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SMART POROUS COLORIMETRIC INDICATORS BASED ON CELLULOSE NANOCRYSTALS-*Calotropis gigantea* FOR NON-DESTRUCTIVE EVALUATION OF FISH FRESHNESS

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Abstract

The development of smart packaging based on colorimetric changes has emerged as an innovative approach for the real-time monitoring of fresh food quality. This study aimed to evaluate a porous colorimetric indicator based on a poly (vinyl alcohol)/poly (vinyl pyrrolidone) (PVA/PVP) composite reinforced with cellulose nanocrystals derived from *Calotropis gigantea* (CNCs-CG) and immobilized with butterfly pea (*Clitoria ternatea*) flower anthocyanins, and to evaluate its potential application as an intelligent packaging system for real-time, non-destructive monitoring of fish freshness. The incorporation of CNCs-CG as a filler (0, 0.50, 0.75, 1.00, and 1.25 g) increased the composite surface area to 0.45, 10.22, 38.66, 162.54, and 40.44 m^2/g, respectively. Based on Brunauer–Emmett–Teller (BET) and scanning electron microscopy (SEM) analyses, the incorporation of 1.00 g CNCs-CG produced the highest porosity and optimal mesoporous structure and was therefore selected for the immobilization of two anthocyanin extracts (AT-1, pH 1, and AT-2, pH 6). The indicator response was evaluated against ammonia vapor and applied to Nile tilapia stored at room temperature and 5°C. The experimental data were analyzed descriptively and comparatively based on morphological characteristics, surface area, indicator color changes, and their relationships with pH and total volatile base nitrogen (TVBN) values. The AT-2 indicator exhibited a faster and more consistent color transition, accompanied by an increase in TVBN from 3.94 to 27.37 mg/100 g after 24 h of storage at room temperature, whereas samples stored at 5°C remained below the freshness threshold until day 14. These findings demonstrate that the AT-2 indicator effectively discriminates fish freshness levels and shows a strong correlation with pH and TVBN values, highlighting its potential application as an intelligent packaging system for the non-destructive monitoring of fish freshness.

Keywords: anthocyanin, cellulose nanocrystals, fish freshness monitoring, intelligent packaging, porous composite

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Indikator Kolorimetri Berpori Berbasis Nanokristal Selulosa dari *Calotropis gigantea* untuk Evaluasi Kesegaran Ikan Secara Non-Destruktif

Abstrak

Pengembangan kemasan cerdas berbasis perubahan warna menjadi salah satu pendekatan inovatif untuk memantau mutu pangan segar secara real-time. Penelitian ini bertujuan mengevaluasi indikator berpori berbasis komposit poli(vinil alkohol)/poli(vinil pirolidon) (PVA/PVP) yang diperkuat nanokristal selulosa dari *Calotropis gigantea* (CNCs-CG) dan diimobilisasi dengan antosianin bunga telang serta mengevaluasi potensi penerapannya sebagai sistem kemasan cerdas untuk pemantauan kesegaran ikan secara real-time dan non-destruktif. Penambahan CNCs-CG sebagai filler (0; 0,50; 0,75; 1,00; dan 1,25 g) meningkatkan luas permukaan komposit menjadi berturut-turut 0,45; 10,22; 38,66; 162,54; dan 40,44 m^2/g. Berdasarkan analisis BET dan SEM, penambahan 1,00 g CNCs-CG menghasilkan porositas dan distribusi mesopori terbaik sehingga dipilih untuk imobilisasi dua jenis antosianin (AT-1, pH 1 dan AT-2, pH 6). Respons indikator diuji terhadap uap amonia serta diaplikasikan pada ikan nila yang disimpan pada suhu ruang dan 5°C. Data hasil pengujian dianalisis secara deskriptif dan komparatif berdasarkan karakteristik morfologi, luas permukaan, perubahan warna indikator, serta keterkaitannya dengan nilai pH dan total volatile base nitrogen (TVBN). Indikator AT-2 menunjukkan perubahan warna lebih cepat dan konsisten, dengan peningkatan TVBN dari 3,94 menjadi 27,37 mg/100 g setelah 24 jam pada suhu ruang, sedangkan pada penyimpanan 5°C nilainya tetap di bawah batas kesegaran hingga hari ke-14. Hasil penelitian menunjukkan bahwa indikator AT-2 mampu membedakan tingkat kesegaran ikan secara efektif dan memiliki korelasi yang baik dengan nilai pH dan TVBN, sehingga berpotensi diaplikasikan sebagai sistem kemasan cerdas untuk pemantauan kesegaran ikan secara non-destruktif.

Kata kunci: antosianin, kemasan cerdas, komposit berpori, nanokristal selulosa, pemantauan kesegaran ikan

INTRODUCTION

Fresh fish is a highly nutritious commodity valued as a rich source of high-quality animal protein. However, it is one of the most perishable food products because of microbial activity and chemical reactions during storage, leading to rapid quality deterioration, safety concerns, and economic losses (Zhai *et al*., 2017; Mardhiah *et al*., 2023). Spoilage is typically associated with the accumulation of volatile basic compounds such as ammonia and trimethylamine, derived from the decomposition of nitrogenous substances. Hence, the reliable monitoring of fish freshness is essential across the supply chain. Conventional methods, such as total volatile basic nitrogen (TVB-N) and total plate count (TPC) analyses, are accurate but destructive, time-consuming, and dependent on laboratory facilities and skilled personnel (Zhai *et al*., 2017; Merz *et al*., 2020). These limitations have encouraged the development of alternative nondestructive technologies that are more efficient, easily applicable, and capable of real-time monitoring.

One widely explored approach is intelligent packaging technology equipped with pH-based colorimetric indicators. These indicators have been identified as effective tools for monitoring fish freshness, operating through visible color changes triggered by pH shifts caused by the accumulation of volatile bases (e.g., ammonia and trimethylamine) during the spoilage. These indicators enable early detection without opening the package (Nagarajarao, 2016; Zhai *et al*., 2017; Merz *et al*., 2020). Several studies have confirmed the effectiveness of natural pigment-based colorimetric indicators, particularly anthocyanins, in monitoring the freshness of food products, including milk (Goodarzi *et al*., 2020), shrimp (Eze *et al*., 2022; Merz

et al., 2020), fish (Moradi *et al*., 2019), and meat (Seftiono *et al*., 2021). However, most of the indicators developed thus far still exhibit limitations in terms of response speed and sensitivity, especially when applied to food systems with rapid pH fluctuations.

Many colorimetric indicators have been fabricated as edible films or biofilms, and they have shown great potential for detecting spoilage in milk (Tirtashi *et al*., 2019), shrimp (Merz *et al*., 2020; Mohammadalinejhad *et al*., 2020), fresh fish (Kuswandi *et al*., 2012; Pourjavaher *et al*., 2017; Amalia *et al*., 2021), and meat (Nurfawaidi *et al*., 2018; Ezati *et al*., 2019; Özkaya & Dağbağlı, 2021). The effectiveness of a colorimetric indicator is primarily determined by its sensitivity to pH changes, which enables rapid color responses. However, color changes in most biofilm-based indicators remain relatively slow, often requiring several minutes (Liu *et al*., 2019; Zhang *et al*., 2019; Mohammadalinejhad *et al*., 2020), which limits their applicability in packaging systems where pH fluctuates rapidly. This issue may be addressed by developing indicators with a porous structure that provides a larger active surface area and enhances gas diffusion. Such porosity facilitates faster interactions between the indicator and volatile compounds, thus improving the sensitivity and responsiveness to pH variations (Li *et al*., 2019; Zia *et al*., 2021).

To address these challenges, porous indicator systems have been developed to increase the active surface area and enhance gas diffusion, thereby improving the sensitivity and responsiveness (Li *et al*., 2019; Zia *et al*., 2021). The incorporation of reinforcing agents, such as microcrystalline cellulose (MCC) and cellulose-based nanomaterials, including cellulose nanocrystals (CNCs), has been reported to improve the porosity, structural stability, and efficiency of color change in indicators. For example, incorporating MCC into the indicator matrix produced porous composite foams with increased porosity (from 64% to 79.6%) and an accelerated response to pH changes (Zia *et al*., 2021). Similarly, integrating CNCs and cellulose nanofibers (CNFs) into PVA-borax matrices resulted in more responsive indicators for monitoring food degradation (Han *et al*., 2017).

Calotropis gigantea (CG), an abundant local plant rich in cellulose fibers, is a promising renewable source of CNCs, which are biodegradable and biocompatible (Maji *et al*., 2013; Handayani *et al*., 2024a; Handayani *et al*., 2025b). CNCs derived from the young bark of this plant can enhance the active surface area of porous indicators, thereby promoting gas diffusion and accelerating the color change. The combination of PVA and PVP polymers is known to form flexible and homogeneous matrices compatible with natural pigments, such as anthocyanins.

This study investigated the incorporation of cellulose nanocrystals derived from *Calotropis gigantea* (CNCs-CG) as a reinforcing filler in porous PVA/PVP composite indicators immobilized with anthocyanins extracted from *Clitoria ternatea.* The developed colorimetric indicator is designed for application in intelligent packaging systems to enable the non-destructive monitoring of nile tilapia (*Oreochromis niloticus*) freshness. Nile tilapia samples were prepared as fillets, as this form provides a more uniform contact surface between the fish matrix and the indicator and facilitates the diffusion of volatile compounds generated during protein degradation, such as ammonia, dimethylamine, and trimethylamine, which contribute to increases in total volatile basic nitrogen (TVB-N) levels. The performance of the developed indicator was evaluated under different storage conditions, namely, room temperature and refrigerated storage, to simulate common handling and distribution conditions in the seafood supply chain. These conditions represent the early handling stages and cold storage commonly used to slow down microbial activity and enzymatic reactions in fish. The findings of this study are expected to support the development of practical and reliable intelligent packaging systems capable of real-time monitoring of fish freshness.

This study is the first to show that cellulose nanocrystals obtained from CNCs-CG can be used as an effective filler to increase the porosity of PVA/PVP

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composites. Their incorporation produces mesoporous colorimetric films with a much larger surface area, which facilitates the faster diffusion of ammonia and other volatile bases. Furthermore, to the best of our knowledge, this is the first report to develop CNC-CG–reinforced PVA/PVP indicators with improved gas-transport characteristics specifically for the real-time monitoring of fish freshness, leading to higher sensitivity and better overall performance of anthocyanin-based colorimetric indicators. This study aimed to evaluate a porous colorimetric indicator based on a poly (vinyl alcohol)/poly (vinyl pyrrolidone) (PVA/PVP) composite reinforced with cellulose nanocrystals derived from *C. gigantea* (CNCs-CG) and immobilized with butterfly pea (*C. ternatea*) flower anthocyanins, and to evaluate its potential application as an intelligent packaging system for the real-time, non-destructive monitoring of fish freshness.

MATERIALS AND METHODS

Preparation of cellulose nanocrystals (CNCs)

The primary raw material used for producing cellulose nanocrystals (CNCs) was the younger stem bark of *C. gigantea* collected from the coastal area of Banda Aceh, Indonesia. This specific part of the plant was selected based on prior studies that demonstrated that fibers from younger bark possess superior characteristics compared to those derived from mature bark or fruit (Handayani *et al.*, 2024b). The CNCs production process began with a series of pretreatment steps for the cellulose fibers, including water retting (degumming) and pulping, followed by delignification, bleaching, and acid hydrolysis. The procedures for water retting, delignification, and bleaching were adapted from a previously published study by the research team (Handayani *et al.*, 2024a). Acid hydrolysis was carried out by refluxing the dried cellulose fibers in a 40% H_2SO_4 p.a Merck (98% v/v) solution (1:20 w/v) at 80°C for 3 h. The resulting suspension was filtered and neutralized with distilled water. The residue was then dried at 40°C until a constant weight was achieved, followed by milling and sieving through a 325-mesh screen. The final product was designated CNCs-CG. The procedure and physicochemical characteristics of the CNCs used in this study have been comprehensively described in our previous study (Handayani *et al.*, 2025a).

Preparation of Anthocyanins

Anthocyanin extraction was performed according to a previously reported method (Handayani *et al.*, 2024b). In this study, two solvent combinations were employed: analytical-grade ethanol (96% v/v, Merck, Germany) combined with analytical-grade HCl (37% v/v, Merck, Germany) and analytical-grade ethanol (96% v/v, Merck, Germany) combined with distilled water. HCl was added to establish an acidic extraction medium, thereby maintaining anthocyanins in their more stable flavylium cation form and facilitating optimal extraction. The different solvent combinations produced anthocyanin extracts with distinct anthocyanin contents and pH values, which were designated AT-1 [ethanol 96%/HCl 1.5 N (85:15, v/v), pH 1] and AT-2 {ethanol 96%/distilled water (30:70, v/v), pH 6]. Both according to total anthocyanins (Handayani *et al.*, 2024c) which are 551.06 mg/L for AT-1 and 125.24 mg/L for AT-2.

Preparation of Colorimetric Indicator

The colorimetric indicator was prepared using a polymer matrix consisting of polyvinyl alcohol (PVA; molecular weight 70,000; fully hydrolyzed), polyvinylpyrrolidone (PVP; molecular weight 40,000), and crystalline sucrose. The colorimetric indicator was prepared according to a previously reported method (Handayani *et al.*, 2024b), with the only modification being the amount of CNC-CG incorporated as a filler. The indicator consists of two essential components that function synergistically within the system. It was fabricated from a poly (vinyl alcohol)/poly (vinyl pyrrolidone) (PVA/PVP) composite matrix reinforced with different concentrations of CNCs-CG (0.50, 0.75, 1.00, and 1.25 g). The resulting composites were characterized for surface morphology using scanning electron

microscopy (SEM) and specific surface area using the Brunauer–Emmett–Teller (BET) method, respectively. The composite with the highest specific surface area was selected for anthocyanin immobilization. Subsequently, 4 mL of AT-1 or AT-2 was dissolved in 25 mL of distilled water, and the immobilization process was carried out for 24 h, following the procedure described by Handayani *et al.* (2024b). The experimental design is presented in Tables 1 and 2, respectively.

Respons to Ammonia Vapour

A 25% ammonium hydroxide (NH_4OH) solution was used as the ammonia vapor source to assess the pH sensitivity of the colorimetric indicator. The response of each colorimetric indicator to ammonia vapor was evaluated using a static headspace system. Each indicator was placed in a beaker containing 50 mL of 8 mmol/L NH_4OH solution. The beaker was tightly sealed with plastic wrap to retain the ammonia vapor in the headspace. The color changes in the indicators were recorded at three exposure times: 20 min, 40 min, and 4 h. The 8 mmol/L NHOH solution (0.008 mol/L) was prepared by diluting 0.14 mL of 25% concentrated ammonia (NH_3) in a 250 mL volumetric flask and filling it to the calibration mark with distilled water (Handayani *et al.*, 2024b).

Colorimetric Indicator Response

Nile tilapia in a pre-rigor mortis state were used as test specimens. The fish sample was placed in a beaker containing a colorimetric indicator, and the beaker was sealed with plastic wrap. One group of samples was stored at 5°C in a refrigerator and evaluated on day 14, while another group was stored at room temperature and observed after 24 h. The evaluation included visual observation of colorimetric indicator color changes, pH measurement, and determination of total volatile base nitrogen (TVBN) levels (Handayani *et al.*, 2024b).

Surface Morphology Analysis Using SEM

Surface morphology analysis of the composite was conducted to observe the structural features of the surface of the material. Scanning electron microscopy (SEM) was employed to examine the surface details at both the macro and submicron levels, providing a three-dimensional visualization of the sample. SEM is an electron microscope capable of producing high-resolution images

Table 1 Experimental design for composite fabrication

Filler (g)	Type of filler	Matrix	Indexing	Analysis
0.00	Without filler	-	T0	SEM and BET
0.50	CNCs-CG	PVA/P /PVP	T0.5	
0.75			T0.75	
1.00			T1.00	
1.25			T1.25	

Table 2 Design of colorimetric indicator fabrication

Composite		Anthocyanins	Image of colorimetric indicator
Filler CNCs-CG	Matrix		
1.00 g	PVA/PVP	AT-1	
		AT-2	

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of solid surfaces. In this study, the surface morphology was analyzed using SEM (JSM-6360LA, JEOL Ltd., Tokyo, Japan) (Handayani *et al.*, 2025a).

Surface Area and Pore Analysis Using BET

The surface area and pore characteristics of the composite were determined using the Brunauer-Emmett-Teller (BET) method. BET theory was applied after performing measurements using a Surface Area Analyzer (SAA). This instrument analyzes the specific surface area and porosity based on the physical adsorption (physisorption) of inert gases, such as nitrogen (N_2), at cryogenic temperatures (77 K) under a vacuum. Physisorption depends solely on the surface area and pore structure, not the chemical properties of the sample. The surface area is calculated from the number of adsorbed molecules forming a monolayer, whereas the pore size is determined from the condensation (or evaporation) pressure of the gas within the pores. The total surface area of the sample can be estimated by knowing the volume of the adsorbed gas and the theoretical cross-sectional area of a single gas molecule. The surface area refers to the total pore area per unit sample area, whereas the specific surface area is expressed as the surface area per gram of material. Surface area and pore volume analyses were performed using an instrument from Quantachrome Corporation with NOVA Data Analysis Package Ver. 2.00 software (Handayani *et al.*, 2025a).

pH and TVBN Analysis

The pH and total volatile base nitrogen (TVBN) levels were measured to assess the freshness of the fish samples at the beginning and end of the storage period. An increase in TVBN levels is typically accompanied by an increase in pH, indicating a decline in fish quality and consumer acceptability. Elevated TVBN levels indicate protein degradation and spoilage. pH and TVBN measurements (using the Conway microdiffusion method) were performed as described in our previous study (Handayani *et al.*, 2024c). A schematic illustration of the experimental workflow from start to finish is presented in Figure 1.

RESULTS AND DISCUSSION

Characteristics of the Composites as Colorimetric Indicators

Surface microstructure

Figure 2 shows no significant difference in the surface morphology between the composites with and without CNCs-CG filler; both exhibited visible pore formation on the surface. However, further analysis using the BET method (Figure 3 and Table 2) revealed that the addition of the CNCs-CG filler influenced the surface area and pore characteristics of the resulting composite. This finding aligns with previous studies that reported that incorporating cellulose-based fillers leads to the formation of porous composites (Handayani *et al.*, 2024b). Similarly, the use of microcrystalline cellulose as a filler in porous composite fabrication

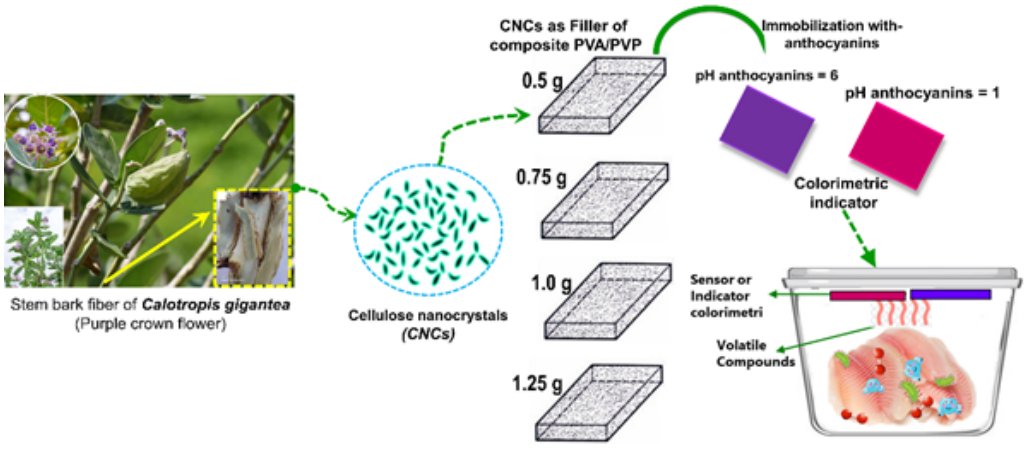

Figure 1 Research illustration

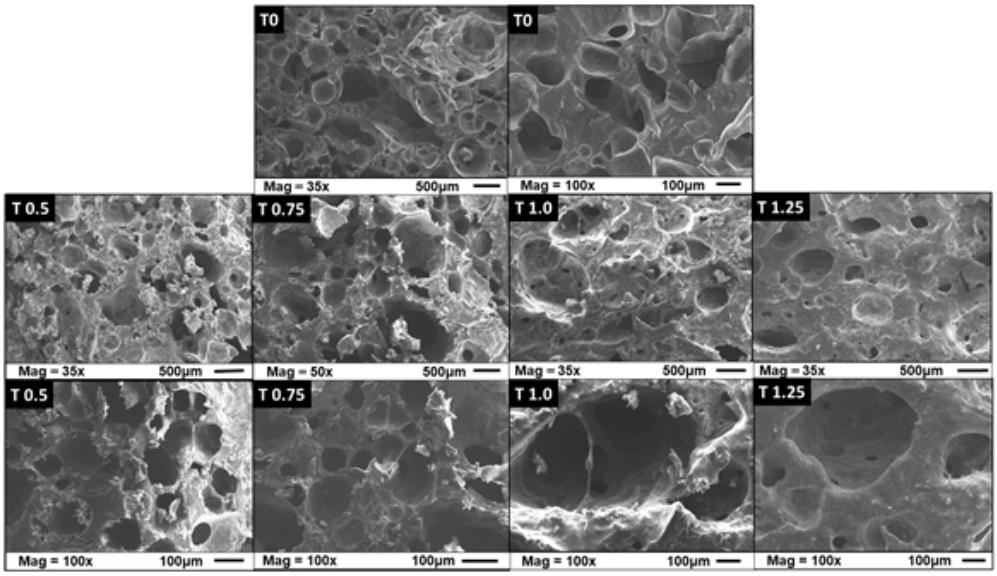

Figure 2 Surface morphology of the colorimetric indicators with CNCs: SEM images of (T0) control, (T0.5) 0.5 g CNCs, (T0.75) 0.75 g CNCs, (T1.0) 1.0 g CNCs, (T1.25) 1.25 g CNCs

achieved optimal porosity with the addition of 3 g of filler (Zia *et al.*, 2021). The CNC-CG fillers interacted with the PVA matrix, aided by the dispersing agent PVP, ensuring a uniform distribution without clumping or agglomeration. These results were further supported by the BET analysis data shown in Figure 3 and Table 2.

Surface area

The surface area and pore characteristics were analyzed using inert N_2 gas adsorption–desorption. Nitrogen gas was selected as the adsorbate because of its high purity and strong interaction with various solids. The data of adsorbed and desorbed gas volumes relative to pressure were processed into isotherm curves. As shown in Figure 3 (right), the resulting graph corresponds to a type-IV isotherm. According to the International Union of Pure and Applied Chemistry (IUPAC) classification, a type IV isotherm indicates the presence of mesoporous structures (1.5–50 nm), consistent with the BET pore diameter values listed in Table 2, which range from 1.6 to 2.0 nm.

The pore size distribution of the prepared composites is shown in Figure 3(A), which illustrates the variation in pore volume as a function of pore width for different

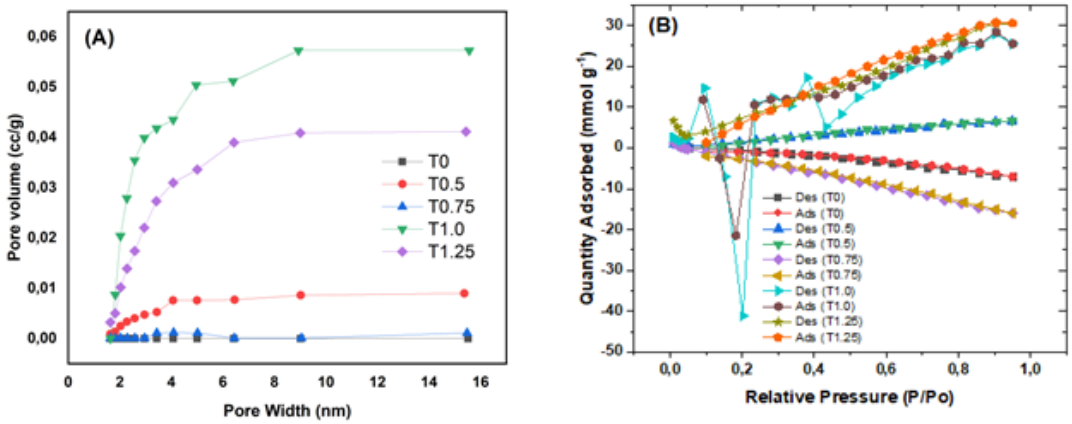

Figure 3 Pore size distribution of the composites (A); N_2 adsorption and desorption isotherms at at 77.3 K (B)

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sample compositions. These results suggest that changes in the material composition significantly affect the development of the pore structure within the composite matrix. In addition, the textural characteristics of the materials were further analyzed using nitrogen adsorption–desorption isotherms at 77.3 K**,** as shown in Figure 3(B)**.**

As shown in Figure 3 (left)**,** mesopores (2–100 nm) dominated all the composites compared to micropores (<2 nm). However, the T1.0 composite exhibited the highest pore number and volume among all the samples. This can be attributed to the addition of 1.0 g CNCs-CG, which represents the optimal amount for the matrix volume used. The filler was well integrated into the PVA/PVP matrix, ensuring compatibility with the CNC-CG content. The addition of 0.2 g PVP and 1.0 g PVA complemented the 1.0 g filler, whereas lower filler amounts resulted in unbound matrix portions, leading to reduced porosity (Jin *et al.*, 2020). Conversely, excessive filler amounts hindered proper dispersion by PVP. In this system, PVA primarily functions as a matrix-forming polymer owing to its strong intermolecular hydrogen bonding capability, which enables the formation of a stable polymeric network that effectively accommodates filler particles. Meanwhile, PVP acts as a dispersing and stabilizing agent, as the carbonyl groups along its polymer backbone can interact with particle surfaces through polar interactions and hydrogen bonding, thereby improving particle stabilization and preventing agglomeration of the particles. Consequently, the ratio of the polymer matrix to the filler plays a critical role in determining the homogeneity and structural integrity of the composite (Shen *et al.*, 2010; Jiang *et al.*, 2020).

PVP acts as a nanoparticle dispersant, stabilizing the surface and preventing agglomeration through repulsive forces generated by hydrophobic carbon (Kyrychenko *et al.*, 2015). PVP is also known to assist in pore formation (Shen *et al.*, 2010; Jiang *et al.*, 2020) and plays a crucial role in dispersing reinforcements (Jin *et al.*, 2020). A previous study using a PVA/PVP matrix reinforced with microcrystalline cellulose fibers showed that the resulting composites had high porosity, smaller pore sizes, greater pore quantity, and improved pore interconnectivity (Zia *et al.*, 2021).

The textural properties of the prepared composites were characterized by nitrogen adsorption–desorption analysis using the Brunauer–Emmett–Teller (BET) and Barrett–Joyner–Halenda (BJH) methods. The BET method was used to determine the specific surface area, while the BJH method was used to calculate the pore volume and pore size distribution. The textural parameters obtained, including the specific surface area, pore volume, average pore diameter, and correlation coefficient (R^2), are presented in Table 4. These results demonstrate how different CNCs-CG–CG loadings modify the pore structure of the composites, which in turn influences gas diffusion, anthocyanin immobilization within the polymer matrix, and the overall performance of the colorimetric indicator.

Table 3 presents the textural properties of the composites obtained from the BET and BJH analyses. The results show that the incorporation of CNCs-CG significantly affected the pore characteristics of the composite. Among all the formulations, T1.0 exhibited the highest BET surface area (162.54 m^2/g) and the largest BJH pore volume (0.06 cm^3/g), indicating the formation of a well-developed porous structure. In contrast, the control sample (T0) exhibited the lowest surface area despite having the largest average pore diameter, suggesting the presence of fewer but larger pores. These observations indicate that variations in CNCs-CG loading influenced the pore architecture of the composites by modifying their surface area, pore volume, and pore size distribution. Overall, the T1.0 formulation exhibited the most favorable textural properties, with the highest porosity among all samples. Based on these characteristics, T1.0 was selected for anthocyanin immobilization and further developed as a colorimetric indicator matrix.

Porous materials are classified based on their pore size. The pore size can be defined as the distance between two boundaries in slit-shaped pores or the cavity distance within

Table 3 Textural properties of the composites obtained from N_2 adsorption–desorption analysis

Properties	T0	T0.5	T0.75	T1.0	T1.25
BJH surface area (m^2/g)	0.00	6.21	0.00	42.88	28.20
BJH pore volume (cm^3/g)	0.00	0.01	0.00	0.06	0.04
BJH average pore diameter (nm)	48.95	2.00	1.28	0.49	23.32
BET surface area (m^2/g)	0.45	10.22	38.66	162.54	40.42
BET pore diameter (nm)	1.63	1.64	1.64	2.03	2.03
Correlative coefficient (R2)	0.91	0.95	0.84	0.87	0.97

*The specific surface area was calculated using the Brunauer–Emmett–Teller (BET) method, while the pore volume and pore size distribution were determined using the Barrett–Joyner–Halenda (BJH) method based on nitrogen adsorption–desorption isotherms.

the composite (Rouquerol *et al*., 1994). This spacing affects the number of pores per unit surface area of the catalyst. A larger cavity distance resulted in a lower surface area, as observed in T0, which had the largest pore diameter (48.95 nm) and thus the lowest surface area. In contrast, T1.0 had the smallest pore size (0.49 nm), resulting in the largest surface area. The pore volume reflects the capacity of the material to absorb adsorbates, such as nitrogen gas. Based on the surface area analyzer (SAA) results, the T1.0 composite exhibited the highest pore volume of 0.06 cm^3/g.

BET analysis helps clarify how the structural properties of a material influence its sensing performance. An increase in the surface area and mesoporous volume improves the accessibility of volatile basic compounds, especially $NH_{3,}$ to the anthocyanin active sites embedded within the composite matrix. In addition, a more interconnected pore structure reduces the diffusion path and supports faster gas transport throughout the material (Thommes *et al*., 2015; Sharma *et al*., 2024). This explains why the T1.0 composite, which exhibited the highest BET surface area (162.54 m^2/g), exhibited a faster interaction between ammonia molecules and pH-sensitive anthocyanins, leading to a shorter response time and a more distinct color change (Sing, 1982; Hamzah *et al*., 2024). In contrast, composites with lower surface areas tend to have fewer available adsorption sites and slower mass transfer, resulting in a delayed color response. Overall, these results indicate that pore architecture plays an important role not only as a structural characteristic but also as a functional factor that directly affects the sensitivity and response kinetics of the indicator sensor.

Response of Colorimetric Indicator to Ammonia Vapor

A preliminary experiment was conducted to examine the response of the colorimetric indicator to alkaline conditions before applying it to fish samples. Ammonia vapor was generated from a 25% ammonium hydroxide (NH_4OH) solution to simulate the alkaline environment produced during protein degradation in spoiled fish. In aqueous solution, NH_4OH exists in equilibrium with dissolved ammonia (NH_3), allowing NH_3 to diffuse into the sealed headspace and interact with the pH-sensitive anthocyanins immobilized in the indicator matrix, resulting in a visible color change (Ip *et al*., 2001; Kim *et al*., 2023).

The indicator was prepared using a PVA/PVP polymer matrix reinforced with 1.0 g of CNCs-CG, selected based on its superior textural properties obtained from BET and BJH analyses. Two anthocyanin extracts were incorporated into the matrix: AT-1, extracted with ethanol/HCl, and AT-2, extracted with an ethanol/water mixture, as described in Tables 1 and 3, respectively. The extraction solvent influences both the concentration and composition of anthocyanins, which

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subsequently affects the color intensity and sensitivity of the indicator to changes in pH (Handayani *et al.*, 2024c). Therefore, the differences in the responses of the two indicators to ammonia vapor were expected to reflect the characteristics of the anthocyanins extracted using each solvent system.

As shown in Figure 4, the AT-2–based indicator demonstrated a faster and more uniform response to ammonia vapor than AT-1. Within 20 min, localized color changes appeared at the edges, and by 40 min, a uniform green tone was visible across the entire surface. In contrast, the AT-1 indicator exhibited only a partial discoloration over the same period.

This difference in performance can be attributed to the initial pH of the anthocyanin extracts. AT-2, with an initial pH near neutrality (~pH 6), required fewer hydroxide ions to reach the alkaline threshold (pH ≥ 8) that triggers a chromatic transition. Conversely, AT-1, with a highly acidic starting pH (~pH 1), required a significantly greater OH^- concentration to initiate the same pH shift.

The observed color change is attributed to the pH-dependent structural transformation of anthocyanin molecules from the flavylium cation under acidic conditions to the quinonoidal or anhydrobase forms under alkaline conditions. As the pH increased following exposure to ammonia vapor, this structural conversion resulted in a distinct color transition from purple to green. A similar response has been reported for colorimetric indicators developed using different anthocyanin sources. Zia *et al.* (2021) observed the same purple-to-green transition in indicators prepared from red cabbage anthocyanins, whereas Handayani *et al.* (2024b) reported comparable results using butterfly pea (*C. ternatea*) anthocyanins. Although the botanical sources differed, both studies demonstrated that anthocyanins exhibit a consistent response to alkaline conditions, confirming their suitability as natural pH-sensitive pigments for colorimetric sensing. In this study, the AT-2–based indicator exhibited a faster and more uniform color change than the AT-1–based indicator, indicating a higher responsiveness to ammonia vapor. This enhanced performance highlights the potential of AT-2 as an active sensing component for smart packaging systems designed for the real-time freshness monitoring of highly perishable foods, such as fish.

Application of Colorimetric Indicators in Smart Packaging

The use of colorimetric indicators in smart packaging is being developed as an innovative solution for monitoring food product quality. This technology offers convenience for both producers and consumers in detecting quality changes, especially in fresh fish products, which are highly perishable in nature. Fish are particularly susceptible to spoilage because of their high water content and nutrient composition, which support the growth of spoilage microorganisms. In addition to microbial activity, autolysis caused by endogenous enzymes (enzymes present in the cells) accelerates tissue degradation after fish die, ultimately reducing their nutritional value and safety.

These results indicate that spoilage

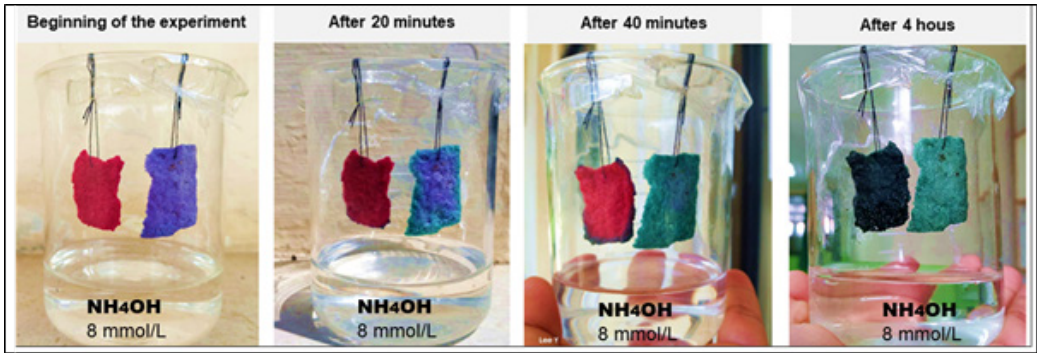

Figure 4 Response of colorimetric indicators to ammonia vapor: red (AT-1); purple (AT-2)

under both room temperature and refrigerated storage conditions can be successfully detected using the developed colorimetric indicator, as indicated by the corresponding changes in TVBN and pH. As illustrated in Figure 5, the AT-2 indicator (purple) exhibited a rapid and distinct color transition to green within 24 h at room temperature (±28°C), reflecting the accumulation of volatile basic nitrogen compounds, primarily ammonia and trimethylamine (TMA), generated by proteolytic enzymes and spoilage bacteria. In contrast, at 5°C, both indicators maintained color stability until day 14, with AT-1 (red) showing minimal change.

Anthocyanin stability remains a key challenge in the development of natural pigment-based indicators, particularly under high-humidity conditions such as refrigerated storage. Moisture condensation within the packaging system can accelerate anthocyanin degradation, as reflected by a decrease in color intensity. This behavior is closely associated with the hydrolysis of the anthocyanin structure, partial hydration of the polymer matrix, and structural transformation of the pigment molecules. Consequently, the indicator color fades, and the sharpness of the color response declines (Castañeda-Ovando *et al*., 2009; Alizadeh-Sani *et al*., 2020; Handayani *et al*., 2024a). This phenomenon became evident in the AT-2 indicator after prolonged storage at 5°C, as indicated by the slight discoloration appearing in the upper region of the indicator. These observations are consistent with those of previous studies on anthocyanin-based intelligent packaging systems, which have shown that relative humidity significantly influences color stability during chilled storage (Prietto *et al*., 2017; Yan *et al*., 2021; Handayani *et al*., 2024c). Therefore, future studies should focus on developing effective moisture control strategies to improve the stability and durability of colorimetric indicators under practical application conditions.

The slower response of AT-1 can be attributed to its strongly acidic initial pH (~pH 1, Table 1), which requires a higher OH^- concentration to initiate chromatic transition. AT-2, with a near-neutral starting pH (~pH 6), is inherently more sensitive to pH increases. The primary driver of pH increase during fish storage is the enzymatic reduction of trimethylamine oxide (TMAO) to TMA, a weak base whose nitrogen lone pair elevates the local surface pH. This process occurs more rapidly at ambient temperatures because of intensified microbial and enzymatic activity.

By day 14 at 5°C, minor discoloration appeared at the top of the AT-2 indicators, likely caused by condensation inside the packaging. This fading is consistent with the lower anthocyanin content (Table 1), which may compromise pigment stability under humid conditions. Anthocyanin stability is influenced by the solvent composition, pigment concentration, and storage environment. Overall, while AT-2 offers higher pH sensitivity and faster spoilage detection, its moisture resistance requires enhancement. The optimization of formulation parameters, particularly anthocyanin concentration, is essential for reliable performance across diverse storage environments.

In addition to sensitivity, the practical

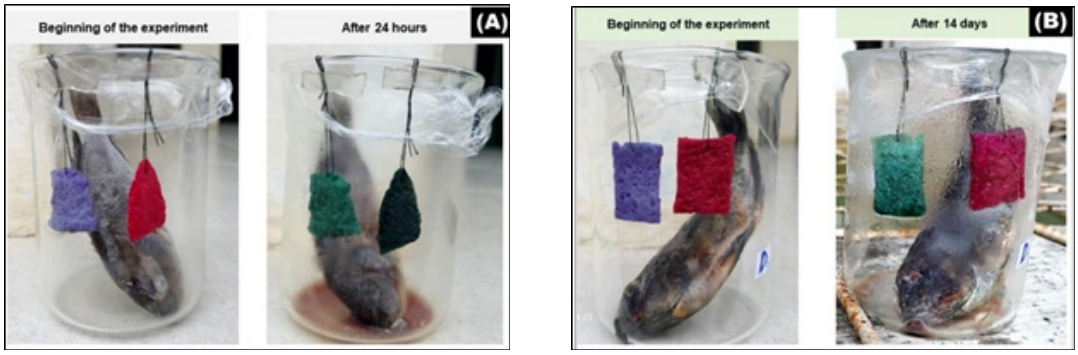

Figure 5 CNC-reinforced colorimetric indicator response to fish freshness under different storage conditions: (A) room temperature; (B) 5°C. Red: AT-1; Purple: AT-2

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use of intelligent indicators is determined by their stability during storage before application (Xue *et al.*, 2024). The shelf life of these indicators is strongly influenced by environmental factors, such as light, oxygen, temperature, and humidity, which can accelerate pigment degradation and reduce their responsiveness (Tang *et al.*, 2023; Remedio, 2024). As the present study focused on proof-of-concept performance during fish freshness monitoring, extended storage tests of unused indicators were not conducted. Further research is required to evaluate the functional shelf life of the indicator under various packaging and storage conditions, particularly in terms of maintaining the initial color, response rate, and color intensity over time.

pH and Total Volatile Base Nitrogen (TVBN)

Changes in pH and TVBN values in Nile tilapia stored under two different temperature conditions showed a strong correlation with fish freshness. Fish stored at room temperature (±25°C) for 24 h exhibited an increase in pH to 8.2, indicating alkaline conditions. This value is above the freshness threshold and reflects spoilage caused by intensive microbial activity and enzymatic autolysis at room temperature. The developed colorimetric indicator successfully detected this condition through a significant color change, as shown in Figure 5.

In contrast, fish stored at 5°C for 14 d maintained a pH of 7.1, which remained within the acceptable range for fresh and consumable fish. The slower quality deterioration under refrigerated conditions can be attributed to the inhibition of spoilage microorganisms and reduced activity of endogenous enzymes responsible for protein degradation and tissue breakdown. Consequently, the formation of alkaline metabolites, such as ammonia and other volatile basic nitrogen compounds, was effectively delayed, thereby maintaining a relatively stable pH throughout the storage period of the fish. This observation demonstrates that low-temperature storage effectively retards biochemical and microbiological spoilage processes, thereby extending fish freshness and shelf life.

TVBN analysis further supported these pH results. TVBN represents the concentration of volatile nitrogenous compounds, including ammonia, trimethylamine (TMA), and dimethylamine (DMA), which are generated during protein degradation by microbial metabolism and endogenous enzymatic activities. After 24 h of storage at room temperature, the TVBN value increased to 27.37 mg/100 g, exceeding the acceptable spoilage threshold. In contrast, fish stored at 5°C for 14 d exhibited a TVBN value of 12.16 mg/100 g, which remained within the fresh category. The initial TVBN value immediately after harvest was only 3.94 mg/100 g, indicating excellent product freshness. Fish are classified as very fresh when TVBN is below 10 mg/100 g, fresh at 10–20 mg/100 g, and spoiled when TVBN exceeds 20 mg/100 g. Similarly, Riyanto *et al.* (2010) reported

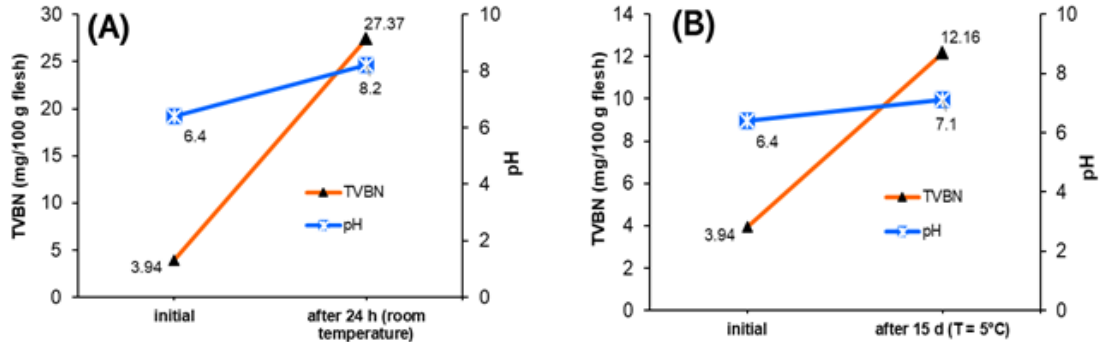

Figure 6 Changes in pH and total volatile base nitrogen (TVBN) in Nile tilapia stored under different conditions: (A) room temperature for 24 h and (B) refrigerated storage at 5 °C for 15 days. The results demonstrated that spoilage under both storage conditions could be detected using the developed colorimetric indicator

that Nile tilapia with TVBN values exceeding 30 mg/100 g had reached the final stage of spoilage (Farber, 1965).

The increase in TVBN was positively correlated with the increase in total bacterial count (TBC) during the storage period. Riyanto *et al.* (2010) demonstrated that the decline in Nile tilapia freshness from 0 to 15 h of storage was accompanied by simultaneous increases in both TVBN and TBC, indicating a close relationship between microbial proliferation and the formation of volatile basic nitrogen compounds. Similarly, Suharto *et al.* (2024) reported that prolonged storage significantly increased the TPC of deboned milkfish, confirming that bacterial growth is strongly influenced by storage duration. As microbial populations increase, bacterial metabolism accelerates the degradation of proteins and other nitrogen-containing compounds, resulting in the formation of volatile basic compounds, such as ammonia, TMA, and DMA, which accumulate as TVBN. Therefore, TVBN is widely recognized as a reliable chemical indicator of microbial growth and fish quality.

In Nile tilapia the increase in TVB-N during storage is primarily driven by microbial growth and endogenous enzymatic activity, which accelerates the degradation of proteins and amino acids, producing volatile alkaline compounds, predominantly ammonia. As these compounds accumulate, the TVB-N content increases, reflecting the progressive deterioration of fish freshness. A similar trend was reported by Anissah *et al.* (2019), who observed a gradual increase in TVB-N during frozen storage due to the natural degradation of fish proteins. Therefore, TVB-N is widely recognized as a reliable indicator of fish freshness, as it reflects both microbial spoilage and protein degradation.

The TVB-N results obtained in this study were in good agreement with the performance of the developed CNCs-CG-reinforced PVA/PVP colorimetric indicator. Fish stored at room temperature reached a TVB-N value of 27.37 mg/100 g and showed a distinct color change, whereas samples stored at 5°C maintained a much lower TVB-N value (12.16 mg/100 g) and exhibited only minor color changes. The close agreement between the indicator response, pH, and TVB-N values demonstrates that the developed intelligent packaging system can reliably monitor fish freshness in real time and non-destructively.

Low temperatures effectively retard the accumulation of total volatile basic nitrogen (TVB-N) by inhibiting the proliferation of spoilage bacteria and autolytic processes induced by endogenous enzymes, thereby minimizing the breakdown of proteins (Lan *et al.*, 2023). Under freezing conditions (<0°C), most of the water in fish tissue crystallizes into ice, leading to a substantial reduction in molecular mobility, as well as microbial and enzymatic activities. However, biological tissues do not freeze uniformly because a fraction of the water remains unfrozen owing to its association with macromolecular surfaces such as proteins and cellular structures.

Studies on the freezing behavior of water in biological tissues indicate that part of the tissue water can remain in an unfrozen state even at subzero temperatures, mainly due to interactions with macromolecular surfaces, confinement within microscopic pores, and solute concentration effects within the biological matrix of the tissue. In fish muscle, this unfrozen water fraction may still account for approximately 9–37% of the total tissue water, even at temperatures between −40°C and −80°C, allowing limited chemical or enzymatic reactions to occur. Therefore, although the fish matrix may contain relatively high levels of non-protein nitrogen, frozen storage generally slows rather than completely halts the formation of volatile basic nitrogen compounds such as TVB-N, resulting in a much slower increase compared to storage at chilled or ambient temperatures (Cameron *et al.*, 2013). Conversely, storage at ambient temperature allows bacterial growth and autolysis to progress without hindrance. The concentration of TVB-N is closely associated with the level of non-protein nitrogen in fish, which is influenced by factors such as diet composition, fishing season, and fish size (Goulas & Kontominas, 2007).

CONCLUSION

A porous PVA/PVP colorimetric indicator reinforced with cellulose nanocrystals from *C. gigantea* (CNCs-CG) and immobilized with butterfly pea flower anthocyanins was successfully developed for intelligent fish packaging. The incorporation of 1.0 g CNC-CG produced an optimal porous structure, enhancing gas diffusion and colorimetric responsiveness. The AT-2 indicator exhibited a faster and more distinct color transition in response to ammonia vapor than AT-1. When applied to Nile tilapia, the indicator effectively differentiated between fresh and spoiled samples under different storage conditions, showing color changes that correlated with pH and TVB-N values. These findings demonstrate that the developed indicator is sensitive and suitable for real-time, non-destructive monitoring of fish freshness, highlighting its potential for intelligent packaging.

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