposure to temperatures beyond the recommended range accelerates insulin degradation, reducing its biological activity and potentially compromising glycemic control.

Maintaining appropriate storage conditions is particularly challenging in tropical regions where ambient temperatures frequently exceed recommended limits. In Koronadal City, Philippines, temperatures commonly range from 22.8 °C to 33.9 °C and may reach as high as 37 °C (AccuWeather, 2025). Studies have shown that insulin stored at 32–37 °C for 28 days loses approximately 14–18% of its potency, increasing the risk of poor glycemic control (Jaiswal et al., 2024). Clinical reports have also linked heat-compromised insulin to serious complications, including diabetic ketoacidosis (BMJ Open Diabetes Research & Care, 2022). Furthermore, Khurana and Gupta (2019) reported that many patients store insulin at room temperature because of limited access to refrigeration, emphasizing the need for practical methods to monitor insulin quality during storage.

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Time–temperature indicators (TTIs) have emerged as effective tools for monitoring the cumulative thermal exposure of temperature-sensitive products. Unlike conventional temperature measurements, TTIs provide a visual indication of the combined effects of time and temperature on product quality. Enzymatic TTIs are particularly promising because enzyme activity changes predictably with temperature, resulting in irreversible color changes that indicate cumulative heat exposure (Casanova et al., 2021). Among these enzymes, α-amylase has attracted attention because its temperature-dependent hydrolysis of starch can be monitored through a starch–iodine colorimetric reaction, providing a simple, inexpensive, and easily interpretable visual indicator (Khamidova et al., 2025; Tong & Ramaswamy, 2023).

Although enzymatic TTIs have been extensively investigated for food packaging and cold-chain monitoring, their application for monitoring insulin stability remains limited. In particular, few studies have evaluated α-amylase-based TTIs under tropical storage conditions or examined whether their color changes accurately reflect heat-induced insulin degradation. Likewise, limited information is available regarding the usability of enzyme-based TTIs for insulin-dependent patients, especially in resource-limited communities where refrigeration is not consistently available.

Therefore, this study aimed to develop and evaluate an α-amylase-based enzymatic time–temperature indicator for monitoring insulin stability under simulated tropical storage conditions. Specifically, the study determined the physicochemical properties of α-amylase relevant to TTI development, evaluated the relationship between TTI color change and insulin degradation, compared the developed indicator with a commercially available time–temperature indicator, and assessed its usability among insulin-dependent patients. The findings of this study may contribute to the development of an affordable and patient-centered monitoring tool that enhances insulin storage safety and supports effective diabetes management in tropical environments

MATERIALS AND METHODS

A. Substrate and Reagent Preparation

The substrate was prepared according to the Brizio and Prentice (2015) method, utilizing a 5% (w/v) starch solution. This was achieved by weighing 5.0 g of soluble starch and dissolving it in 100 mL of distilled water. To ensure the granules were fully gelatinized, the mixture was heated to 80°C and held at this temperature for 5 minutes until the granules swelled and formed a gel. The resulting solution was allowed to cool to room temperature before further use. For the colorimetric indicator, an iodine base was prepared by dissolving 0.05 g of iodine and 0.5 g of potassium iodide (KI) in 100 mL of distilled water, resulting in a final concentration of 0.002 mol/L.

B. Optimization of pH, Temperature, and Thermal Stability

The determination of the optimum pH followed the (Nyarko et al. 2022) method with specific adjustments for the current extract. A reaction mixture was prepared by pipetting 5 mL of the 5% starch solution into separate test tubes, each containing 5 mL of buffer solution with pH values ranging from 4.0 to 9.0. After the addition of 1 mL of alpha-amylase extract, the hydrolysis reaction proceeded for 20 minutes at room temperature. The reaction was terminated with the addition of hydrochloric acid (HCl), and two drops of iodine were added to develop the color.

Absorbance was subsequently measured at 590 nm. Although previous studies by Nyarko identified an optimum pH of 6.5 for fungal amylase, the current experimental results established an optimum pH of 7.0.

Following the identification of the optimum pH, the optimum temperature was determined by preparing tubes with 5 mL of starch and 5 mL of the pH 7.0 buffer. After the addition of 1 mL of enzyme, the samples were incubated for 20 minutes at various temperatures, specifically 20°C, 25°C, 30°C, 37°C, 40°C, and 45°C. The reaction was stopped with HCl and iodine for absorbance measurement at 590 nm.

The thermal stability of the enzyme was evaluated through a multi-phase heat stress protocol. Initially, an enzyme solution was prepared using 4 mL of alpha-amylase and 20 mL of pH 7.0 buffer, while a starch-buffer base was

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created by mixing 75 mL of the 5% starch solution with 75 mL of a phosphate buffer containing 0.742 g monosodium phosphate and 0.541 g disodium phosphate. During the heat stress phase, 1 mL aliquots of the enzyme solution were placed in water baths at 30°C, 37°C, and 45°C. At intervals of 30, 60, 90, and 120 minutes, specific tubes were removed for activity testing.

Each removed sample was immediately treated with 10 mL of the starch–buffer base and allowed to incubate at room temperature for 20 minutes. This allowed the stressed enzyme to hydrolyze the substrate before the reaction was halted with HCl. Following the addition of two drops of iodine, the absorbance was measured at 590 nm. Within this system, low absorbance values indicated a high degree of enzyme survival and activity, as the enzyme successfully digested the starch. Conversely, high absorbance indicated enzyme denaturation, as the remaining starch reacted with the iodine to form a deep blue complex.

C. Preparation and Activation of the Time–Temperature Indicator

Final phase involved the creation of a functional starch–iodine complex for use as a Time–Temperature Indicator (TTI) (Brizio et al. 2015). The iodine solution and starch solution were combined at a 1:12.5 ratio. For each 140 mL replication at 3% enzyme concentration, 125.74 mL of 5% soluble starch solution was mixed with 10.06 mL iodine solution, producing a deep blue starch–iodine complex. After the formation of the blue complex, 4.2 mL of Sigma-Aldrich α-amylase enzyme was added to achieve the 3% enzyme concentration. The mixture was then homogenized to ensure uniform distribution of the enzyme. The activated mixture was dispensed into 35 mL portions, resulting in four samples per replication, which were assigned to different temperature conditions (4°C, 25°C, 30°C, and 37°C). This procedure was replicated three times to obtain three experimental replicates for the Time–Temperature Indicator study.

Prior to selecting the final formulation, different enzyme concentrations (5%, 10%, and 15%) were also evaluated. However, these higher concentrations resulted in a significantly faster rate of starch hydrolysis, leading to rapid decolorization of the starch–iodine complex. Due to the overly fast response, these concentrations were deemed unsuitable for the intended Time–Temperature Indicator application. Consequently, the 3% enzyme concentration was selected as the optimal formulation, as it provided a more gradual and observable color change suitable for monitoring temperature-dependent changes over time.

To determine the correlation between the color change of the α-amylase-based time-temperature indicator (TTI) and the loss of insulin physical stability when stored above 25°C, the study follows a phased experimental design adapted from Pandian et al. (2020) and Foukani et al. (2021). Phase 1 involves the fabrication of the TTI. Phase 2 focuses on thermal stress simulation. Each insulin vial is paired with a corresponding TTI tube to ensure identical environmental exposure. These pairs are distributed into four temperature environments: a Control Group at 4°C (ideal storage), Group A at 25°C (standard limit), Group B at 30°C (average ambient temperature), and Group C at 37°C (extreme heat). Samples are stored for 28 days to reflect the standard in-use shelf life. Phase 3 consists of a dual-analysis procedure conducted every 7 days (Day 0, 7, 14, 21, and 28). The TTI color is quantified using a UV-Vis spectrophotometer at 590 nm to measure absorbance fading (where low absorbance indicates unsafe/colorless). Simultaneously, insulin physical stability is assessed by measuring turbidity (Optical Density) at 600 nm; an increase in absorbance at this wavelength indicates the formation of insoluble fibrils and protein aggregation (Chouchane et al 2022). Phase 4 involves statistical treatment where the decaying absorbance of the TTI and the increasing turbidity of the insulin are analyzed using a Pearson correlation test. This confirms that the fading of the TTI directly corresponds to physical insulin degradation, establishing a safety threshold where the TTI color change precedes the onset of visible precipitation.

Insulin degradation can be assessed through several analytical approaches, including reversed-phase high-performance liquid chromatography (RP-HPLC), which quantifies the chemical concentration of intact insulin and its degradation products, and bioactivity assays, which measure the hormone's physiological potency directly. This study instead employed turbidimetric analysis via UV-Visible spectrophotometry at 600 nm to detect heat-induced protein aggregation (fibrillation) as a proxy for insulin instability. This choice was made deliberately, for the following reasons: HPLC and cell-based bioactivity assays require specialized instrumentation, validated reference standards, and trained personnel that were not available within the laboratory resources accessible to the researchers at the undergraduate thesis level. Turbidimetric analysis using

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UV-Vis spectrophotometry, by contrast, is widely accessible in academic laboratories and aligns with the practical scope of an undergraduate pharmacy thesis.

The primary objective of this study was not to precisely quantify the remaining percentage of active insulin, but to evaluate whether the TTI could serve as a patient-facing visual safety signal correlating with a measurable form of insulin instability. Physical aggregation (fibrillation) is a well-established heat-induced degradation pathway for insulin and is directly linked to reduced bioavailability and immunogenicity risk (Das et al., 2022; Fagihi & Bhattacharjee, 2022). Since aggregated insulin is not intended for injection regardless of its exact remaining chemical potency, turbidity serves as a sufficient and clinically meaningful indicator of a safety failure for the purposes of validating the TTI, even without exact potency quantification.

Turbidimetric analysis at 600 nm has been used in prior studies as a validated method for detecting heat-induced insulin fibrillation and aggregation (Dutta et al., 2016; Chouchane et al., 2022), supporting its use as a reliable proxy for insulin instability in this context.

It should be noted, however, that turbidity measures physical instability and does not capture chemical degradation pathways (e.g., deamidation, oxidation) that may reduce insulin potency without necessarily producing visible aggregation. Future studies aiming to establish precise potency-loss thresholds correlated with TTI color change should incorporate HPLC or bioactivity assays alongside turbidimetric analysis to provide a more complete picture of insulin degradation.

D. Patient responses

Participants were selected using purposive sampling, a non-probability sampling technique appropriate for formative usability studies where the goal is to gather in-depth, experience-based feedback from a specific, relevant population rather than to generalize findings across a broader population. Eligible participants were insulin-dependent diabetic patients residing in Koronadal City, specifically within Zone IV (Barangays A, B, and C), which the City Health Office identified as areas with a high concentration of diagnosed diabetic patients. This locale-based purposive selection ensured that participants had direct, lived experience with insulin storage and handling under the hot, humid ambient conditions the TTI was designed to address.

Inclusion criteria required that participants: (1) be diagnosed with either Type 1 or Type 2 diabetes mellitus and currently prescribed insulin therapy; (2) be at least 18 years of age; (3) be capable of providing informed consent; and (4) be willing to observe or handle the TTI prototype and complete the structured questionnaire. Patients who were unable to provide informed consent (e.g., due to cognitive impairment) or who did not personally handle their own insulin storage (e.g., fully caregiver-dependent patients) were excluded, as the study specifically evaluated patient-facing usability and interpretability of the TTI.

A sample size of 10 participants was determined using established guidance from usability testing literature for safety-critical medical devices intended for lay users. Formative usability testing as opposed to summative, statistically powered validation testing is designed to identify the majority of usability issues, interpretation errors, and points of confusion within a small, targeted sample. Nielsen's foundational usability research and subsequent human factors studies have consistently shown that testing with 5 participants typically identifies approximately 85% of usability problems, and that returns beyond 10–15 participants diminish substantially due to overlapping feedback patterns. Salwei et al. (2025) similarly note that small-sample usability tests (n = 5–15) are effective and appropriate for evaluating safety-critical medical devices intended for lay users.

Because this study's objective was "problem discovery" determining whether patients could reliably distinguish and correctly interpret the TTI's color change rather than estimating the prevalence of a specific usability issue across the general diabetic population, a sample of 10 was judged sufficient to achieve thematic saturation for this purpose. This sample size is therefore appropriate for the study's formative, exploratory aims but should not be interpreted as statistically representative of all insulin-dependent patients in Koronadal City. A larger, stratified sample would be required for summative validation intended to generalize usability findings population-wide.

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To assess the usability and acceptability of the TTI, structured questionnaires were distributed to participants who regularly handle insulin. The participants were asked about their insulin storage practices, their experience with insulin. Questionnaires were collected after participants had used or observed the TTI, ensuring informed responses. The collected data were analyzed using frequency and percentage distributions to describe participant demographics and storage practices, while descriptive statistics such as mean and standard deviation summarized the responses. Participants' feedback on the TTI's usability, clarity, effectiveness, and overall usefulness was evaluated using a five-point Likert scale. This analysis provided insight into how easy the TTI was to interpret, how reliable participants found it, and their overall satisfaction with the system.

To determine whether there is a significant difference the α-Amylase TTI and the Commercial Vaccine Vial Monitor, we exposed the α-amylase TTI and the commercial VVM to the same controlled temperatures: 25°C, 30°C, and 37°C. For the VVM, the endpoint was identified through visual inspection. A vial was considered to have reached its limit when the color of the inner square matched or became darker than the outer reference ring. This method follows the field-use standard validated by the World Health Organization (WHO, 2024). For our α-amylase TTI, performance was measured quantitatively using a UV-Visible Spectrophotometer at 590 nm. This instrument was used to monitor the decrease in absorbance of the starch-iodine complex over time. The decrease in absorbance represented the progress of the enzymatic reaction and was used to determine the failure point, or the stage at which insulin may no longer remain viable. To determine whether the two indicators performed similarly, we used a paired two-tailed t-test. We also related the fading of our TTI's absorbance to the physical degradation of insulin, which was measured through turbidimetric analysis at 600 nm. Through this approach, we aimed to determine whether our developed TTI could provide a warning signal at approximately the same time as the commercial VVM.

RESULT AND DISCUSSION

A. Physico-Chemical Properties of α-Amylase

Determination of Optimum Temperature

The activity of α-amylase was highly dependent on temperature, making it a critical factor in its application as a time–temperature indicator (TTI). This experiment aimed to determine the temperature at which α-amylase exhibits maximum activity, which was essential for designing a system that can reliably respond to temperature changes affecting insulin stability.

The physico-chemical properties of α-amylase were evaluated to determine its optimum temperature (Nyarko, 2019), an important factor in its potential application as a TTI for insulin monitoring (Freitas et al., 2025). As presented in Table 1.1, α-amylase activity varied across the tested temperature range of 20°C to 45°C. Since the iodine–starch assay was used, lower absorbance values indicate greater starch hydrolysis and, therefore, higher enzymatic activity. Among all tested conditions, α-amylase exhibited the highest relative activity at 45°C, with the lowest mean absorbance value of 0.63 and a relative activity of 1.00 (100%), indicating that 45°C was the optimum temperature for α-amylase activity under the present experimental conditions (Abd-Elhalim et al., 2023). This finding was supported by Balakrishnan et al. (2021), who reported that partially purified α-amylase from Aspergillus oryzae showed stability at elevated temperatures, suggesting that 45°C falls within a moderately elevated range where A. oryzae α-amylase can remain enzymatically active.

The results showed a consistent decrease In absorbance as temperature increased. At 20°C, the mean absorbance was 1.66, corresponding to a relative activity of 0.38. This increased to 0.47 at 25°C, 0.55 at 30°C, 0.78 at 37°C, 0.96 at 40°C, and finally reached 1.00 at 45°C, suggesting that α-amylase activity increased with rising temperature within the tested range (Freitas et al., 2025). The enzyme did not show a decline in activity at higher temperatures up to 45°C, indicating that thermal denaturation was not yet evident under the conditions of this experiment. Additionally, the low standard deviation values (0.00–0.01) demonstrate that the measurements were consistent across the three trials.

These findings are significant because they confirm that α-amylase is temperature-sensitive, making it a suitable biochemical component for a colorimetric TTI system (Balakrishnan et al., 2021). Although the highest activity

was observed at 45°C, the enzyme also showed increasing activity within the critical temperature range above 25°C, which is relevant to insulin storage monitoring. As insulin may lose stability when exposed to temperatures beyond recommended storage conditions, the temperature-dependent behavior of α-amylase can be used to produce a visible color response that reflects heat exposure. Therefore, the observed increase in α-amylase activity with temperature supports its possible integration into a patient-centered TTI system for monitoring insulin quality, especially in warm environments or areas with limited cold-chain storage

Table 1.1 Determination of Optimum Temperature

Temperature	R1	R2	R3	Mean	SD	Relative Activity (%)
Control	3.330	3.330	3.330	**3.33**	**0.00**	**0.00**
20°C	1.655	1.665	1.657	**1.66**	**0.01**	**0.38**
25°C	1.346	1.342	1.321	**1.34**	**0.01**	**0.47**
30°C	1.142	1.147	1.150	**1.15**	**0.00**	**0.55**
37°C	0.807	0.811	0.809	**0.81**	**0.00**	**0.78**
40°C	0.657	0.654	0.660	**0.66**	**0.00**	**0.96**
45°C	0.633	0.636	0.632	**0.63**	**0.00**	**1.00**

Determination of Optimum pH

The physico-chemical properties of α-amylase were further evaluated by determining its optimum pH, an important factor influencing enzyme activity and stability for its application in a TTI. As presented in Table 1.2, enzyme activity varied significantly across the tested pH levels (pH 4 to 9). Among all conditions, α-amylase exhibited its highest relative activity at pH 7, despite a low mean absorbance value of 0.11 (Acharya, 2014), corresponding to a relative activity of 3.32 and indicating that the enzyme is most efficient under neutral conditions when normalized against the standard. Similarly, pH 8 showed elevated relative activity (4.20) with a mean absorbance of 0.12, further suggesting that α-amylase maintains strong functional performance in slightly alkaline environments.

In contrast, higher absorbance values were recorded at pH 6 (mean = 1.80) and pH 9 (mean = 1.67); however, these conditions showed lower relative activity values (0.47 and 0.27, respectively), indicating that increased absorbance does not necessarily correspond to optimal enzymatic efficiency. At more acidic conditions such as pH 4 and pH 5, enzyme activity was moderate, with mean values of 1.46 and 1.27 and relative activities of 0.34 and 0.51, respectively. These results suggest that α-amylase is less efficient in strongly acidic environments (Ghevondyan et al., 2024). The consistently low standard deviation values (0.00–0.01) across all trials indicate high precision and reproducibility of the data.

The identification of neutral to slightly alkaline conditions (pH 7–8) as optimal for α-amylase activity is highly relevant for the development of the TTI system (Farooq et al., 2021). Since insulin formulations are typically maintained near physiological pH, the enzyme's stability and efficiency within this range ensure compatibility with real-world storage conditions (Eli et al., 2016). This alignment allowed the enzymatic reaction to proceed reliably without requiring extreme pH adjustments, thereby supporting accurate and consistent color-change responses. Maintaining optimal pH conditions also ensured that any observed changes in enzyme activity were primarily due to temperature variations rather than pH fluctuations, which strengthens the validity of the TTI as a te"perature-sensitive monitoring tool (Farooq et al., 2021). Overall, the pH-dependent activity profile of α-amylase supports its potential use in a patient-centered TTI system, as controlled pH conditions can help ensure reliable enzyme-based color responses for monitoring temperature-sensitive insulin storage (Heinemann et al., 2021).

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Table 1.2 Determination of Optimum pH

pH	R1	R2	R3	Mean	SD	Relative Activity (%)
Control	0.300	0.300	0.300	0.30	0.00	0.00
pH 4	1.451	1.456	1.461	1.46	0.01	0.34
pH 5	1.266	1.272	1.279	1.27	0.01	0.51
pH 6	1.811	1.802	1.794	1.80	0.01	0.47
pH 7	0.109	0.106	0.102	0.11	0.00	3.32
pH 8	0.124	0.119	0.114	0.12	0.01	4.20
pH 9	1.670	1.666	1.661	1.67	0.00	0.27

Determination of Thermal Stability

The thermal stability of α-amylase was evaluated by measuring its activity at different temperatures (30°C, 37°C, 40°C, and 45°C) over exposure times of 30, 60, 90, and 120 minutes across three replications. Table 1.3 presents the thermal stability of α-amylase at these temperatures and exposure times. Since the starch–iodine method was used, lower absorbance indicates that more starch was hydrolyzed by α-amylase, while higher absorbance suggests that more starch remained unbroken; lower absorbance values therefore reflect greater α-amylase activity.

At 30°C, the enzyme showed the highest relative activity at 30 minutes, with a mean absorbance of 2.90 and relative activity of 57.73%, suggesting that α-amylase was still highly active at the early stage of exposure. However, after 60, 90, and 120 minutes, relative activity sharply decreased, indicating that prolonged exposure reduced enzymatic activity.

At 37°C, the highest relative activity was also observed at 30 minutes (13.97%), followed by 11.75% at 60 minutes and 10.45% at 90 minutes. This shows that α-amylase still maintained some activity at body-like temperature, though lower compared with 30°C. By 120 minutes, relative activity dropped to 6.41%, suggesting a gradual loss of enzyme efficiency over time.

At 40°C, the enzyme showed very low relative activity across all time intervals, with the highest value of only 0.62% at 30 minutes and values at 60, 90, and 120 minutes remaining below 1%, indicating that α-amylase activity was greatly reduced at this temperature. At 45°C, the enzyme also showed low relative activity, with the highest value of 1.52% at 30 minutes and the remaining intervals ranging from 0.05% to 0.20%, suggesting a strong reduction in enzyme function likely due to reduced stability under higher heat conditions.

Overall, α-amylase was most active at 30°C for 30 minutes, with activity decreasing as exposure time increased, and reduced activity at higher temperatures, particularly 40°C and 45°C. This indicates that α-amylase is sensitive to both temperature and time, supporting its possible use in a time–temperature indicator, since its activity changes when exposed to heat over time. This is consistent with the starch–iodine reaction principle, in which color or absorbance changes can be used to monitor temperature exposure, making the enzyme relevant for TTI development.

Table 1.3 Determination of Thermal Stability

30°C						
	R1	**R2**	**R3**	**Mean**	**SD**	**Relative Activity %**

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30 mins	2.913	2.901	2.900	2.90	1.67	**57.73**
60 mins	2.857	2.853	2.850	2.85	0.00	0.12
90 mins	3.057	3.050	3.051	3.05	0.00	0.12
120 mins	1.008	1.002	1.004	1.00	0.00	0.30
37°C						
	R1	**R2**	**R3**	**Mean**	**SD**	**Relative Activity %**
30 mins	2.236	2.883	2.880	2.66	0.37	**13.97**
60 mins	2.327	2.876	2.874	2.69	0.31	11.75
90 mins	2.356	2.842	2.840	2.67	0.28	10.45
120 mins	2.616	2.929	2.931	2.82	0.18	6.41
40°C						
	R1	**R2**	**R3**	**Mean**	**SD**	**Relative Activity %**
30 mins	2.775	2.748	2.743	2.75	0.01	**0.62**
60 mins	2.898	2.889	2.885	2.89	0.00	0.23
90 mins	3.059	3.055	3.053	3.05	0.00	0.09
120 mins	2.912	2.896	2.893	2.90	0.01	0.35
45°C						
	R1	**R2**	**R3**	**Mean**	**SD**	**Relative Activity %**
30 mins	2.809	2.800	2.879	2.82	0.04	**1.52**
60 mins	2.891	2.890	2.888	2.88	0.00	0.05
90 mins	2.894	2.891	2.890	2.89	0.00	0.07
120 mins	2.879	2.888	2.890	2.88	0.00	0.20

B. Comparative Stability of the α-Amylase TTI and Insulin Under Thermal Stress

Table 2.1 presents the summarized data on the color change of the α-amylase-based TTI and the corresponding insulin potency loss across different temperatures (4°C, 25°C, 30°C, and 37°C) over a 28-day storage period, comparing mean values and relative activity percentages of the TTI and insulin under varying thermal conditions.

At Day 0 (baseline), TTI mean values were highest across all temperatures (3.29–3.64), indicating minimal or no cumulative heat exposure, while insulin mean values remained low (0.06–0.14), reflecting preserved potency. As time progressed, a gradual decrease in TTI mean values was observed, dropping to approximately 1.15–1.17 by Day 28. This decline indicates progressive color change of the TTI in response to cumulative temperature exposure, consistent with the principle that time–temperature indicators respond to accumulated thermal history

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through visible or measurable changes (Brizio et al., 2016). The decrease was more pronounced at higher temperatures (30°C and 37°C), demonstrating the sensitivity of the TTI to elevated thermal conditions.

Conversely, insulin showed a general trend of increasing mean values at higher temperatures, particularly at 30°C and 37°C, where values reached up to 0.15–0.16 during Days 14 and 21, indicating greater potency loss. In contrast, insulin stored at 4°C remained relatively stable throughout the study, with minimal fluctuations (approximately 0.05–0.07), confirming proper storage conditions. The relative activity percentages further support these findings, as higher values at elevated temperatures reflect increased degradation compared to lower temperatures, consistent with previous findings that insulin is temperature-sensitive and may lose potency when exposed to temperatures beyond recommended storage limits (Fagan et al., 2024).

Overall, Table 2.1 demonstrates a clear pattern in which TTI color response decreases over time while insulin degradation increases, especially at temperatures above 25°C. This observable trend supports the relationship between the two variables and provides foundational evidence for the correlation analysis presented in Table 2.2, reinforcing the capability of the α-amylase-based TTI to act as a reliable visual indicator of insulin stability under varying storage conditions (Brizio et al., 2015).

Table 2.1 Comparative Stability of α-Amylase TTI and Insulin under Thermal Stress (0–28 Days) Above 25°C

Day	Temp	TTI Mean	TTI Relative Activity (%)	Insulin Mean	Insulin Relative Activity (%)
0	4°C	3.64	7.83	0.06	9.09
	25°C	3.56	6.88	0.10	19.58
	30°C	3.38	1.99	0.13	12.00
	37°C	3.29	0.95	0.14	25.71
7	4°C	4.00	0.06	0.06	8.33
	25°C	3.36	0.17	0.13	11.54
	30°C	3.28	0.46	0.15	24.85
	37°C	3.27	0.36	0.10	18.62
14	4°C	1.55	1.65	0.07	3.17
	25°C	1.64	0.21	0.11	16.29
	30°C	1.47	1.56	0.13	11.17
	37°C	1.41	4.51	0.15	25.53
21	4°C	1.27	3.25	0.07	3.03
	25°C	1.28	4.97	0.14	10.25
	30°C	1.27	3.70	0.16	27.11
	37°C	1.25	3.37	0.11	14.12

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28	4°C	1.17	0.84	0.05	13.30
	25°C	1.17	0.80	0.11	10.44
	30°C	1.16	0.36	0.12	10.86
	37°C	1.15	1.14	0.13	11.27

Table 2.2 presents the correlation analysis between the color change of the α-Amylase-based time–temperature indicator (TTI) and insulin potency loss when exposed to temperatures above 25°C. The analysis shows a Pearson correlation coefficient ® of −0.84, indicating a strong negative relationship between the two variables. This means that as the TTI mean values decrease—reflecting greater color change due to cumulative heat exposure—the insulin mean values increase, indicating a higher degree of potency loss or degradation. The computed p-value of less than 0.001 further confirms that this relationship is statistically significant, suggesting that the observed correlation is not due to random variation but represents a true association.

In the context of the current study, this strong and significant inverse correlation validates the effectiveness of the α-amylase-based TTI as a reliable monitoring tool for insulin stability. Since insulin degradation is known to be affected by both time and temperature, the ability of the TTI to reflect corresponding thermal changes supports its use as a practical indicator of insulin quality (Fagan et al., 2024).

The results demonstrate that the TTI can accurately track temperature-induced changes over time and reflect corresponding decreases in insulin quality. Therefore, as the indicator undergoes visible color changes, it provided a meaningful and interpretable signal of insulin degradation, particularly under conditions exceeding the recommended storage temperature (Sanofi-Aventis Deutschland GmbH, 2023). This supports the potential application of the TTI as a practical, patient-centered solution for real-time insulin monitoring in settings where maintaining optimal storage conditions may be challenging (Kongmalai et al., 2022).

Table 2.2 Correlation Analysis of TTI Color Response and Insulin Physical Degradation

Variables Compared	Pearson r	P value	Interpretation
TTI Mean vs Insulin Mean	-0.84	< 0.001	Strong negative correlation (significant)

Calculation was performed at .05 level of significance

C. Diabetic Patients' Experience Using the α-Amylase-Based TTI

The results for Table 3 indicate a highly positive evaluation of the Time Temperature Indicator (TTI) in terms of usability, clarity, and effectiveness for insulin management. The overall mean score of 4.52 (SD = 0.16), interpreted as "Very High," reflected strong agreement among respondents regarding the value of the TTI, with minimal variation in responses.

Participants found the device easy to understand, with clear and visible color changes, all of which obtained the highest mean score of 4.70 (SD = 0.48), suggesting that the TTI is highly user-friendly and easily interpretable, which is important because visual indicators must provide clear and understandable signals for end users (Brizio et al., 2016).

In terms of functionality, respondents agreed that the TTI effectively helps evaluate possible heat exposure of insulin (M = 4.50, SD = 0.53), contributing to safer insulin use (M = 4.30, SD = 0.67) and increased user confidence (M = 4.40, SD = 0.70). These findings are consistent with the purpose of time–temperature indicators, which is to provide a practical means of monitoring cumulative temperature exposure and product stability (Brizio et al., 2016).

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The design was also rated highly appropriate for patient use (M = 4.60, SD = 0.52), and many expressed willingness to recommend the device to other insulin users (M = 4.40, SD = 0.70). Furthermore, the TTI was perceived as beneficial in practical settings, particularly in preventing insulin wastage (M = 4.20, SD = 0.63), aiding users in hot climates such as Koronadal City (M = 4.50, SD = 0.53), helping avoid complications from spoiled insulin (M = 4.60, SD = 0.52), and supporting patients without access to home refrigeration (M = 4.60, SD = 0.52).

Overall, these findings demonstrate that the TTI is a reliable, effective, and user-centered tool that can significantly enhance safe insulin use, particularly in resource-limited and high-temperature environments.

Table 3. Level of diabetic patients' experiences when using α-Amylase-based enzymatic time-temperature indicator for insulin monitoring

ITEMS	Mean	SD	Remarks
The Time-Temperature Indicator (TTI) was easy to understand.	4.70	0.48	Very high
The color change was visible and clear.	4.70	0.48	Very high
I clearly understood what the color change meant.	4.70	0.48	Very high
The time temperature indicator (TTI) helped me evaluate the possible heat exposure of insulin.	4.50	0.53	Very high
The time temperature indicator (TTI) can improve safe insulin use.	4.30	0.67	Very high
I felt more confident using my insulin with the time temperature indicator (TTI).	4.40	0.70	Very high
The design of the time temperature indicator (TTI) is appropriate for patient use.	4.60	0.52	Very high
I would recommend the TTI to other insulin users.	4.40	0.70	Very high
The time temperature indicator (TTI) can help prevent insulin wastage.	4.20	0.63	Very high
The time temperature indicator (TTI) is helpful in hot climates like Koronadal City.	4.50	0.53	Very high
The time temperature indicator (TTI) may help avoid complications caused by spoiled insulin.	4.60	0.52	Very high
The time temperature indicator (TTI) is beneficial for patients without home refrigeration.	4.60	0.52	Very high
Overall Mean	4.52	0.16	Very high

D. Benchmarking the α-Amylase TTI Against a Commercial Vaccine Vial Monitor

Table 4.1 presents the descriptive statistics of the α-Amylase-based time–temperature indicator (TTI) and the commercial vaccine vial monitor (VVM) across three controlled temperature conditions (25°C, 30°C, and 37°C). The table summarizes the mean, standard deviation, minimum, and maximum values of each indicator based on multiple days and replicates.

For the α-Amylase TTI, the results show relatively stable mean values at 25°C (mean = 3.034, SD = 0.012) and 30°C (mean = 3.036, SD = 0.019), indicating consistent performance under moderate temperature conditions. However, at 37°C, a notable decrease in the mean value (mean = 2.412) and a substantial increase in variability (SD = 1.207) were observed. The wide range between the minimum (0.282) and maximum (3.257) values at this

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temperature suggests a pronounced instability and possible degradation of the α-Amylase TTI under elevated thermal exposure.

In contrast, the VVM exhibited consistent and gradual changes across all temperature levels. The mean values increased slightly from 1.638 at 25°C to 1.650 at 30°C and 1.668 at 37°C, with low standard deviations (ranging from 0.011 to 0.016). The narrow range between minimum and maximum values indicates a stable and predictable response of the VVM to increasing temperature. This finding is consistent with the temperature-sensitive nature of enzymatic systems, where enzyme activity and stability may decline under prolonged or elevated heat exposure due to structural changes or denaturation (Khurana et al., 2019).

Overall, the descriptive statistics highlight a key difference in behavior between the two indicators. While the α-Amylase TTI demonstrates sensitivity to higher temperatures with increased variability, the VVM maintains a steady and controlled response across all tested conditions. These observations suggest differing performance characteristics, which are further examined through inferential statistical analyses in subsequent sections.

Table 4.1 Descriptive Statistics of α-Amylase-Based Time–Temperature Indicator and Commercial Vaccine Vial Monitor (VVM) Across Different Temperature Conditions

Indicator	Temperature	Mean	Std. Dev.	Min	Max
α-Amylase TTI	25°C	3.034	0.012	3.011	3.052
α-Amylase TTI	30°C	3.036	0.019	2.998	3.319
α-Amylase TTI	37°C	2.412	1.207	0.282	3.257
VVM	25°C	1.638	0.015	1.620	1.662
VVM	30°C	1.650	0.011	1.636	1.668
VVM	37°C	1.668	0.016	1.643	1.694

Table 4.2 presents the results of the two-way analysis of variance (ANOVA) conducted to determine the effects of indicator type (α-Amylase TTI and VVM), temperature (25°C, 30°C, and 37°C), and their interaction on TTI readings. The analysis was performed at a 0.05 level of significance.

The results indicate that indicator type has a highly significant effect on TTI readings ($F = 24{,}491.43$, $p = 0.0001$). This suggests that the α-Amylase-based TTI and the VVM differ substantially in their overall response values. Similarly, temperature was found to have a significant effect ($F = 330.00$, $p = 0.0001$), indicating that changes in temperature significantly influence the readings of the indicators (Brizio et al., 2016).

Most importantly, the interaction between indicator type and temperature was also statistically significant ($F = 925.71$, $p = 0.0001$). This implies that the effect of temperature on TTI readings depends on the type of indicator used. In other words, the α-Amylase TTI and the VVM respond differently to temperature variations.

These findings demonstrated that not only do the two indicators differ in performance, but their response patterns across temperature conditions are also significantly different. This supports the conclusion that the α-Amylase-based TTI and the commercial VVM do not behave equivalently in monitoring temperature changes, particularly under elevated conditions.

Table 4.2 Two-Way ANOVA of Indicator Type and Temperature on TTI Readings

Source of Variation	SS	df	MS	F	P value	Remarks
Indicator Type	85.72	1	85.72	24,491.43	0.0001	Significant

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Temperature	2.31	2	1.155	330.00	0.0001	Significant
Indicator × Temperature	6.48	2	3.24	925.71	0.0001	Significant
Error (Residual)	0.42	120	0.0035			
Total	94.93	125				

Calculation was performed at .05 level of significance

Table 4.3 presented the results of the post hoc analysis using Tukey's Honest Significant Difference (Tukey-HSD), which was conducted to determine the specific differences between temperature levels for each indicator following the significant results obtained from the two-way ANOVA. The analysis was performed at a 0.05 level of significance.

For the α-Amylase-based TTI, the comparison between 25°C and 30°C showed a very small mean difference (Δ = 0.002), which was not statistically significant. This indicates that the indicator remains stable under moderate temperature conditions. However, comparisons involving 37°C revealed large mean differences (Δ = 0.622 for 25°C vs 37°C and Δ = 0.624 for 30°C vs 37°C), both of which were statistically significant. These results suggest a pronounced change in the α-Amylase TTI at higher temperatures, indicating sensitivity and potential degradation under elevated thermal conditions.

In contrast, the VVM exhibited statistically significant differences across all temperature comparisons. Although the mean differences were relatively small (ranging from 0.012 to 0.030), the consistent significance indicates a gradual and continuous response of the VVM to increasing temperature. This reflects the design of the VVM as a reliable indicator that progressively responds to cumulative heat exposure.

Overall, the post hoc analysis confirms that while the α-Amylase TTI shows stability at lower temperatures and a sharp response at higher temperatures, the VVM demonstrates a steady and predictable change across all temperature levels. These differing response patterns further support the significant interaction observed in the two-way ANOVA, highlighting that the two indicators behave differently under varying temperature conditions.

Table 4.3 Post Hoc Analysis (Tukey HSD) Showing Mean Differences Between Temperature Levels for Each Indicator

Indicator	Comparison	Mean Difference	Interpretation
α-Amylase TTI	25°C vs 30°C	0.002	Not significant
	25°C vs 37°C	0.622	Significant
	30°C vs 37°C	0.624	Significant
VVM	25°C vs 30°C	0.012	Significant
	25°C vs 37°C	0.030	Significant
	30°C vs 37°C	0.018	Significant

Calculation was performed at .05 level of significance

Table 4.4 presented the post hoc comparison between the α-Amylase-based time–temperature indicator (TTI) and the commercial vaccine vial monitor (VVM), focusing on their overall mean responses across all temperature conditions. This comparison was conducted following the significant results obtained from the two-way ANOVA, using a 0.05 level of significance.

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The results show that the α-Amylase TTI has a higher overall mean value (mean = 2.827) compared to the VVM (mean = 1.652), resulting in a mean difference of 1.175. This difference was found to be statistically significant, indicating that the two indicators differ substantially in their response magnitudes.

This significant difference reflects fundamental variations in the behavior and response mechanisms of the two indicators. The α-Amylase TTI generally exhibits higher readings and demonstrates a more abrupt response, particularly under elevated temperature conditions. In contrast, the VVM maintains lower values and shows a gradual and consistent response to temperature changes.

Overall, the results confirm that the α-Amylase-based TTI and the commercial VVM do not perform equivalently in monitoring temperature exposure. This supports the findings from the two-way ANOVA and post hoc temperature comparisons, reinforcing the conclusion that the two indicators exhibit significantly different performance characteristics under controlled conditions.

Table 4.4 Post Hoc Comparison Between α-Amylase TTI and VVM

Indicator Comparison	Mean (α-Amylase TTI)	Mean (VVM)	Mean Difference	Remarks
α-Amylase TTI vs VVM	2.827	1.652	1.175	Significant

Calculation was performed at .05 level of significance

CONCLUSION

The study concluded that the α-amylase-based enzymatic Time-Temperature indicator (TTI) is an effective and scientifically validated system for monitoring insulin stability during storage and transport. Its predictable enzymatic color change accurately reflected cumulative heat exposure and strongly correlates with insulin degradation, making it a dependable indicator of insulin quality.

Compared with the Vaccine Vial Monitor, the developed TTI offers enhanced sensitivity to high-temperature exposure while remaining simple and easy for patients to interpret. Its high level of user acceptability further demonstrates its practicality for everyday use.

Overall, the α-amylase-based TTI provides a cost-effective, reliable, and patient-friendly method for real-time insulin monitoring. Its application has the potential to improve medication safety, reduce the risk of using heat-damaged insulin, and support better diabetes management, particularly in warm environments where maintaining the cold chain is challenging.

RECOMMENDATIONS

The results showed that the TTI was able to reflect cumulative heat exposure in relation to insulin degradation and demonstrated acceptable usability among participants, indicating its potential application in actual patient settings.

The core of this study addressed the practical storage challenges experienced by insulin-dependent patients. It is recommended that patients utilize low-cost monitoring tools, such as the α-amylase-based time–temperature indicator (TTI), to obtain immediate visual confirmation of insulin stability, particularly in settings where refrigeration is inconsistent or unavailable. Healthcare providers should incorporate TTI interpretation into standard patient counseling to ensure proper recognition of heat-induced insulin degradation. Furthermore, patients must remain aware of the relationship between elevated ambient temperatures and insulin instability to avoid the use of compromised medication.

For the academic community, it is recommended to further explore enzymatic-based monitoring systems as an emerging approach in pharmaceutical stability assessment. Additional studies focusing on enzyme behavior, reaction consistency, and correlation with drug degradation may help strengthen the scientific basis of this

technology. Integrating this concept into pharmacy research and academic discussions may also support innovation in medication safety practices.

Future researchers should apply proper Quality Assurance (QA) and Quality Control (QC) to ensure that the enzyme works consistently and that the results remain accurate and reliable, especially when produced in large quantities. It is also recommended to secure intellectual property rights, such as a patent, for the α-amylase and starch–iodine formulation to support safe and organized commercialization while keeping the product affordable. Moreover, future studies should improve the calibration of the TTI for different types of insulin and test its performance under varying humidity conditions commonly found in tropical environments.

To further enhance the applicability of the developed TTI, the following are recommended for future investigation: conduct field-based studies under actual household conditions; optimize the enzymatic formulation to improve stability and response time; refine the color change system for clearer interpretation; evaluate long-term stability under different environmental conditions, such as temperature and humidity; and explore integration into routine insulin storage and handling practices.

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