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DEVELOPMENT AND EVALUATION OF ANTHOCYANIN- BASED FRESHNESS INDICATORS FROM BUTTERFLY PEA FLOWER (*CLITORIA TERNATEA*) FOR REAL-TIME FOOD QUALITY MONITORING

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ABSTRACT

The increasing global emphasis on food safety and waste reduction has driven the demand for real-time, non-invasive methods to detect spoilage. Conventional microbiological and chemical techniques, though accurate, are often time-consuming and unsuitable for continuous monitoring. This study investigates the use of anthocyanins extracted from *Clitoria ternatea* (Butterfly pea flower) as pH-sensitive indicators for intelligent packaging applications. These natural pigments undergo distinct structural and spectral changes in response to pH variations, enabling detection of spoilage-associated metabolites such as ammonia and trimethylamine. The extracted anthocyanins were characterized for their stability, colorimetric responsiveness, and absorbance properties. They were then incorporated into biopolymer-based matrices—agar, gelatin, China grass, and cellulose films—to assess their functionality in real-time spoilage detection. Under simulated seafood spoilage conditions involving *Vibrio*-spiked prawns, the films exhibited progressive, visible colour transitions aligned with microbial growth and pH elevation. These results support the potential of anthocyanin-based matrices as biodegradable, consumer-friendly alternatives to synthetic indicators, offering a promising approach to intelligent food packaging and shelf- life monitoring.

KEYWORDS: Anthocyanins, *Clitoria ternatea*, food spoilage, intelligent packaging, freshness indicator, pH sensor, biodegradable matrix.

1 INTRODUCTION

Food safety is a critical aspect of public health and economic stability, ensuring that food products remain free from contamination and spoilage throughout the supply chain. Globalization and the expansion of food markets have further amplified concerns regarding food quality and shelf life (FAO, 2021). With an increasing consumer demand for fresher, minimally processed, and ready-to-eat foods, food quality assurance faces significant challenges (Nopwinyuwong *et al.*, 2010). These challenges necessitate the development of innovative detection systems that can provide real-time monitoring of food freshness and spoilage.

One of the primary challenges in food safety is the accurate identification of food spoilage. Food deterioration not only affects sensory characteristics such as taste, texture, and appearance but also increases the risk of microbial contamination, which can lead to foodborne illnesses.

Traditional methods of spoilage detection, such as microbiological testing and sensory evaluation, are often time-consuming and expensive. Consequently, there is a growing need for rapid, reliable, and cost-effective detection technologies that provide real-time assessments of food freshness (Genovese *et al.*, 2021).

Additionally, the confusion over date marking—such as "use-by," "sell-by," and "best before"—leads to significant food waste, as consumers often discard food that is still safe to eat (Weston et al., 2020). The lack of scientific validation and standardized global regulations further contributes to this issue, with many labels reflecting peak flavour rather than actual safety. In developed countries, household waste makes up nearly 50% of food loss, with date marking alone responsible for about 20% (Weston et al., 2020). Incorporating real-time freshness indicators alongside conventional labels could offer a more accurate shelf-life assessment and help reduce food waste.

Intelligent packaging, especially interactive indicators, offers innovative ways to monitor food quality by detecting chemical and microbiological changes (Kuswandi, 2017). These include dye-based sensors for temperature, oxygen, and spoilage-related gases like ammonia and CO_2 (Barska & Wyrwa, 2017). Smart labels such as TTIs, CTIs, freshness, and leak indicators, along with RFID systems, help ensure product safety and extend shelf life. Among them, pH-sensitive dyes are key freshness indicators, changing colour in response to microbial activity that alters pH levels during spoilage (Balbinot-Alfaro et al., 2019; Moradi et al., 2019). Traditionally, synthetic dyes like bromocresol green and methyl red have been used, but many pose health and environmental risks (Kobylewski & Jacobson, 2012). Safer, biodegradable alternatives using plant pigments—such as anthocyanins, curcumin, and chlorophyll—are now being developed in biopolymer films (Kumar et al., 2024).

Anthocyanins are pH-sensitive plant pigments responsible for the red, purple, and blue colours in many fruits, vegetables, flowers, and grains. As flavonoids, they help plants attract pollinators, resist stress, and protect against UV damage (Khoo et al., 2017). Their ability to change colour with pH makes them ideal for use in food freshness indicators and smart packaging (Kumar et al., 2024). Rich sources include berries, red cabbage, purple sweet potatoes, black rice, and hibiscus flowers, with key anthocyanidins like cyanidin and delphinidin offering antioxidant and health benefits (Mattioli et al., 2020; Handayani et al., 2024).

Clitoria ternatea (Butterfly pea flower) is a rich, stable source of delphinidin-based anthocyanins with strong pH sensitivity, making it ideal for real-time food freshness indicators (Khoo *et al.*, 2017 +& Handayani *et al*., 2024). These pigments shift colour across a wide pH range, signalling spoilage, especially in protein-rich foods (Handayani *et al.*, 2024). They are also antioxidant and antimicrobial, adding food safety benefits. Easily extracted using acidified ethanol, these anthocyanins can be embedded in biodegradable materials for intelligent packaging (Khoo *et al.*, 2017). This natural, eco-friendly solution offers a promising alternative to static expiry labels, helping reduce food waste and improve consumer safety (Kumar *et al.*, 2024).

2 MATERIAL AND METHOD

Dried butterfly pea flowers were purchased from D.G. Sons Ayurvedic House of Herbs. Ethanol (analytical grade), hydrochloric acid (HCl), sodium hydroxide (NaOH), potassium chloride (KCl), boric acid, acetic acid, phosphoric acid, and glycerol. Food-grade agar, gelatin, and China grass were sourced from local suppliers, Whatman No. 1 filter paper. Nutrient agar media were obtained from HiMedia Laboratories. Fresh prawns were purchased from local market. Pre-sterilized Petri plates and transparent storage containers were used for experimental procedures.

2.1 Extraction & Characterization of Anthocyanin

2.1.1 Extraction of Anthocyanin from Butterfly Pea Flower (BFP)

Anthocyanins were extracted from butterfly pea flowers using a modified method. Dried butterfly pea flowers were destemmed and grinded into fine powder. Approximately 75 gms of this powder was soaked in 280 mL of an ethanol-water mixture (7:3 ratio). The mixture was then subjected to sonicated for 15 minutes in dark conditions at ambient temperature. After sonication, 50 ml distilled water was added to the extract which was then filtered to remove any plant residual material. The clear violet supernatant is separated and stored at 5ºC for 24 hours in the dark (Narayanan *et al.*, 2023).

2.1.2 Confirmatory test for Anthocyanin

The presence of anthocyanins in the extract was confirmed using standard qualitative tests. In the sulfuric acid test, 1 mL of concentrated sulfuric acid (H_2SO_4) was added to 2 mL of the extract, and the formation of a reddish-orange coloration at the interface indicated the presence of anthocyanins. In the sodium hydroxide test, two drops of 1N sodium hydroxide (NaOH) were added to 2 mL of the extract, resulting in a colour change from blue to bluish-green, confirming the presence of anthocyanins (Agunos *et al.*, 2020).

2.1.3 Drying Of BFPE

The crude extract was acidified to pH 2 using 1.5M HCl to enhance anthocyanin stability and prevent degradation. It was then subjected to controlled drying to facilitate the evaporation of residual ethanol, ensuring the removal of excess solvent while preserving the integrity of the anthocyanin compounds. This step was essential for obtaining a concentrated and stable anthocyanin-rich extract. Finally, the pH of the resulting extract is adjusted to 7 using 2.5 mol/L sodium hydroxide suitable for further characterization and application (Narayanan *et al.*, 2023).

2.2 Determination of Total Anthocyanin Content (TAC) in BPE

The total anthocyanin content (TAC) was determined using the pH differential method (Azlim *et al.*, 2022) and (Wijaya *et al.*, 2021).

pH sensitivity of the BPFE

The color response of the anthocyanin extract at different pH values was evaluated with modifications to assess its pH sensitivity. The extract was exposed to buffer solutions ranging from pH 1 to 14, and the resulting color variations were observed. For pH 1 to 3, a hydrochloric acid (HCl) and potassium chloride (KCl) buffer was used, while acetate buffer was used for pH 4 to 6. pH 7 was maintained as neutral without any buffer. Borate buffer was used for pH 8 to 10, and sodium hydroxide (NaOH) solutions were used for pH 11 to 13. This analysis helped determine the stability and responsiveness of the anthocyanin extract across a wide pH range. The color change was photographed. The color intensity was expressed as L* value showing lightness, a* value representing red to green, and b* value yellow- blue to estimate the colour difference in all the buffer solutions (Narayanan *et al*., 2023). ΔE was calculated from an equation as shown below.

$$\Delta E = \sqrt{\ (Ls - L)^2 + (as - a)^2 + (bs - b)^2}$$

The values from the white screen used as a back plate were: Ls (98.87), as (−2.54), and bs (3.95) (Khezerlou *et al*., 2023).

2.3.1 Spectral Analysis of Anthocyanin across various pH

The peaks for BPE for pH 1-13 in the range of 400 to 700 nm were recorded using a colorimeter spectrophotometer. 5ml of diluted BPE was dissolved in 25 mL of buffer solutions (pH 1–13) for 5 minutes and were scanned on colorimeter (Narayanan *et al*., 2023).

2.3.2 Liquid Chromatography Mass Spectrometry (LCMS) analysis of the extract

LC-MS analysis was performed using a High-Resolution Liquid Chromatography–Mass Spectrometry Quadrupole Time-of-Flight (HRLC-MS QTOF) system (Agilent Technologies, USA).

Data processing and compound identification were carried out using Agilent MassHunter Qualitative Analysis B.0 software.

2.4 Development of Anthocyanin incorporated Matrix & Its Evaluation

2.4.1 Anthocyanin Incorporated Matrix Preparation

For the agar-gelatin (AGel) composite matrix, a modified protocol based on (Azlim *et al*. 2021) was followed. A blend of 4.5% gelatin and 2% agar was dissolved in a 30% glycerol solution under continuous stirring and gentle heating until fully homogenized. The solution was then cooled slightly, and 1 mL of 1:10 diluted BFPE was incorporated with thorough mixing. The total volume was adjusted to 10 mL, poured into a sterile Petri plate, and allowed to solidify at room temperature, forming a flexible and structurally stable hydrogel.

For the China grass (CG) matrix, 2 g of China grass was soaked in 70 mL of 30% glycerol solution for 1 hour to allow complete hydration and dissolution. The mixture was then subjected to controlled heating with continuous stirring until fully digested and homogenized. After digestion, it was allowed to cool slightly to prevent the degradation of anthocyanins. Once the temperature was appropriate, 1 mL of 1:10 diluted butterfly pea flower extract (BFPE) was incorporated and mixed thoroughly for uniform distribution. The prepared solution was then cast into a sterile Petri plate and left undisturbed at room temperature to solidify, forming a stable and flexible hydrogel matrix for pH-responsive applications.

The paper-based indicator labels were prepared by incorporating the dye extract into a cellulose matrix. Whatman No. 1 filter paper (Cat. No. 1001) was used as the base material and cut into 1.5 cm × 1.5 cm squares. These paper labels were then immersed in butterfly pea flower extract (BFPE) for 30 seconds, allowing the dye to be absorbed. After immersion, the labels were air-dried at room temperature until fully dry and ready for use (Listyarini *et al.*, 2018).

2.4.2 pH Sensitivity of Anthocyanin incorporated Matrix

To evaluate the pH responsiveness of the developed matrices, uniform blocks were carefully cut from each type—4% Agar, 2.86% China grass, 2% Agar + 4.5% Gelatin (all plasticized with 30% glycerol), and paper-based labels. These matrix samples were immersed in a series of pH solutions ranging from 1 to 14, ensuring a comprehensive assessment of their colorimetric response (Narayanan *et al.*, 2023).

2.4.3 Dimensions and Physical Properties of Anthocyanin incorporated Matrix

2.4.3.1 Thickness of Matrix

The thickness of each matrix was measured at five random points using a micrometer screw gauge to ensure accuracy and uniformity. The average thickness was calculated to account for any variations across the samples, providing a reliable assessment of the matrix structure (Khezerlou *et al.*, 2023).

2.4.3.2 Moisture content of Matrix

The moisture content of the films was determined following the protocol described by (Azlim *et al.*, 2022).

2.4.3.3 Swelling Index (SI) of Matrix-The swelling index of the films was determined gravimetrically following the method described by (Taherkhani *et al.*, 2020).

2.5 Stability of Matrix

The pH-responsive colorimetric ability of the matrices was evaluated after 30 days of storage at 4°C following a modified protocol of (Shukla *et al.*, 2016). The stored matrices were immersed in pH 2, pH 7, and pH 10 buffer solutions, and the resulting color changes were visually observed to assess the stability of anthocyanin incorporation. The ability of the matrices to retain their pH-dependent color transitions after prolonged storage was used to determine their suitability for long-term application in food freshness monitoring.

2.6 Testing in simulatory conditions of food spoilage

The sensitivity of the matrices to volatile ammonia was assessed by exposing the films to ammonia vapors in a controlled environment. The matrices were securely fixed in the headspace of an airtight container containing a source of ammonia to ensure uniform vapor exposure without direct contact with liquid ammonia. The films were monitored for 50 minutes, with optical changes recorded at 10-minute intervals to evaluate the progression of colorimetric response over time. This method allowed for the assessment of the matrices' ability to detect pH shifts induced by ammonia, which is relevant for food spoilage detection applications (Hu *et al.*, 2020).

2.7 Testing On Real Food Products (Prawns)

2.7.1 Initial density of *Vibrio* culture for spiking of prawns sample

The optical density (OD) of the *Vibrio* culture was adjusted to 0.1 at 600 nm to standardize the bacterial concentration. Serial dilutions were performed to determine the viable cell count. The culture density was then further adjusted to 100 CFU/mL by performing appropriate serial dilutions in sterile saline. The cell count for spiking was adjusted in accordance with the guidelines established by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) Executive Secretariat (2009) to ensure appropriate microbial load for spoilage evaluation. This standardized bacterial suspension was used for controlled spiking of prawn samples to evaluate spoilage detection using the developed pH-responsive matrices.

2.7.2 Evaluation of Prawn Spoilage Using pH-Sensitive Anthocyanin-Based Indicator

The prawns, after being purchased, were cleaned, deveined, and directly transported to the laboratory for immediate preparation. For test preparation, shrimp samples weighing 10 g each were treated with vinegar to minimize microbial load and then washed with sterile distilled water to remove any traces of vinegar (De Rezende *et al.*, 2024). The prepared shrimp samples were placed in pre-sterilized transparent containers. The anthocyanin-incorporated matrix was positioned on the lid of each container, ensuring that it did not come into direct contact with the shrimp. The distance between the matrix and the shrimp was maintained at approximately 5 cm. The containers were then tightly sealed.

Separate containers were maintained for each type of matrix, along with their respective control samples, which were not spiked with Vibrio, to compare natural spoilage progression. Similar samples were prepared under identical conditions, with storage tests conducted at both room temperature and freezing conditions. The microbial load, pH changes, and texture variations of the shrimp were evaluated at storage intervals of 0 h, 24 h, 48 h, and 72 h under each temperature condition. The colorimetric response of the matrix was recorded alongside these parameters to assess its effectiveness in real-time spoilage detection (Listyarini *et al.*, 2018).

3 RESULTS

3.1.1 Extraction of Anthocyanin

The anthocyanins from *Clitoria ternatea* were extracted using a **solid-liquid extraction** method with **70% ethanol (v/v)** as the solvent. Ultrasonication was applied to enhance cell wall disruption and maximize anthocyanin yield. The extract was then filtered to remove plant residues, obtaining a purified anthocyanin-rich solution. To prevent degradation, the process was conducted under controlled temperature conditions, with minimal exposure to light and oxygen to preserve pigment stability and prevent oxidation.

3.1.2 Confirmatory Test

The presence of anthocyanins in the extract was confirmed using **acid-base analysis**. Confirmatory Test for Anthocyanins

Test	**Result**	**Interpretation**
1ml Conc. H_2SO_4 + 2ml crude extract	Reddish orange ring at the interface	Anthocyanin Present
2 drops of NaOH + 2ml crude extract	Greenish colour	Anthocyanin Present

3.1.3 Drying Of BFPE

The **crude extract** was **acidified to pH 2** using **1.5M HCl** to enhance anthocyanin stability and prevent degradation.

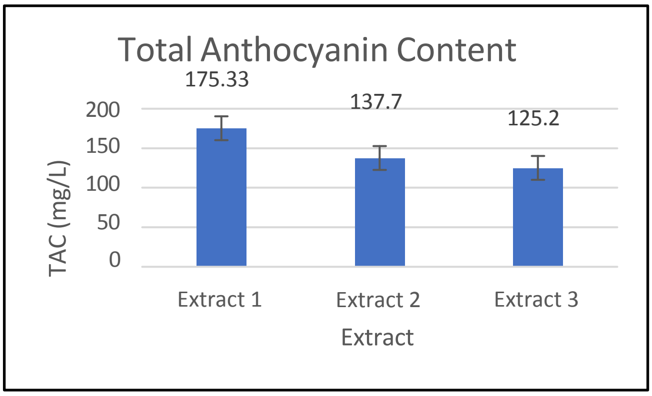
3.2.1 Determination of Total Anthocyanin Content (TAC) in BPE (pH Differential method) The total anthocyanin content (TAC) was determined using the pH differential method

Absorbance of BPFE of pH 1 and 4.5 at 510 nm and 700 nm

Extract	pH	Absorbance at 510 nm	Absorbance at 700 nm
Extract 1	1	0.753	0.023
	4.5	0.623	0.033
Extract 2	1	1.12	0.1
	4.5	1.04	0.13
Extract 3	1	1.11	0.09
	4.5	1.04	0.13

Total Anthocyanin Content (TAC) of different BPFE

Sample	TAC (mg/L)
Extract 1	175.33
Extract 2	137.7
Extract 3	125.2

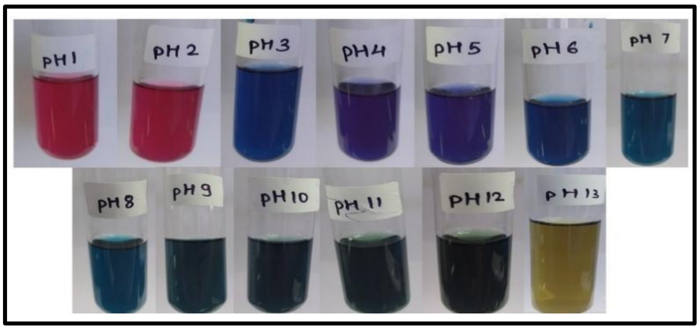


TAC of different BPFE

Extract with highest TAC ie. **Extract 1** was proceeded for further analysis and in matrix development.

3.2.2 pH sensitivity of the BPFE

The Butterfly Pea Flower Extract (BPFE) with the highest anthocyanin yield was subjected to buffer solutions ranging from pH 1 to pH 13 to evaluate its colourimetric response.

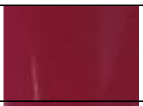
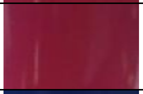
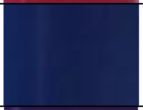
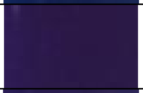
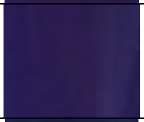
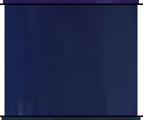
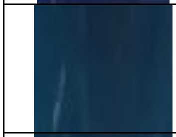
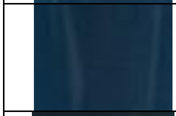
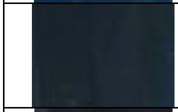
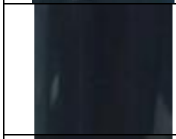
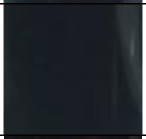
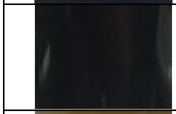
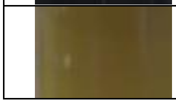


Colour change of BPFE across different pH.

3.2.3 Determination of L* a* b* values of the extract at various pH

The colour properties were analyzed using CIELAB colour space. The colour intensity was expressed as L* value showing lightness, a* value representing red to green, and b* value yellow- blue to estimate the colour difference in all the buffer solutions (Narayanan *et al.*, 2023).

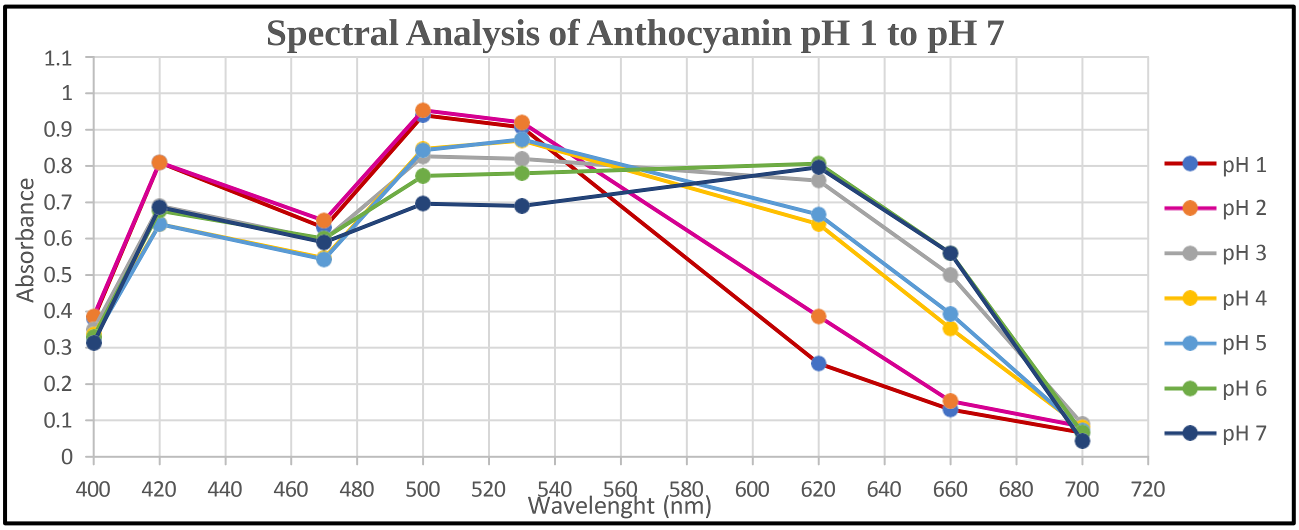
Colour properties of BPFE at different pH ranging from pH 1 to pH 13

Extract	pH	L*	a*	b*	ΔE
	1	28	43	15	84.96
	2	30	41	10	81.7
	3	33	1	-27	72.86
	4	19	-6	-14	81.94
	5	41	8	-29	67.42
	6	47	-10	-28	61.38
	7	50	-16	-17	54.85
	8	52	-18	-14	52.52
	9	33	-7	-2	66.29
	10	54	-6	-2	45.39
	11	42	-11	4	57.5
	12	46	-10	8	53.55
	13	59	-5	22	43.83

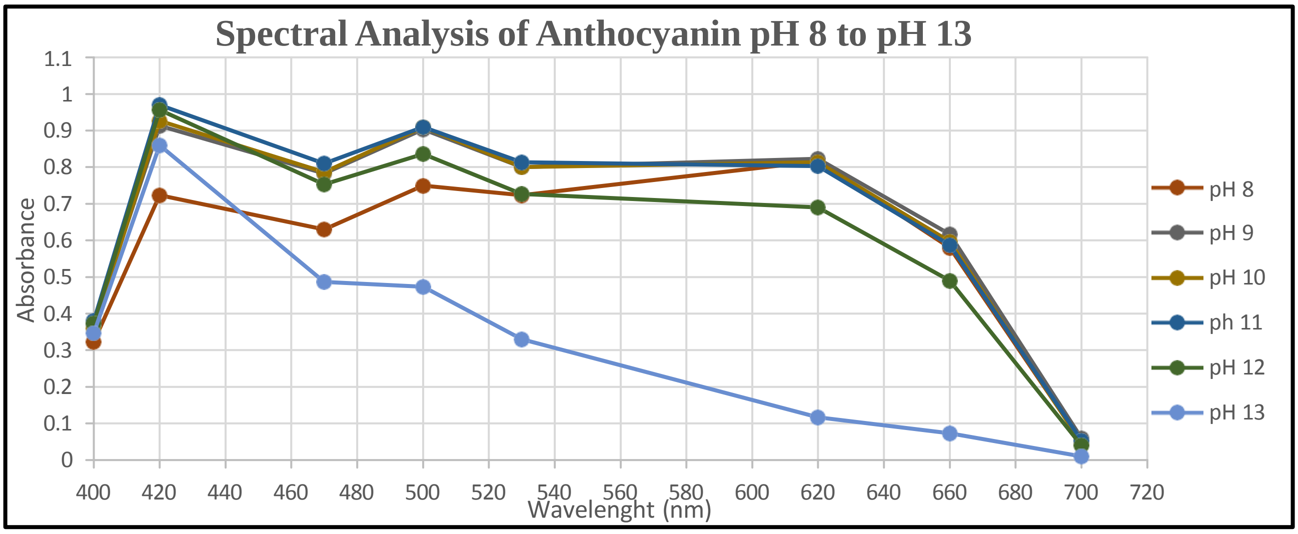
A one-way ANOVA showed a significant difference ($p < 0.05$) in colour across pH levels, confirming its potential as a natural pH indicator.

3.2.4 Spectral Analysis of Anthocyanin across various pH

The absorbance spectrum of the extract was recorded across different pH levels at varying wavelengths to determine the λmax (maximum absorbance wavelength) corresponding to each pH.



Spectral analysis of anthocyanin pH 1 to pH 7



Spectral analysis of anthocyanin pH 8 to pH 13 λmax of extract at different pH

pH	1	2	3	4	5	6	7	8	9	10	11	12	13
λmax	500	500	500	530	530	620	620	620	420	420	420	420	420

To assess the statistical significance of colour variation, two-way ANOVA was performed, considering pH and wavelength as independent factors. A p-value < 0.05 confirmed a significant difference in colour expression across different pH levels and wavelengths, highlighting the pH-dependent spectral shifts of anthocyanins.

3.2.5 LCMS analysis of the extract

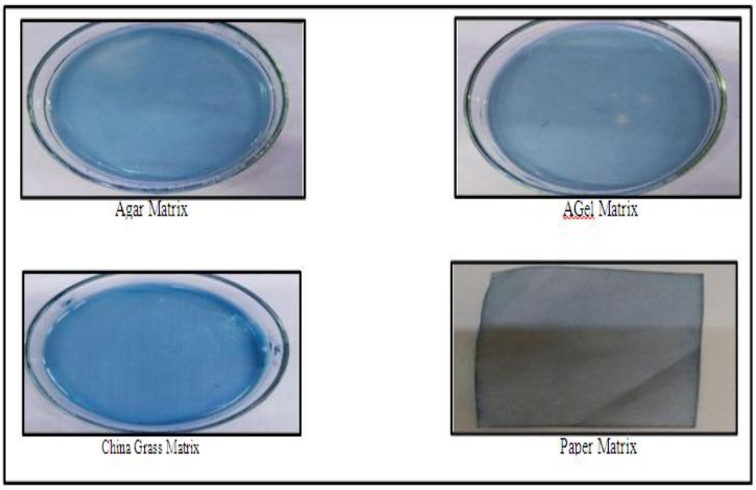
The LC-MS/QTOF analysis of the extract in positive ionization mode revealed the presence of various flavonoid glycosides and anthocyanin-related compounds. Key compounds identified include **rutin**, **trifolin**, **kaempferol**, **Quercetin-3-β-D-glucoside**, and high molecular weight glycosides such as **$C_{27}H_{30}O_{15}$** and **$C_{33}H_{40}O_{19}$**, which are consistent with **Ternatin-like anthocyanins** commonly reported in *Clitoria ternatea*.

Sr. No.	Compound Name	Molecular Formula	RT (min)	m/z
1	Rutin	$C_{27}H_{30}O_{16}$	8.84, 9.13, 9.24	611.1612
2	Trifolin	$C_{21}H_{20}O_{11}$	9.19, 9.81	449.1033
3	Quercetin-3-β-D-glucoside	$C_{21}H_{20}O_{12}$	8.84, 9.12, 9.45	465.0982
4	Kaempferol	$C_{15}H_{10}O_{6}$	9.14, 9.41, 9.66, 9.81	287.0550
5	Kaempferol-3-Galactoside-Rhamnoside	$C_{33}H_{40}O_{19}$	8.86, 9.10, 9.41	741.2192
6	Ternatin-like glycoside (tentative ID)	$C_{27}H_{30}O_{15}$	9.18, 9.41	595.1506

3.3 Development of Anthocyanin incorporated Matrix & Its Evaluation

3.3.1 Development of different matrix

Four matrices were developed to test anthocyanin-based pH-responsive color change: 4% agar,



2.86% China grass (CG), 2% agar + 4.5% gelatin (AGel), all with 30% glycerol, and a paper-based matrix for stable, porous anthocyanin immobilization.

Matrix incorporated with BFPE

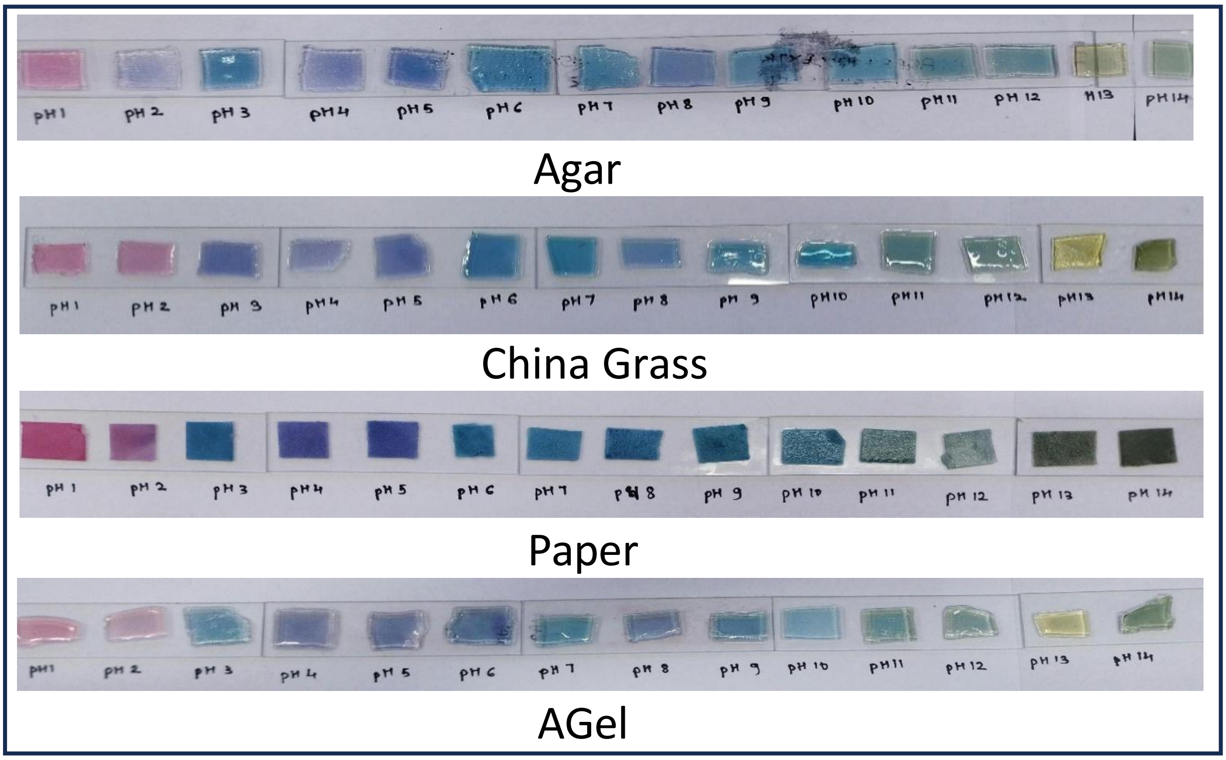
Colour parameters including L*, a*, b* and ΔE of the anthocyanin incorporated matrix.

Matrix	**L***	**a***	**b***	**ΔE**
Agar	51.67	-7	-24	55.1
AGel	51	-5	-17.67	52.6
CG	51	-7	-35	60.1
Paper	50.33	-7	-29.33	59.1

To assess the statistical significance of colour variation, a one-way ANOVA was performed on the ΔE values of different matrices. The analysis yielded a p-value of 0.003281, which is less than 0.05, confirming a significant difference in colour variation among at least one of the matrices. This indicates that the matrices exhibit distinct colour properties, validating the influence of their composition on colour expression.

3.3.2 pH Sensitivity of Anthocyanin incorporated Matrix

The pH sensitivity of the developed matrices was evaluated by incorporating anthocyanin extract and observing the colourimetric response across a range of pH conditions.



Colour change in the matrix when exposed to different pH buffers.

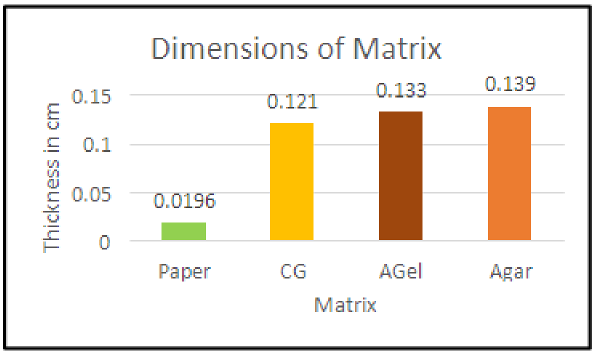
3.4 Dimensions and Physical Properties of Anthocyanin incorporated Matrix

3.4.1 Thickness of Matrix

The thickness of the developed films was measured using a micrometer screw gauge to ensure precision and uniformity across different matrix formulations.

Thickness of the matrix.

Matrix	Point 1 (cm)	Point 2 (cm)	Point 3 (cm)	Point 4 (cm)	Point 5 (cm)	Average (cm)
Paper	0.019	0.02	0.019	0.02	0.02	0.0196 cm
CG	0.122	0.122	0.12	0.12	0.122	0.121 cm
AGel	0.135	0.134	0.134	0.134	0.134	0.133 cm
Agar	0.14	0.139	0.139	0.14	0.139	0.139cm



Thickness of the film

To evaluate the statistical significance of thickness variation among matrices, a one-way ANOVA was conducted. The analysis resulted in a p-value of **2.01E-15**, which is significantly lower than **0.05**, indicating a statistically significant difference in thickness across at least one of the matrices. This confirms that the thickness of the matrices varies significantly.

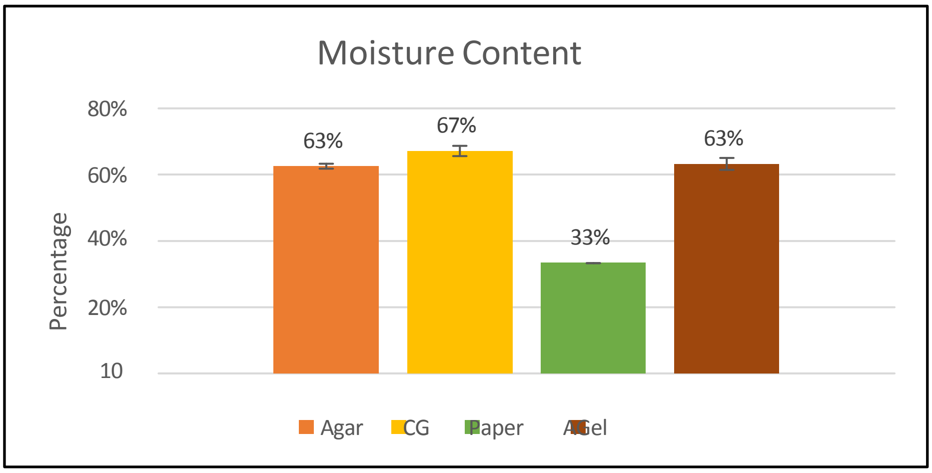
3.4.2 Moisture content of Matrix

The moisture content was determined by measuring the weight of the film before and after drying at 105^{0}C for 24 Hrs.

Moisture Content of Matrix.

Matrix	Weight before drying (W_1) (gms)	Weight after drying (W_2) (gms)	Moisture Content $((W_1-W_2)/W_1)\times100$ (%)
Agar	0.67	0.25	63% ±1
CG	0.44	0.14	67% ±2
Paper	0.06	0.04	33% ±0
AGel	0.59	0.21	63% ±2

Here the values are expressed as Mean±Standard deviation (SD)



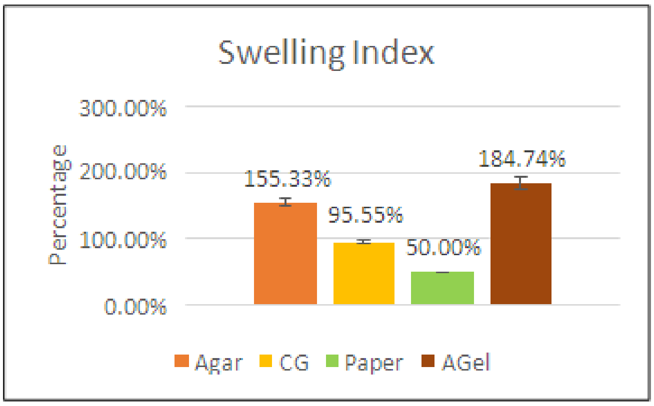
A one-way ANOVA was conducted to analyze moisture content variation among different matrices. The p-value (**2.4E-09**) is significantly lower than **0.05**, indicating a statistically significant difference in moisture content across at least one of the matrices, rejecting the null hypothesis.

3.4.3 Swelling Index (SI) of Matrix

The swelling index was calculated as the percentage weight gain after 24-hour water immersion, indicating the film's water absorption, stability, and suitability for aqueous use. Swelling Index (SI) of Matrix.

Matrix	**Weight before immersion (W1) (gms)**	**Weight after immersion (W2) (gms)**	**Swelling Index (SI) $(W2-W1)/W1\times 100$ (%)**
Agar	0.25	0.64	155.33%±0.05
CG	0.14	0.28	95.55%±0.038
Paper	0.04	0.06	50.00%±0
AGel	0.21	0.61	184.74%±0.09

Here the values are expressed as Mean±Standard deviation (SD)

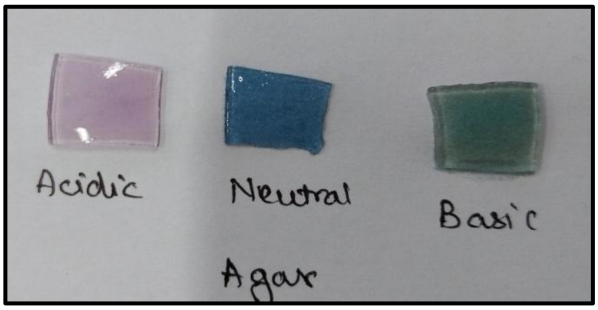


Swelling Index (SI)

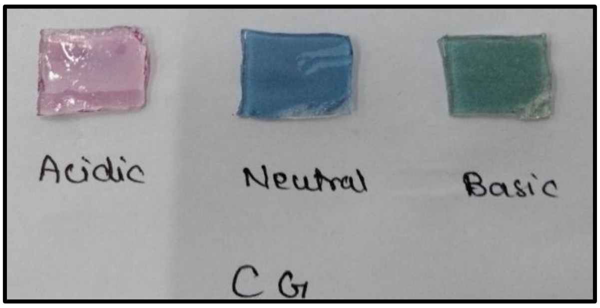
A one-way ANOVA was performed to evaluate differences in swelling index among matrices. The p-value (**8.81E-09**) is significantly below **0.05**, confirming a statistically significant difference in swelling index across at least one matrix, leading to the rejection of the null hypothesis.

3.5 Stability of Matrix

The stability of the matrices and their pH-responsive colorimetric ability were evaluated after 30 days of storage at 4°C. Each matrix was examined for structural integrity, colour retention, and responsiveness to pH changes following the storage period.

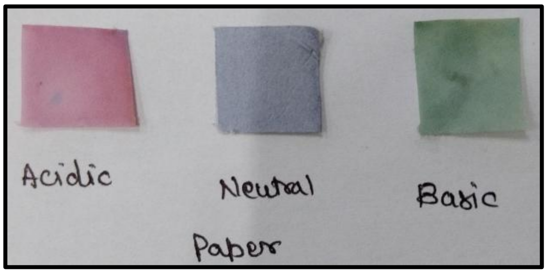


Colour change in the Agar matrix at different pH levels after 30 days of storage



Colour change in the China Grass matrix at different pH levels after 30 days of storage

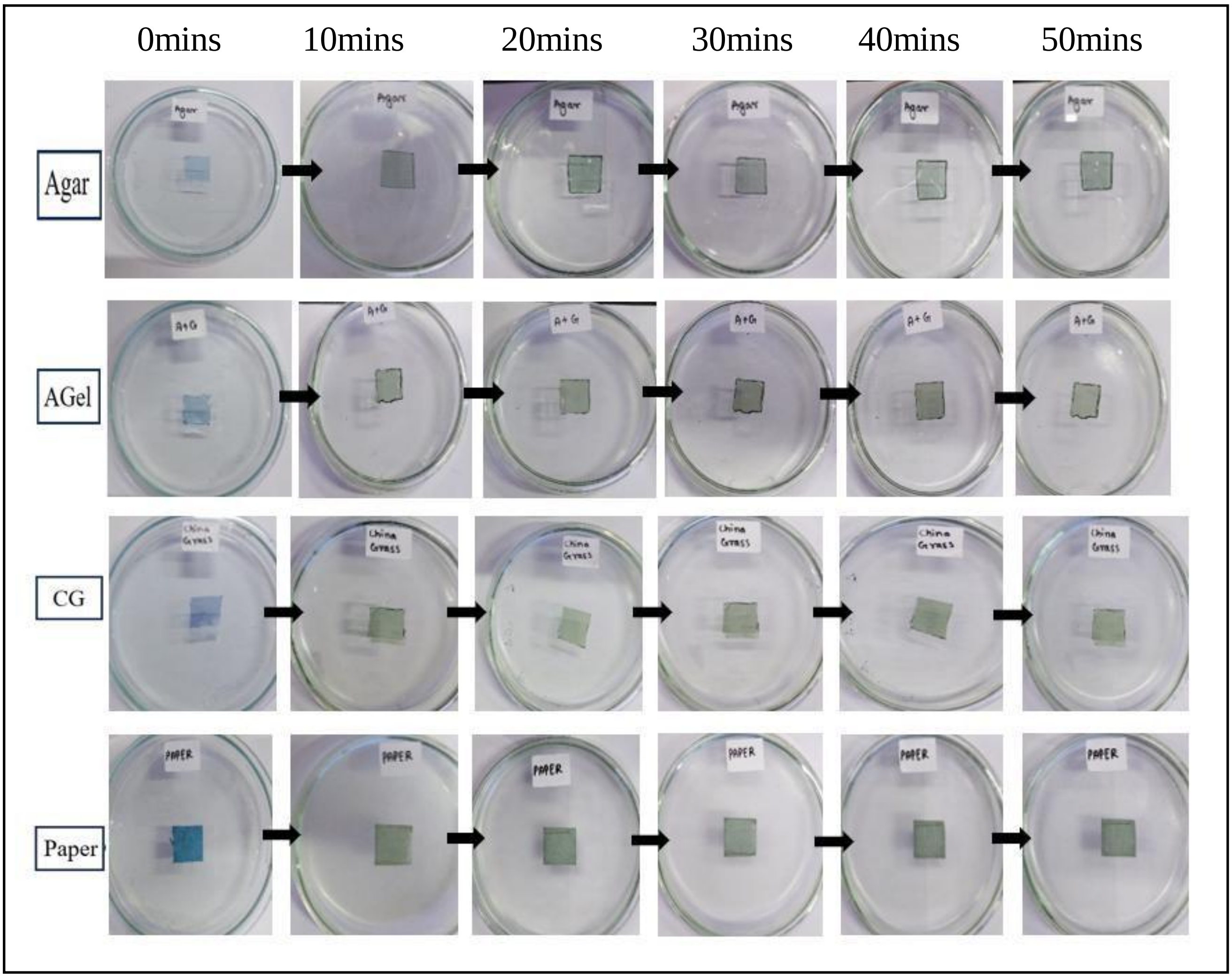
Colour change in the AGel matrix at different pH levels after 30 days of storage



Colour change in the Paper matrix at different pH levels after 30 days of storage

3.6 Testing in stimulatory conditions of food spoilage

The sensitivity of the matrices to volatile ammonia was evaluated by exposing the films to ammonia vapours for 50 minutes. Optical changes were recorded at 10-minute intervals to monitor the colourimetric response over time.

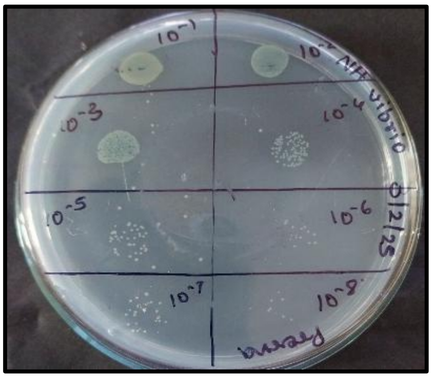


Colour change in the matrix after exposure to Ammonia vapour.

3.7 Testing On Real Food Products (Prawns)

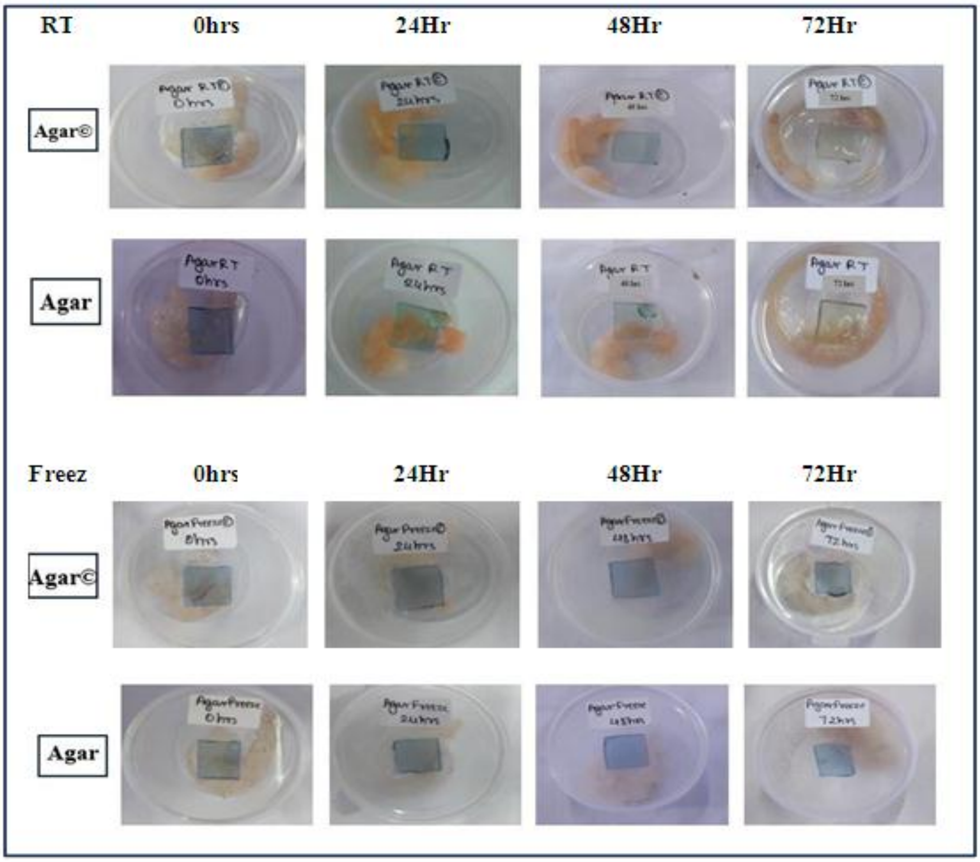
Initial density of *Vibrio* culture for spiking of prawns sample

The optical density (OD) of the *Vibrio* culture was adjusted to 0.1 at 600 nm to standardize the bacterial concentration. Serial dilutions were performed to determine the viable cell count, which was calculated as 3.925 × 10^{10} CFU/mL. This standardized culture was then appropriately diluted and used to spike the prawn samples.



Viable count of *Vibrio* using Miles & Misra Method

4.2 Evaluation of Prawn Spoilage Using pH-Sensitive Anthocyanin-Based Indicator

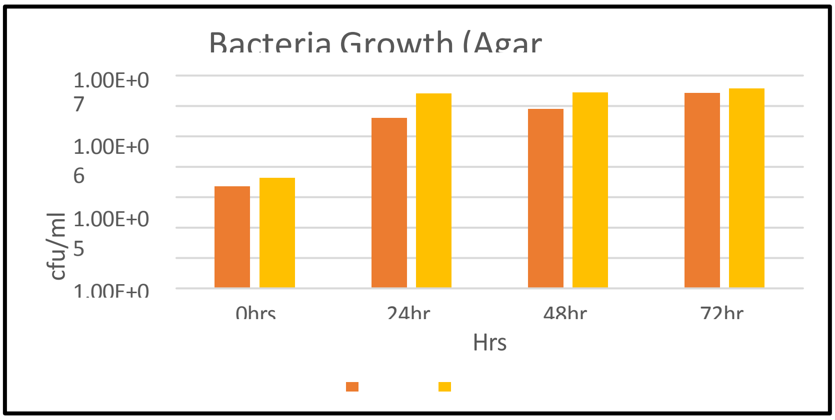


Anthocyanin incorporated Agar Matrix Response to Prawn Spoilage

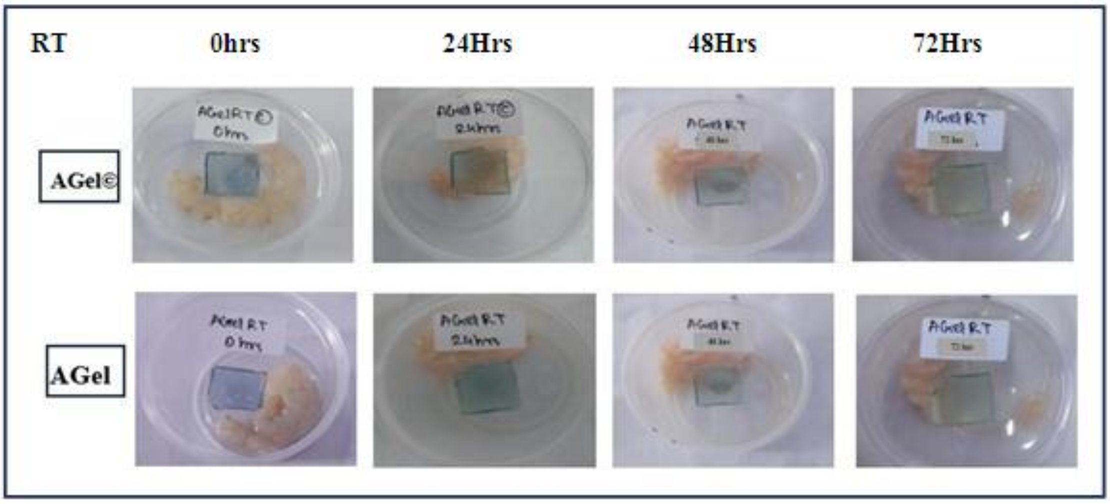
Bacterial load (cfu/ml) in prawns exposed to the agar matrix.

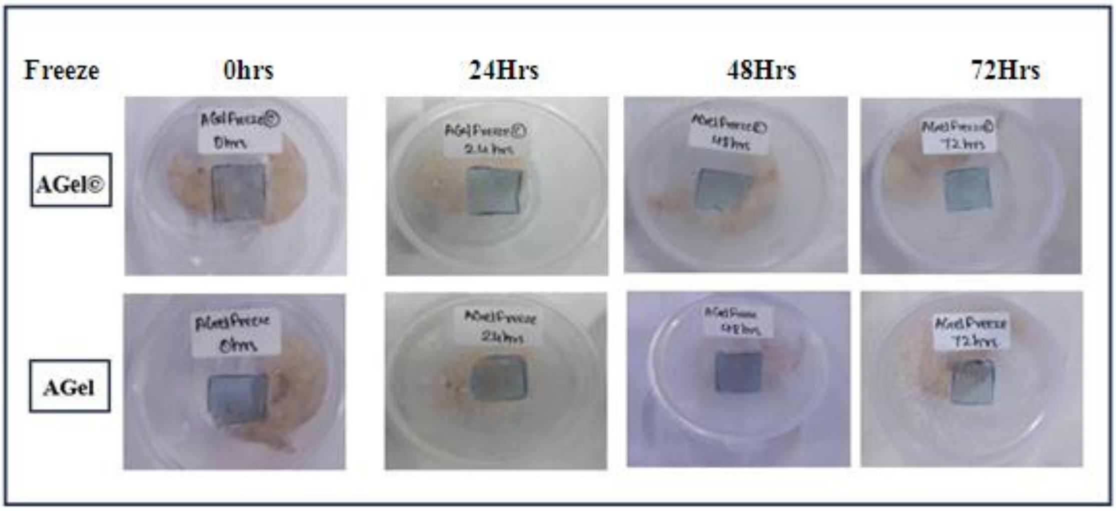
Matrix	**0hrs**	**24hrs**	**48hrs**	**72hrs**
Agar Control	2.30×10^3	4.00×10^5	8.00×10^5	2.65×10^6
Agar	4.30×10^3	2.60×10^6	2.80×10^6	3.75×10^6

Key: Control=Unspiked



Bacterial load (cfu/ml) in prawns exposed to the agar matrix



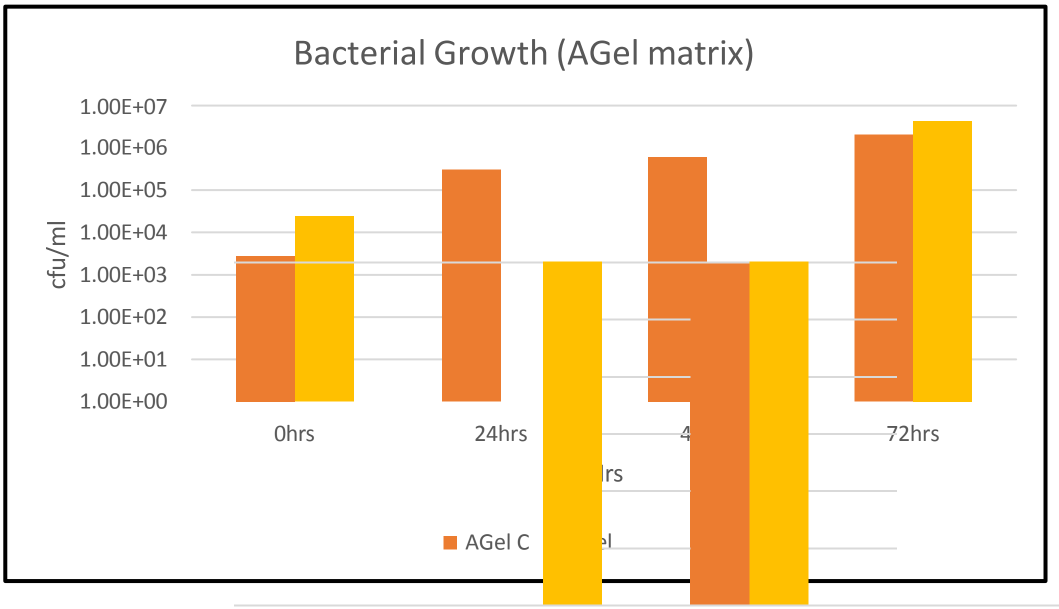


Anthocyanin incorporated AGel (Agar+ Gelatin) Matrix Response to Prawn Spoilage

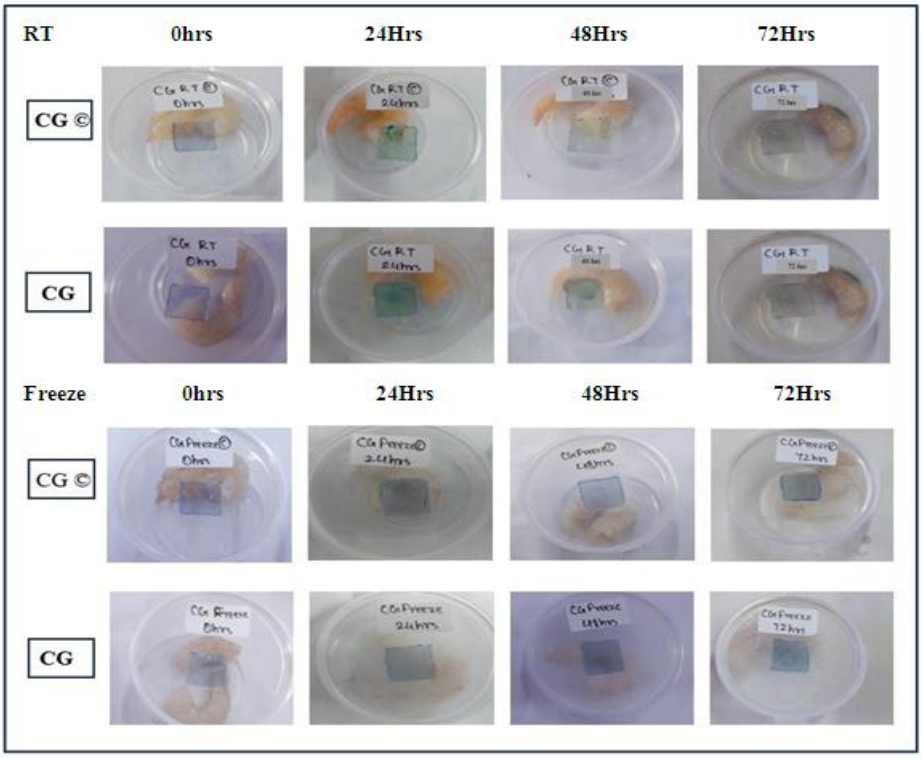
Bacterial load (cfu/ml) in prawns exposed to the AGel matrix

Matrix	**0hrs**	**24hrs**	**48hrs**	**72hrs**
AGel Control	$2.80x10^3$	$3.00x10^5$	$6.00\ x10^5$	$2.05\ x10^6$
AGel	$2.40x10^4$	$1.20x10^6$	$1.20x10^6$	$4.35\ x10^6$

Key: Control=Unspiked



Bacterial load (cfu/ml) in prawns exposed to the AGel matrix

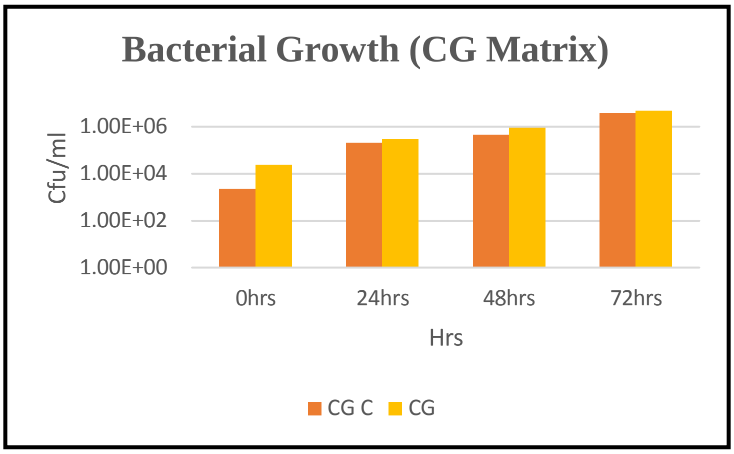


Anthocyanin incorporated CG (China Grass) Matrix Response to Prawn Spoilage.

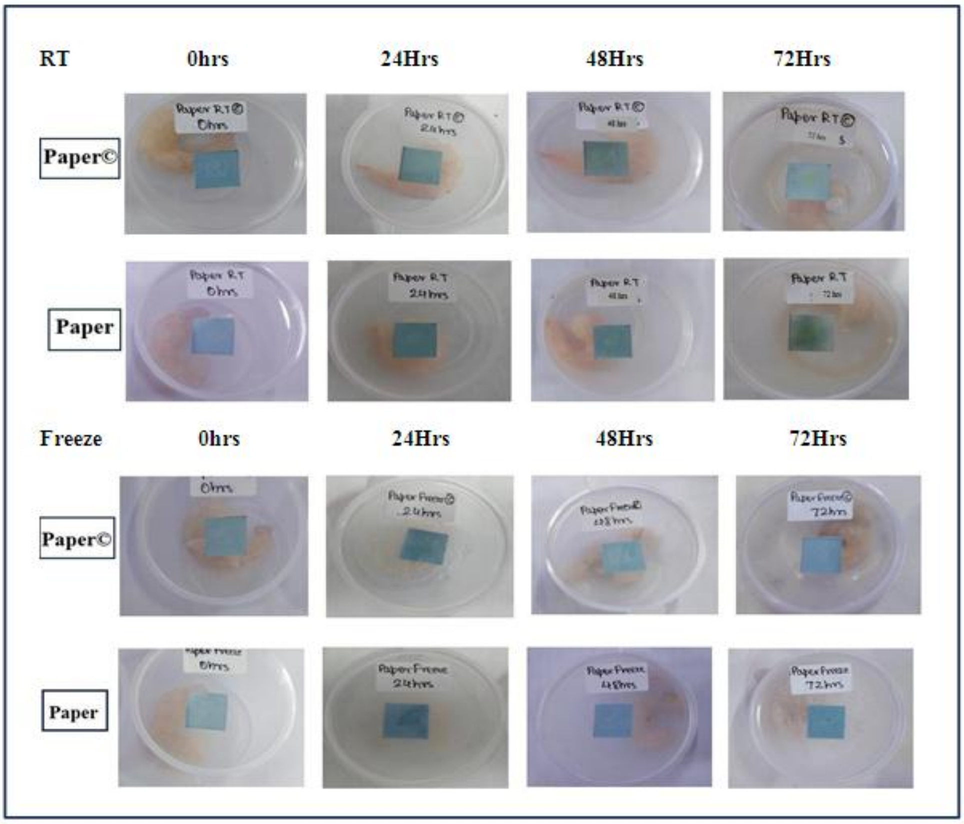
Bacterial load (cfu/ml) in prawns exposed to the China Grass matrix.

Matrix	**0hrs**	**24hrs**	**48hrs**	**72hrs**
CG Control	2.30×10^3	2.00×10^5	4.50×10^5	3.75×10^6
CG	2.40×10^4	3.00×10^5	9.00×10^5	4.90×10^6

Key: Control=Unspiked



Bacterial load (cfu/ml) in prawns exposed to the China Grass matrix

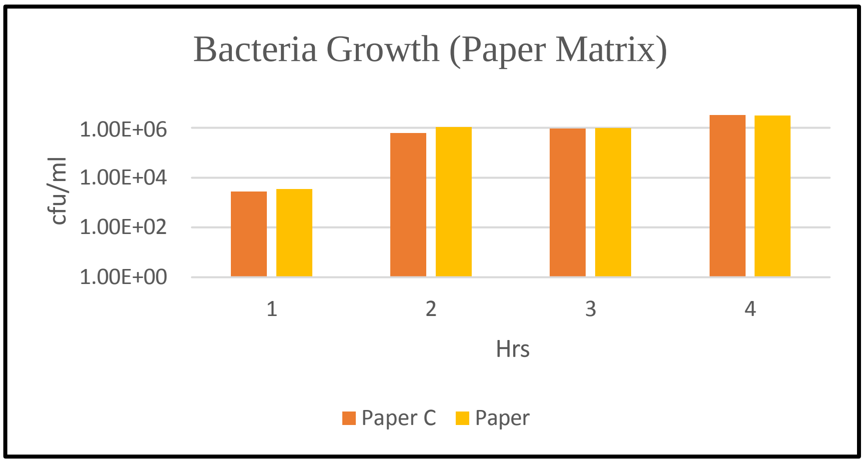


Anthocyanin incorporated Paper Matrix Response to Prawn Spoilage

Bacterial load (cfu/ml) in prawns exposed to the Paper matrix

Matrix	**0hrs**	**24hrs**	**48hrs**	**72hrs**
Paper Control	$2.70\text{x}10^3$	$9.00\text{x}10^5$	$6.00\text{x}10^5$	$3.30\text{x}10^6$
Paper	$3.45\text{x}10^3$	$1.00\text{x}10^6$	$1.10\text{x}10^6$	$3.10\ \text{x}10^6$

Key: Control =Unspiked



Bacterial load (cfu/ml) in prawns exposed to the Paper matrix

DISCUSSION

Anthocyanins from *Clitoria ternatea* were extracted using solid-liquid extraction with 70% ethanol and ultrasonication, enhancing pigment recovery through improved solvent penetration and cell wall disruption (Narayanan *et al.*, 2023). This method, proven effective in extracting anthocyanins from red cabbage and black rice (Silva-Pereira *et al.*, 2020; Sharma *et al.*, 2021), offers greater efficiency and pigment preservation compared to conventional maceration (Zeng *et al.*, 2023). Acidification to pH 2 using 1.5M HCl stabilized the extract, preventing degradation and pigment loss (Wijaya *et al.*, 2021; Mary *et al.*, 2020). Low-temperature drying further retained anthocyanin integrity (Agunos *et al.*, 2020).

Anthocyanin presence was confirmed via acid-base tests showing characteristic color changes (Azlim *et al.*, 2022; Choi *et al.*, 2016; Zhai *et al.*, 2017). Total anthocyanin content (TAC) was determined using the pH differential method, with Extract 1 yielding the highest TAC (175.33 mg/L), consistent with findings in saffron petals and dragon fruit skin (Khezerlou *et al.*, 2023; Azlim *et al.*, 2021). pH sensitivity tests across a pH range of 1–13 revealed predictable color shifts due to structural transformations (Shukla *et al.*, 2016), supported by spectral analysis showing λmax shifting from 500 nm (acidic) to 420 nm (alkaline), similar to hibiscus anthocyanins (Zhai *et al.*, 2017).

Further, LC-MS analysis in positive ion mode confirmed the presence of flavonoid and anthocyanin-related compounds such as rutin, kaempferol, trifolin, and quercetin-3-glucoside, along with high molecular weight ternatin-like glycosides typical of *Clitoria ternatea*.

Four matrices—agar, agar-gelatin (AGel), China grass (CG), and paper—were developed for use as pH-responsive food freshness indicators. Hydrogel matrices showed better pigment retention and uniform colour response, while paper exhibited faster but less stable transitions (Listyarini *et al.*, 2018). AGel had the highest swelling index (184.74%), while CG showed the greatest moisture retention (67%) (Garcia *et al.*, 2019; Lee & Kim, 2020). After 30 days at 4°C, all matrices retained pH responsiveness, confirming stability (Shukla *et al.*, 2016).

Ammonia vapor tests showed intensified colour change, with CG displaying the most pronounced response (Hu *et al.*, 2020). In prawn spoilage tests, room temperature storage triggered distinct colour transitions within 24–72 hours, while frozen samples showed minimal change, consistent with microbial suppression at low temperatures (Taherkhani *et al.*, 2020). CG matrix performed best, followed by agar and AGel; paper showed delayed response due to lower porosity (Weston *et al.*, 2020).

These findings support the use of anthocyanin-based matrices as sustainable, visual, and non-invasive indicators for food spoilage, offering advantages over synthetic indicators and conventional assays (Andretta *et al.*, 2019). Further enhancements in matrix formulation and sensor integration could improve performance and enable commercial application.

CONCLUSION

This study successfully developed anthocyanin-based biopolymer matrices using *Clitoria ternatea* extracts for real-time food freshness detection. Incorporating natural pigments into agar, agar-gelatin, China grass, and paper matrices enabled clear, pH-responsive colour transitions, confirmed via UV-Vis spectroscopy and influenced by total anthocyanin content. Structural and physical evaluations showed that agar-based matrices offered superior stability due to higher thickness and moisture retention.

Storage studies demonstrated that the matrices retained functionality for up to 30 days at 4°C. When exposed to ammonia vapours and during prawn spoilage testing, the matrices exhibited distinct, progressive colour changes correlating with microbial growth, validating their effectiveness in detecting spoilage-related volatiles.

These findings highlight the potential of these eco-friendly, non-toxic matrices as smart indicators for food packaging. Future work should aim to enhance mechanical strength, broaden food compatibility, scale production, and integrate sensor-based technologies to improve food safety and reduce waste.

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