



Optimization and characterization of colorimetric composite film based on biodegradable polymers (Gluten and Carboxymethyl Cellulose) and Roselle (*Hibiscus Sabdariffa*) anthocyanins as a TVN-B indicator in fish fillet

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Abstract

Colorimetric indicators are essential in intelligent packaging for food freshness monitoring. This study utilized *Hibiscus sabdariffa* extract as a colorimetric indicator, confirming the presence of anthocyanins, phenols, flavonoids, and antioxidant activity. Anthocyanins displayed color changes from red to blue and yellow as pH increased from 2 to 12. The effects of anthocyanin (10–30% w/w) on the physical properties of gluten (1–3% w/w) and CMC (0.5–1.5% w/w) composite films were analyzed using response surface methodology (RSM). The addition of anthocyanins increased film thickness, tensile strength, and modulus of elasticity while decreasing elongation at break ($p < 0.05$). Higher anthocyanin levels (30% v/w) reduced moisture content and increased solubility. All three variables significantly lowered water vapor permeability ($p < 0.05$). Redness (a^*) was influenced by anthocyanin, gluten, and the interaction of CMC $\times$ anthocyanin, while the yellowness index (1.85 to 2.93) increased with more gluten and anthocyanin. The optimal composition—3% gluten, 1.5% CMC, and 10% anthocyanin—offered the highest thermal stability. FT-IR and SEM confirmed the successful incorporation and compatibility of anthocyanins within the film. XRD showed that anthocyanins did not affect film crystallinity. These gluten/CMC films with *Hibiscus sabdariffa* indicator exhibited significant color changes in fish fillets, aligning with TVB-N variations and Different colorimetric indicators are employed for various intelligent packaging applications depending on the product type effectively indicating spoilage.

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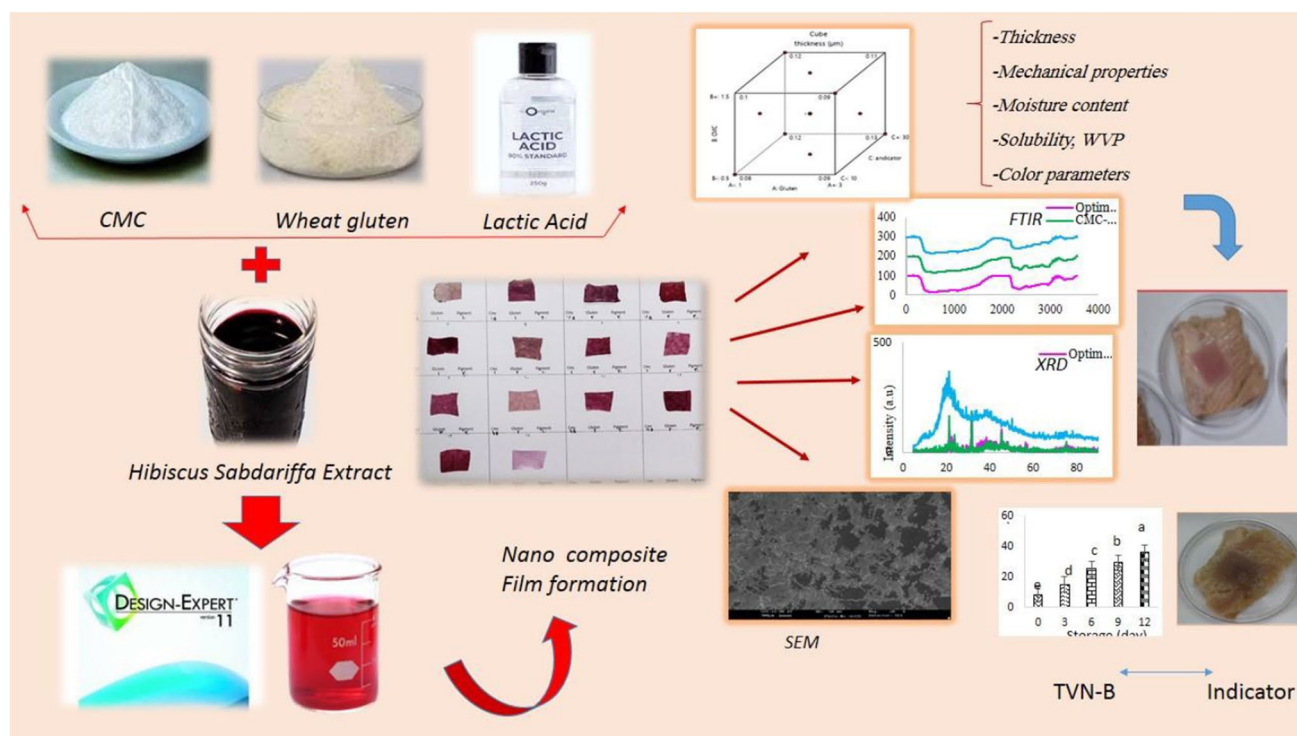
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Graphical abstract



Keywords Colorimetric Indicators · Edible film · Gluten · *Hibiscus Sabdariffa* · Intelligent Packaging

Abbreviations

WG	Wheat gluten
CMC	Carboxymethylcellulose
TPC	Total phenolic compounds
TFC	Total flavonoid content
TAC	Total anthocyanin content
RSM	Response surface methodology
MC	Moisture content
TS	Tensile strength
EB	Elongation at break
ME	Modulus of elasticity
SW	Solubility in water
WVP	Water vapor permeability
YI	Yellowness index
WI	Whiteness index
FT-IR	Fourier transform infrared spectroscopy
SEM	Scanning electron microscope
XRD	X-ray diffraction
DSC	Differential scanning calorimetry

Introduction

Intelligent packaging systems interact with products and/or the environment to detect specific analytes in packaged foods, thereby informing manufacturers, retailers, and consumers about food quality [1]. These systems are divided into three main categories: indicators, sensors, and data carriers. Indicators include freshness indicators, temperature indicators, gas indicators, and colorimetric indicators [2, 3]. Freshness indicators monitor food quality by detecting spoilage reactions such as microbial growth and chemical degradation [4–6]. Time and temperature indicators (TTIs) signal temperature deviations beyond critical thresholds, changing color or physical properties to indicate spoilage [3]. Gas indicators monitor the headspace gas composition, identifying the presence of volatiles, microbial metabolites, and enzymatic autolysis products [7, 8]. Colorimetric indicators are composed of reactive dyes or pigments that respond to physicochemical

stimuli by changing color within the visible spectrum [3, 9, 10]. These indicators are crucial for detecting changes in product quality. Depending on the product type, different colorimetric indicators are employed for various intelligent packaging applications. Natural food colorants, such as anthocyanins [3, 11], are extensively studied as colorimetric indicators due to several advantages over non-food colorants: low toxicity, high consumer acceptance, diverse color range, excellent sensitivity, wide pH range, and abundant sources [3, 12, 13]. These benefits make natural colorants, particularly anthocyanins, ideal for use in intelligent packaging systems to ensure food safety and quality.

Extensive research has underscored the significant health benefits of anthocyanins, which include protection against neurological disorders, reduction in heart disease risk, mitigation of chronic diseases, and prevention of obesity, diabetes, and cancer, alongside potent anti-inflammatory effects [14–16]. A prominent source of anthocyanins is Roselle (*Hibiscus sabdariffa*) from the Malvaceae family [17], which contains anthocyanins ranging from 1.7–2.5% of its dry weight, though this content varies among species [18].

Biodegradable polymers, encompassing proteins, lipids, polysaccharides, polyesters, and their derivatives, are pivotal as carriers for colorimetric indicators in intelligent packaging [11, 19, 20]. Wheat proteins or wheat gluten (WG) are particularly notable for their application in protein-based films due to their exceptional viscoelastic properties, tensile strength, and gas barrier performance [21, 22]. Despite these strengths, WG films face challenges related to hydrophilicity and high moisture permeability [23]. To overcome these limitations, composite films have been innovatively developed by incorporating materials such as soy protein isolate (SPI)-zein [24, 25], Persian gum-guar gum [26], vanillin-salicylic acid-montmorillonite cloisite [23], and SPI. Polysaccharides like carboxymethyl cellulose (CMC) [27, 28] are integral to biofilm formulations due to their excellent film-forming capability, non-toxicity, biodegradability, and biocompatibility [29, 30]. These properties make CMC an ideal component in the development of sustainable and effective packaging solutions. Such advancements not only enhance the functional properties of the films but also contribute significantly to the development of intelligent packaging systems that ensure food safety and quality while being environmentally friendly.

Fish is a globally cherished and highly nutritious food, yet it is exceptionally prone to rapid spoilage during transportation and storage due to its high protein content and short shelf life [6, 31]. Accurate and early detection of spoilage is paramount for consumer safety [32]. Extensive research [33–35] has demonstrated that fish is swiftly impacted by bacterial and microbial activity, which leads to the breakdown of protein compounds and amino acids. This process

results in the production of ammonia, amines, and other alkaline nitrogenous compounds, such as trimethylamine (TMA) and dimethylamine (DMA). These compounds, collectively known as total volatile base nitrogen (TVB-N), are critical indicators of fish spoilage [36, 37]. The detection of TVB-N is vital for assessing fish spoilage, and smart packaging that can identify these volatile nitrogenous compounds is both convenient and attractive to consumers.

This study explores the use of anthocyanin-rich indicators derived from *Hibiscus sabdariffa*, which are stabilized with gluten and carboxymethyl cellulose (CMC), to serve as colorimetric indicators. Anthocyanins are natural pigments that change color in response to pH changes, making them ideal for detecting spoilage. These innovative colorimetric indicators work by exhibiting a visible color change when exposed to the alkaline conditions produced by the release of nitrogenous compounds during fish spoilage. The anthocyanins in *Hibiscus sabdariffa* respond to these pH changes by transitioning through a spectrum of colors, thus providing a clear visual signal of spoilage. This method not only ensures early and accurate detection of spoilage but also enhances food safety and quality control. Moreover, the integration of these indicators into biodegradable polymer films made from gluten and CMC addresses environmental concerns associated with packaging waste. Gluten provides excellent viscoelastic properties, tensile strength, and gas barrier capabilities [23, 26, 38], while CMC offers good film-forming ability, nontoxicity, biodegradability, and biocompatibility. Combining these materials with anthocyanins results in a robust, eco-friendly packaging solution.

Herin, this research highlights the potential of *Hibiscus sabdariffa*-based anthocyanin indicators, incorporated into gluten and CMC films, to revolutionize the monitoring of fish spoilage. By providing a reliable, early-warning system for detecting spoilage through color changes, these indicators enhance consumer safety and contribute to sustainable packaging practices.

Material and methods

Materials

High-protein wheat gluten ($\geq 90\%$) was supplied by Shahdineh Aran Company (Isfahan, Iran). Additional materials, including carboxymethyl cellulose (CMC), glycerol, and other chemical reagents, were procured from Merck (Darmstadt, Germany).

Preparation of *Hibiscus sabdariffa* extract

Dried flowers of *Hibiscus sabdariffa* were obtained from a local vendor in Isfahan, Iran. The flowers were thoroughly

cleaned, dried at 40 °C for 4 h, and stored under ambient conditions [39]. For extract preparation, 40 g of powdered flowers were mixed with 400 mL of water, and the mixture was heated at 60 °C for 2 h. The extract was separated by centrifugation at 4500 rpm for 10 min, followed by filtration using Whatman filter paper. The filtrate was concentrated using a rotary evaporator and then freeze-dried with a lyophilizer (Virtis Co., Gardiner, NY, USA). The resulting powder was stored at −20 °C for further use [40].

Extract characterization

The phenolic and flavonoid contents, along with antioxidant activity (at 200 ppm), were assessed using the Folin-Ciocalteu method, an aluminum chloride colorimetric assay, and DPPH free radical scavenging, respectively, based on established methodologies [41].

Anthocyanin isolation

To isolate anthocyanins, 1 g of the freeze-dried extract was dissolved in 15 mL of ethanol (80%) and adjusted to pH 2 with HCl. The solution was heated at 50 °C for 1 h, centrifuged at 3000 rpm for 6 min, and the solvent was removed using a rotary evaporator (Heidolph Hei-VAP, Germany) at 50 °C. The concentrated residue was dried and preserved for analysis [42].

Total anthocyanin content (TAC)

The total anthocyanin content was determined by adding 0.5 mL of the extract to 4.5 mL of potassium chloride buffer (0.025 M, pH 1) and 4.5 mL of sodium acetate buffer (0.4 M, pH 4.5). The absorbance of the solution was measured at 522 nm (A522) and 699 nm (A699) using a Jasco V-550 spectrophotometer (Japan). The TAC (mg/100 g) was calculated using Eq. (1), with distilled water serving as the blank [39, 43].

$$\text{TAC} = \frac{[(A_{522\text{nm}} - A_{699\text{nm}})_{\text{pH} = 1} - (A_{522\text{nm}} - A_{699\text{nm}})_{\text{pH} = 4.5}] M_w DF}{\epsilon L} \quad (1)$$

where M_w represents the molecular weight of cyanidin-3-glucoside (449.2 g/mol), DF is the dilution factor (10), ϵ is the molar extinction coefficient (29,600 Lcm^{−1}mol^{−1}), and L is the optical path length (1 cm).

Color changing and pH sensitivity of extract

The extract (400 mL) was mixed in 10 mL of citrate-phosphate buffer. For 20 mL buffer, 0.2 M Na₂HPO₄ and 0.1 M citric acid were mixed. The extract was dissolved in ethanol

(42.8 mg/mL) and filtered through a 0.2 µm membrane. The color of the extract was analyzed 30 min after mixing in different pH buffers using a spectrophotometer. Extract images were captured using a high-resolution digital camera [39, 40].

pH sensitivity of extract powder

The extract powder was dissolved in ethanol (42.8 mg/mL) and filtered through a 0.2 µm membrane. At room temperature, 200 µL of extract powder was added to 5 mL of 0.2 M buffers, including citrate-phosphate buffer (pH 8.2), glycine-NaOH buffer (pH 10), and potassium chloride-NaOH buffer (pH 12). After mixing for 15 min, the UV-Vis spectrum of the extract powder was recorded in the 200–800 nm range [40].

Experiment design, optimization, and statistical analysis

Response surface methodology (RSM) with central composite design (CCD) was used to evaluate the effect of independent parameters including gluten (X_1) (1–3 wt %), CMC (X_1) (0.5–1.5 wt %), and indicator (X_1) (10–30 wt %) on dependent variables in Design-Expert software (V. 11.0.10). A total of 14 treatments (Table 1) were performed, including six replicates at the central point to evaluate pure error and data repeatability, following the Central Composite Design (CCD). The responses (y_i) were fitted to Eq. (2).

$$y_i = \beta_0 + \sum_{i=1}^3 \beta_i x_i + \sum_{i=1}^2 \sum_{j=2}^3 \beta_{ij} x_i x_j + \sum_{i=1}^3 \beta_{ij} x_i x_j^2 + \epsilon_i \quad (2)$$

where β_0 represent the intercept (β_0), (β_i) main effects, (β_{ij}) interaction effects of independent variables in the model, and x_{ij} is the predictors or factors affecting the response variable (y_i) [44, 45]. The suitability of the equations and models for all the responses, which are the

dependent variables, was evaluated through analysis of variance (ANOVA) with a significance level set at 0.05 ($p < 0.05$), as shown in Table 2.

Preparation of colorimetric indicator films

Since in most cases, film preparation was based on alkaline conditions, to evaluate the efficiency of the indicator at low pHs, the composite gluten-based films containing CMC and anthocyanin of *H. Sabdariffa* extract were

Table 1 Experimental design with process variables and observed responses

Run	A: Gluten	B: CMC	C: Indicator	Thickness (μm)	SW (%)	MC (%)	L*	a*	b*	YI	WI	TS (N)	EB (mm)	ME (MPa)	WVP ($\text{g m}^{-1} \text{d}^{-1} \text{pa}^{-1}$)
1	2	0.5	20	0.09	90.12	1.69	61.5	13.12	13.98	2.38	57.03	3.49	154.88	0.026	8.86
2	3	1	20	0.1	97.25	1.78	52.28	15.74	17.85	2.78	46.71	3.29	163.01	0.023	7.31
3	2	1	20	0.1	87.27	1.67	64.89	12.25	12.32	2.25	60.87	3.98	150.97	0.029	9.09
4	2	1	20	0.09	83.12	1.61	62.37	12.1	12.45	2.34	58.6	3.86	146.15	0.029	9.2
5	1	1.5	30	0.12	68.69	1.43	73.25	16.87	8.67	2	67.25	2.63	167.09	0.019	9.54
6	3	1.5	10	0.09	99.6	1.81	55.38	2.08	16.05	2.63	52.58	2.91	165.01	0.021	7.58
7	2	1	20	0.09	84.35	1.63	63.87	11.68	12.52	2.29	50.06	3.92	148.12	0.029	9.01
8	2	1	30	0.12	82.09	1.6	58.75	17.23	15.78	2.48	52.63	4.26	144.08	0.033	7.91
9	2	1	20	0.1	85.21	1.64	64.22	11.9	12.72	2.27	60.25	3.83	149.18	0.029	8.53
10	2	1	10	0.09	92.87	1.75	71.48	3.45	9.98	2.05	69.63	3.7	152.04	0.027	9.12
11	1	1	20	0.1	72.57	1.5	76.77	5.98	7.25	2.01	74.98	2.74	168.55	0.019	9.98
12	1	0.5	10	0.08	76.89	1.53	79.32	0.02	5.46	1.85	78.65	2.5	170.55	0.018	10.7
13	2	1.5	20	0.09	87.88	1.72	68.22	8.25	11.23	2.14	65.34	3.45	156.18	0.025	8.28
14	3	0.5	30	0.13	93.87	1.75	49.5	18.23	19.36	2.93	42.97	3.08	159.01	0.021	6.98

produced by casting method at acidic conditions. WG (1–3% based on solid matter) was added to distilled water. 3–5 drops of lactic acid using a magnetic stirrer at 150 rpm was added to adjust the pH to 4–4.5 and heated to 70 °C. 2% (v/v) glycerol as a plasticizer was added to the solution and the resulting suspension was mixed slowly for 20 min [23].

CMC (0.5–1.5%) was dissolved in 35 mL of distilled water and heated in a water bath at 75 °C for 20 min. After adding the extract of *H. sabdariffa* (10–30%) as an indicator, the film-forming solutions were mixed for 30 min at 20 °C poured into molds, and dried in an oven at 20 °C for 48 h [38, 39]. Gluten-film and CMC-film without *H. Sabdariffa* extract was used as a control.

Physicomechanical characteristics of film

Film thickness

The film thickness was measured using a digital micrometer (No. 103–137, Mitutoyo Co. Ltd, Japan) with an accuracy of ± 0.001 mm and the mean of ten different points of the film was calculated [39, 46].

Mechanical properties

Tensile strength (TS), elongation at break (EB), and modulus of elasticity (ME) were analyzed using a tensile device (SANTAM-STM1) with a force of 200 N and at the speed of 50 mm/min [26].

Moisture content (MC)

First, 40 mg of film was dried at 105 °C for 24 h and weighed after cooling at room temperature. Then, MC was calculated from the weight difference between the undried and dried film [39, 46].

Solubility in water (SW)

Film discs (W_0) were submerged in 50 mL of distilled water and subjected to continuous stirring at 100 rpm at a temperature of 20 °C for 24 h. After immersion, the discs were dried at 105 °C for 24 h to achieve a constant weight (W_1). The swelling ratio (SW%) was determined using the equation provided in previous studies [39, 46, 47].

$$\text{SW} = \frac{W_0 - W_1}{W_0} \times 100 \quad (3)$$

Water vapor permeability (WVP)

WVP was calculated according to Najafian et al. procedures [26]. The films were fixed on the top of the cups containing 10 mL of distilled water and were placed in a desiccator containing silica gels at 25 °C for 7 h. WVP was calculated with Eqs. (4–5):

$$\text{WVP}(\text{gm}^{-1}\text{h}^1\text{Pa}^{-1}) = \frac{\text{WVTR} \times h}{\Delta P} \quad (4)$$

$$\text{WVP}(\text{gm}^{-2}\text{h}^1) = \frac{\Delta m}{A \times \Delta t} \quad (5)$$

Table 2 ANOVA results to determine the fit of model, β , adj. R^2 , and F value of predicted second-order polynomial models for the responses

Factor	Coefficient (β)											
	Thickness	SW	MC	L*	a*	b*	YI	WI	TS	EB	ME	WVP
Intercept	0.0950	85.84	1.65	64.41	11.73	12.54	2.29	59.83	3.88	149.83	0.0288	8.72
Linear												
X ₁ : Gluten (% w/w)	-	12.10	0.1467	-12.24	4.88	5.30	0.3850	-13.10	0.2750	-2.77	0.0020	-1.39
X ₂ : CMC (% w/w)	-	-0.7850	-0.0017	3.36	-2.44	-1.38	-0.1200	1.09	-0.0200	0.6500	-0.0005	-0.1900
X ₃ : Indicator (% w/w)	0.0150	*** -4.12	** -0.0517	-6.36	6.89	2.90	***0.2150	** -6.34	0.2800	-3.98	***0.0030	*** -0.4950
Interaction												
X ₁ X ₂	-0.0050	-	-	** -3.38	* -1.36	***1.27	*0.102	-	*0.2050	-1.61	***0.0028	-
X ₁ X ₃	-	-	-	***3.41	** -2.61	***-1.35	*-0.0825	-	-0.0100	0.0150	-0.0008	-
X ₂ X ₃	-0.0050	-	-	-0.3225	**4.02	-0.0200	-0.0425	-	0.0600	0.6350	0.0007	-
Quadratic												
X ₁ ²	0.0050	-	-	-	-0.6207	-	0.1102	-	0.8425	***14.72	0.0075	-
X ₂ ²	0.0050	-	-	-	*-0.7957	-	-0.0248	-	-0.3875	*4.47	***-0.0030	-
X ₃ ²	***0.0100	-	-	-	** -1.14	-	-0.0198	-	*0.1225	-3.00	0.0015	-
Adj. R ²	0.8793	0.9381	0.8842	0.9887	0.9936	0.9961	0.9882	0.8602	0.9858	0.9078	0.9936	0.9346
F-value (model)	11.52	66.68	34.08	191.05	225.07	550.73	122.34	27.67	101.05	15.23	226.18	62.89
F-value (lack of fit)	0.0000	1.87	2.82	0.6040	11.31	3.06	0.0137	0.4458	0.9944	4.07		0.7257
P-value (model)	0.0156	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	<0.0001	0.0002	0.0093	<0.0001	<0.0001

Level of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Δp represents the difference in water vapor pressure across the two sides of the film, while h indicates the thickness of the sample.

Color measurement

The film color was measured with a Color Meter (TES-135 A, Taiwan) to the measurement of L^* (lightness), a^* (red-green), and b^* (yellow-blue). The color difference (ΔE), whiteness index (WI), and yellowness index (YI) were calculated from the Eqs. (6), (7), and (8) [23].

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \quad (6)$$

$$WI = 100 - [(100 - L)^2 + a^2 + b^2]^{1/2} \quad (7)$$

$$YI = \frac{142.86}{L} \quad (8)$$

Antioxidant properties of the film

In the 2–2-diphenyl-1-picrylhydrazyl (DPPH) method, small pieces (1 g) of the optimal film were cut, weighed, and mixed with 10 mg/ml methanol for 24 h. 150 μ L supernatant was added to the 96-well microplates. After adding 150 μ L DPPH solution (0.1 mM,) in methanol and incubation for 1 h, the absorbance was read at 517 nm. Radical inhibition percentage was calculated from Eq. (9) [40].

$$\% \text{inhibition of DPPH} = \left(\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right) \times 100 \quad (9)$$

FTIR - spectroscopy

The optimal and control films were dried at 50 °C for 48 h and the changes of the functional groups were evaluated using a Fourier transform infrared spectrometer (PerkinElmer Spectrum 65, USA) in the wave number range of 4000–650 cm^{-1} [26, 48].

Scanning electron microscope (SEM)

The microstructure of the optimal and control films after coating with gold and immersion in liquid nitrogen was analyzed using a scanning electron microscope (Philips XL 30 FEG, Netherlands) at a voltage of 5–15 kV [49].

X-ray diffraction (XRD)

X-ray diffractometer (XRD, Karlsruhe, Germany) with Cu Ka radiation (1.54 Å) in the range of $2\theta = 4-40$ and a scanning time of 53 s with a speed of $0.05 \text{ deg} \times \text{s}^{-1}$ was used to evaluate the physical condition of the optimal and control films [50].

Thermal properties

Thermal properties of the optimal and control films were determined using differential scanning calorimetry (DSC, Mettler Toledo, Switzerland). 5 mg of film was placed in a DSC pen, and an empty aluminum pen was used as a reference. The samples were scanned at a heating rate of 10 °C/min between 30 and 260 °C with a nitrogen flow rate of 20 ml/min [40].

Colorimetric film and volatile nitrogen compounds (TVB-N) analysis

Salmon samples were purchased from a local store and quickly transported to the laboratory under refrigerated conditions. After separating skin and scales, 75 g of fillets were cut and placed separately in glass Petri dishes. The colorimetric indicator films (2 × 4 cm) were fixed on the lid of Petri dishes sealed with cellophane to prevent contamination and stored in the refrigerator (4 °C) for 12 days. For determination of TVB-N, 10 g of fish meat, 2 g of magnesium oxide, and 500 mL of distilled water were poured into the Kjeldal balloon containing boiling stone and paraffin as anti-foam. 25 ml of 2% boric acid and methyl red reagent was placed in a flask. After titration with boric acid, TVB-N was calculated as mg/100 g fish meat from Eq. 10 [51].

$$\text{TVB} - \text{N} = 14 \times V_{\text{acid}} \quad (10)$$

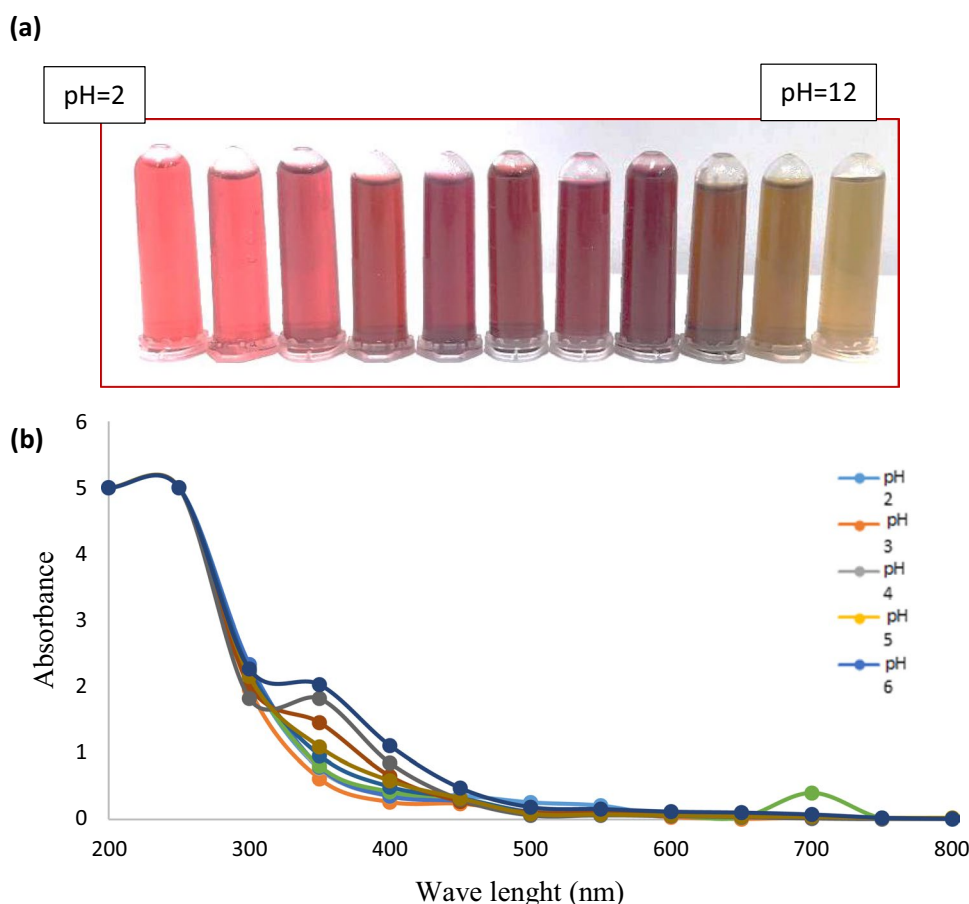
Results and discussion

Characteristics of *H. Sabdariffa* extract

TPC, TAC, TFC, and DPPH inhibition of *H. Sabdariffa* extract was 3.05 ± 0.02 mg GAE/g, 2.24 ± 0.03 mg/100 g, 69.81 ± 2.81 mg QE/g, and $89.09 \pm 0.69\%$, respectively.

Many researchers have confirmed the antioxidant activity and the presence of anthocyanins and phenolic compounds in *H. Sabdariffa*. The phenolic acids (protocatechuic acid), organic acid (hydroxy citric acid), and anthocyanins (delphinidin-3-sambubioside and cyanidin-3-sambubioside) have been confirmed [18, 52–56]. The TAC of *H. Sabdariffa* was 2.52 mg/100 g in the report of Wong et al. (2002) [57], which is similar to our results.

Fig. 1 Color (a) and UV–Vis spectrum (b) of *H. Sabdariffa* anthocyanin at pH =2–12



UV–Vis analyses

The color changing and pH sensitivity of anthocyanin of *H. Sabdariffa* at pH 2–12 are shown in Fig. 1. Anthocyanins changed from red to yellow when the pH increased from 2 to 12. The maximum absorption peak was observed at 200–250 nm (Fig. 1b). The absorption decreased at pH 2–3 and increased at pH 6–7. When the pH was higher than 5, the maximum absorption appeared at 700 nm.

At pH < 5, 5–6, 8–9, and 11–12, the anthocyanins are red, pink, purple, and yellow, respectively. These color changes may be due to their different chemical structure [54, 58]. At pH 2–3, anthocyanins exist in the form of yellow salt ions, the solution looks red, and at pH 4–6 their structure transforms to quinoid form so that the redness decreases. At pH 7–9, their structure becomes colorless and the color gradually becomes blue. Finally, when the pH value is higher than 9, anthocyanins are decomposed in alkaline conditions, and their color changes to yellow-green. The sensitivity of anthocyanins to pH is proven in many studies [10, 11, 59–61].

Fitting model according to RSM

The appearance of gluten films was yellow and became red and purple with the addition of anthocyanins (Fig. 2). The values of independent and dependent variables are shown in Table 1. F value, coefficient of determination (R^2), and coefficient of determination (adj. R^2) were used to predict the suitability of the model based on the ANOVA (Table 2). P-value, lack of fit, and model type were evaluated at $p < 0.05$. A lower p-value shows a significance of the coefficient, and $p \leq 0.05$ shows a high significance of the coefficient, and all insignificant terms with $p > 0.05$ eliminated. Final regression equations (Eqs. 10–21) were developed according to the ANOVA Table.

Thickness

The thickness affects the mechanical properties and gas permeability and therefore is very important [26]. The thickness was 0.08–0.13 μm and there was a significant difference between the thicknesses of different films ($p < 0.05$). The highest thickness was observed in the run of 14 (Table 1).



Fig. 2 Gluten-based films incorporating CMC and indicator designed by RSM. (Anthocyanin (10–30% w/w) gluten (1–3% w/w) and CMC (0.5–1.5% w/w) conditions

The ANOVA results (Table 2) indicated that the model's R^2 value was 0.87, demonstrating that the experimental data can be reliably aligned with the model data. Additionally, thickness significantly influences the amount of independent variables, as presented in Table 2. The thickness was linearly affected by anthocyanin ($p < 0.05$). Additionally, according to the predicted model (Eq. 11), anthocyanin concentration had a positive effect on the response, while the interactions of gluten $\times$ CMC and CMC $\times$ indicator had negative effects. The quadratic and interaction effects of the independent variables on thickness were found to be significant ($p < 0.05$).

$$\begin{aligned} \text{Thickness : } y = & 0.0950 + 0.0450X_3 - 0.0050X_1X_2 \\ & - 0.0050X_2X_3 + 0.0050X_{12} \\ & - 0.0050X_{22} + 0.0100X_{32} \end{aligned} \quad (11)$$

As the results showed, high levels of gluten and CMC increased the film thickness (Fig. 3). By increasing the gluten up to 2%, the thickness decreased, but a further increase in the gluten concentration increased the thickness. The thickness increased with increasing CMC and indicator.

These results are in opposite with Najafian et al. [26] who have reported that the increase in gluten led to a decrease in the thickness of the film. Because *H. Sabdariffa* extract acts as

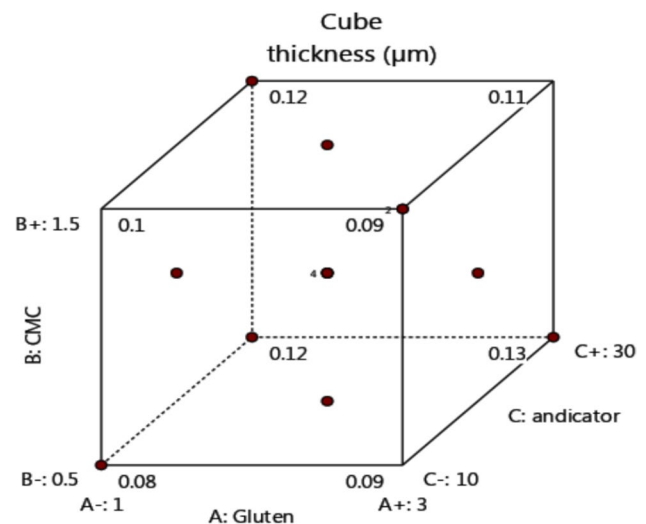


Fig. 3 Cubic plots of the interaction effects of process variables on thickness

a plasticizer and although the gluten has increased, the effect of the extract is more.

In agreement with our results, with the increase of roselle anthocyanins in the polyvinyl alcohol/hydroxypropyl

methylcellulose film, a significant increase in film thickness was observed, because anthocyanins penetrate the space of polymer chains and affect the thickness [62].

Mechanical properties

The values of mechanical parameters were 4.2–26.5 N, 170.55–144.08 mm, and 0.033–0.018 mPa, respectively (Table 1). The ANOVA results indicated that the linear, interaction, and quadratic effects of the independent variables on the mechanical properties are significant ($p < 0.05$), and the lack of fit is not significant, as shown in Table 2. According to the proposed model (Eqs. 12–14), the positive coefficient for X_3^2 confirms the strong influence of anthocyanin on TS and ME ($p < 0.05$). Also, the coefficients of Eq. 12 show that EB was affected by the variables in the following order: CMC > gluten > anthocyanin.

$$\begin{aligned} \text{TS} : y = & 3.88 + 0.2750X_1 - 0.0200X_2 + 0.2800X_3 \\ & + 0.2050X_1X_2 - 0.0100X_1X_3 + 0.0600X_2X_3 \\ & - 0.8425X_1^2 - 0.3875X_2^2 + 0.1225X_3^2 \end{aligned} \quad (12)$$

$$\begin{aligned} \text{EB} : y = & 149.83 - 2.77X_1 + 0.6500X_2 \\ & - 3.98X_3 - 1.61X_1X_2 + 0.0150X_1X_3 + 0.6350X_2X_3 \\ & + 14.72X_1^2 + 4.47X_2^2 - 3.00X_3^2 \end{aligned} \quad (13)$$

$$\begin{aligned} \text{ME} : y = & 0.0288 + 0.0020X_1 - 0.0005X_2 + 0.0005X_3 \\ & + 0.0030X_3 + 0.0028X_1X_2 - 0.0008X_1X_3 \\ & + 0.0007X_2X_3 - 0.0075X_1^2 - 0.0030X_2^2 + 0.0015X_3^2 \end{aligned} \quad (14)$$

According to Fig. 4, increasing gluten up to 2% increased TS and ME and decreased EB, but higher gluten concentration had an inverse effect. Increasing CMC up to 1% increased TS and ME and decreased EB, but its higher

concentration had an inverse effect. With increasing indicators, TS and ME increased and EB decreased.

The packaging industry needs the desired strength and elasticity to maintain the integrity of the packaging [23]. TS increasing with the increase of gluten and CMC is due to the gel properties and strong hydrogen bonds between protein-polysaccharide chains. The ME values depend on the film matrix network and interactions between the film components. The increase of protein/polysaccharide interactions decreased ME and increased flexibility. CMC penetrates the matrix of gluten and reduces the intermolecular interactions and cohesive force in the gluten structure, therefore, flexibility is increased [26].

Anthocyanin influence on the mechanical properties has been reported in many studies, for example, the presence of red barberry anthocyanins in the colorimetric film based on chitin and methyl-cellulose nanofibers [11], red cabbage anthocyanins in chitosan/oxidized chitin nanocrystals composites [10], anthocyanins of *Clitoria* extract in gellan film [50], Rosell anthocyanin in the polyvinyl alcohol/hydroxypropyl methylcellulose film [62] and red cabbage anthocyanin in colorimetric films based on polyvinyl-alcohol/sodium carboxymethyl cellulose [63].

Moisture content (MC)

MC values of films were 1.43–1.81% (Table 1). Based on ANOVA results, the R^2 of the model for MC was 0.88 and this response significantly changes the concentration of all independent variables (Table 2). MC was linearly affected by gluten ($p < 0.05$). Also, anthocyanin concentration had a positive effect on MC (Eq. 15). MC increased with increasing gluten and decreasing indicator (Fig. 5A).

$$\text{MC} : y = 1.65 + 0.1467X_1 - 0.0017X_2 - 0.0517X_3 \quad (15)$$

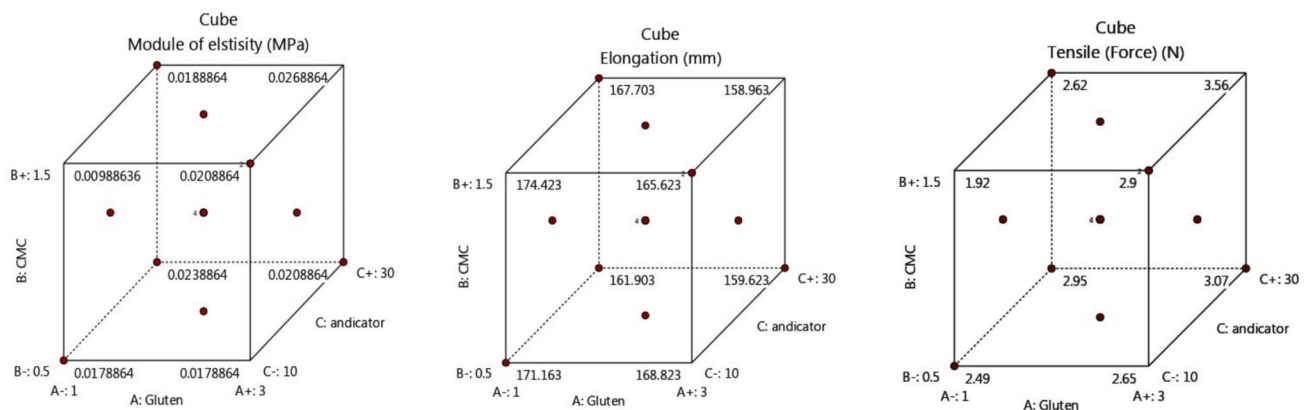


Fig. 4 Cubic plot of the interaction effects of process variables on mechanical properties

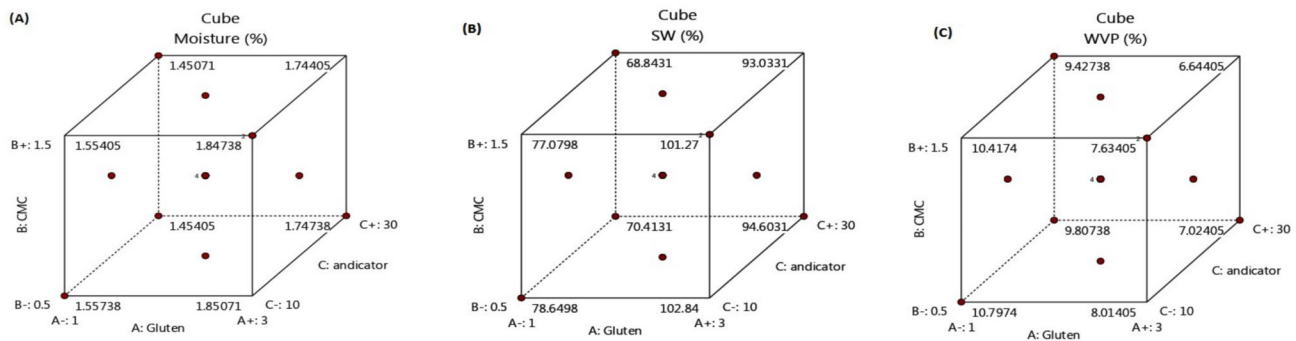


Fig. 5 Cubic plot of the interaction effects of process variables on moisture content, solubility, and WVP

The food packaging must be moisture-resistant to prevent undesirable properties. Moisture absorption of a film depends on the hydrophilic property of the biopolymer and the empty spaces between the chains. CMC due to its hydroxyl groups is a hydrophilic substance. The strong hydrogen bonds between gluten create a strong structure that limits the absorption and penetration of water molecules. The higher effectiveness of the film from gluten concentration than CMC can be seen in higher F-Value of gluten.

The replacement of the colorants between the biopolymer chains reduces the free spaces between the chains for water absorption. For example, the addition of curcumin to agar-polyvinyl-alcohol film decreased the MC. On the contrary, Asadi and Pirsia (2019) [64] showed that lycopene increased the MC of the polylactic acid film.

Water solubility (SW)

SW of films was between 68.69 and 99.6% (Table 1) with a significant difference between treatments ($p < 0.05$). The highest SW was observed in the treatment containing high levels of gluten and CMC and low amounts of indicator (run = 6). The lowest SW was observed in the treatment containing high levels of indicator and CMC and low amounts of gluten (run = 5). Table 2 shows that the fitted linear model for SW is significant ($p < 0.05$) SW increased with increasing gluten and indicator and decreasing CMC (Fig. 5B). The most important effect on SW was related to gluten with the highest coefficient, which increases SW (Eq. 16).

$$\text{SW} : y = 85.84 + 12.10X_1 - 0.7850X_2 - 4.12X_3 \quad (16)$$

SW is an important factor for composite biopolymer films and food packaging. Higher SW reflects lower water resistance and higher hydrophilicity of film components, thus low SW is desirable for food packaging [23, 65].

The hydrophilic substances i.e. CMC in the film matrix increase SW. In films with high concentrations of CMC (carboxymethyl cellulose) and gluten, the interactions between proteins and polysaccharides reduce the interaction of hydroxyl groups with water. Consequently, this leads to a decrease in solubility in water (SW) [26]. Similar to MC results, the F-value of gluten is higher than CMC, so the effectiveness of SW from gluten is higher than CMC. These results are contradictory to those obtained by Najafian et al. [26] in which it was reported that by increasing gluten, SW decreases. They stated that the insolubility of gluten is because of the chemical nature of gluten, non-polar amino acids with aliphatic groups, which are very little ionized, hydrophobicity of amino acids and their sequence and free OH groups reducing after reaction with gluten [23, 26, 66, 67].

SW increasing with anthocyanin agreed with [63, 68] findings, who also reported that red cabbage anthocyanin increased SW of polyvinyl-alcohol/sodium-carboxymethyl-cellulose films.

Water vapor of film (WVP)

The mean values of WVP were 6.98–10.7 g m⁻¹ h⁻¹ Pa⁻¹ (Table 1) and all three variables decreased WVP. WVP was significantly influenced by the linear effects of all independent variables ($p < 0.05$). R² of the model was 0.93 (Table 2). The concentration of all three variables had a negative effect on this response (Eq. 17, Fig. 5C).

$$\text{WVP} : y = 8.72 - 1.39X_1 - 0.1900X_2 - 0.4950X_3 \quad (17)$$

The packaging of food products should have a minimum WVP. The lower water diffusion and high glutamine content of gluten may manage the hydrogen bonds between chains of protein and protein–protein interactions [26]. The results

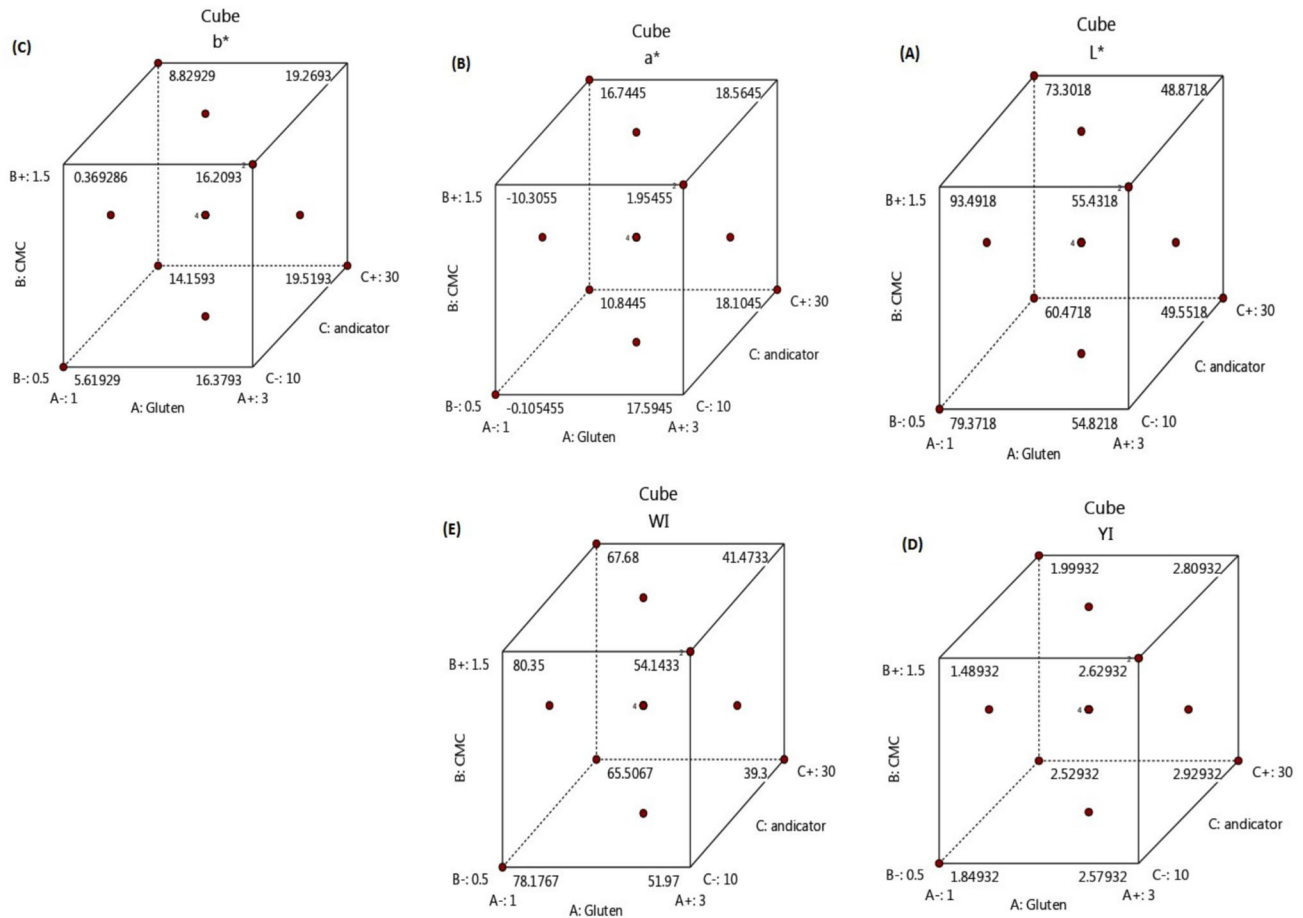


Fig. 6 Cubic plot of the interaction effects of process variables on color parameters

of Najafian et al. [26] showed that gluten improve the WVP of the films.

Anthocyanins probably prevent the diffusion of water molecules by filling the polymer spaces and reducing WVP. In agreement with our results, anthocyanins of red cabbage [10], anthocyanins of *Clitoria* extract [50], and lycopene [64] improved the WVP of bio-composite films.

Color

With the increase of gluten and indicator, L^* and WI decreased and a^* , b^* , and YI increased. The increase of CMC decreased b^* and YI and increased WI (Table 1, Fig. 6).

ANOVA results showed that the P values for L^* , a^* , b^* , YI , and WI are insignificant (< 0.0001) and the R^2 values for these parameters are 0.98, 0.99, 0.99, 0.98, and 0.86, respectively (Table 2). The linear effects of gluten, CMC, and anthocyanin on L^* , a^* , b^* , YI , and WI , the quadratic

effect on YI , and the interaction effect on L^* , a^* , b^* , and YI were significant ($p < 0.05$).

The lowest effect for L^* was related to gluten and anthocyanin, which had the lowest coefficients in the model (Eq. 18). a^* is affected by the linear effect of anthocyanin and gluten and the interaction of CMC $\times$ anthocyanin (Eq. 18).

$$L^* : y = 64.41 - 12.24X_1 + 3.36X_2 - 6.36X_3 - 3.38X_1X_2 + 3.41X_1X_3 - 0.3225X_2X_3 \quad (18)$$

$$a^* : y = 11.73 + 4.88X_1 - 2.44X_2 + 6.89X_3 - 1.36X_1X_2 - 2.61X_1X_3 + 4.02X_2X_3 - 0.6207X_{12}^2 - 0.7957X_{22}^2 - 1.14X_{32}^2 \quad (19)$$

$$b^* : y = 12.54 + 5.30X_1 + 1.38X_2 + 2.90X_3 + 1.27X_1X_2 - 1.35X_1X_3 - 0.0200X_2X_3 \quad (20)$$

$$WI : y = 59.83 - 13.10X_1 + 1.09X_2 - 6.34X_3 \quad (21)$$

$$\begin{aligned}
 \text{YI} : y = & 2.29 + 0.3850X_1 - 0.1200X_2 + 0.2150X_3 \\
 & + 0.1025X_1X_2 - 0.0825X_1X_3 - 0.0425X_2X_3 \quad (22) \\
 & + 0.1102X_1^2 - 0.0248X_2^2 - 0.0198X_3^2
 \end{aligned}$$

The color of films is influenced by the properties of their additives, particularly the cross-linking of polymer chains, which affects the film's structural coherence and can lead to color darkening [23]. Among the various parameters, gluten plays a crucial role in determining film color due to its interaction with polysaccharides. Gluten, which has a yellow hue, significantly impacts the color characteristics of the films, leading to increases in both the b^* value (which measures yellowness) and the yellowness index (YI) [26, 69]. A low YI is generally preferred for the organoleptic acceptability of biopolymers and edible films, as it signifies less undesirable coloration [23]. The effects of gluten and other compounds on YI include linear, quadratic, and interaction effects, with YI increasing as the concentrations of gluten and the colorimetric indicator rise (Eq. 22). This observation is consistent with findings from Dong et al. [27] and Najafian et al. [26], who reported that higher gluten concentrations led to increased YI. This highlights the need to balance additive concentrations to achieve desirable film properties while minimizing undesirable color changes.

Optimization

In Response Surface Methodology (RSM), the desirability point is utilized to identify the optimal combination of variables that enhances multiple responses in order to achieve the most favorable outcomes [70]. The optimal film was achieved with the lowest YI, WVP, SW, and ME and the highest TS and a^* . Compliance analysis was performed under identical test conditions to evaluate the validity of the correlations derived from the mathematical model. The optimal conditions were suggested with 3% gluten, 1.5% CMC, and 10% indicator with 100% desirability. The results from the optimal sample are displayed in Table 3.

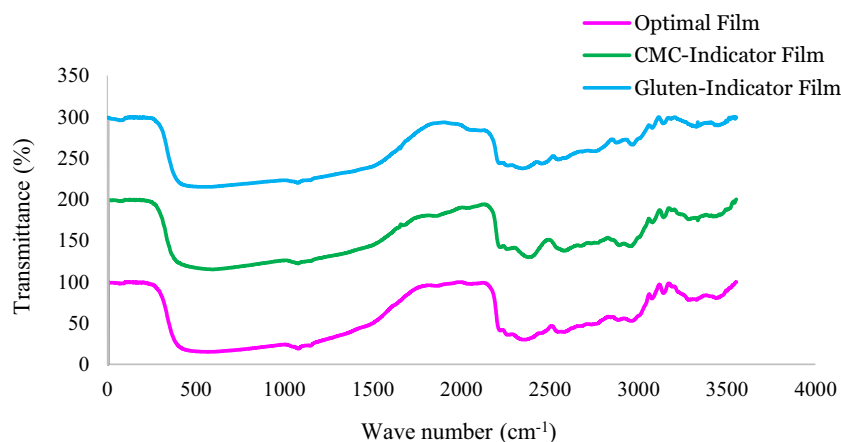
Antioxidant properties

The optimal film inhibited DPPH radical by approximately $61.452 \pm 0.23\%$. The effect of phenolics, flavonoids, and anthocyanins on the antioxidant properties of *H. Sabdariffa* has been evaluated previously [55, 56, 61]. Also, the improvement of antioxidant properties of the composite films with another source of anthocyanins such as red cabbage [10], red barberry [11], and Roselle [42, 62] was observed previously.

Table 3 The results of optimized treatment and experimental tests

	Thickness	L	a	b	WI	YI	MC	SW	TS	EB	ME	WVP
Experimentally determined	0.09 ± 0.005	5.385 ± 0.93	2.08 ± 0.46	16.05 ± 0.24	52.58 ± 3.92	2.63 ± 0.033	1.81 ± 0.037	99.6 ± 2.22	2.91 ± 0.066	165.01 ± 2.68	0.021 ± 0.00	7.58 ± 0.26
Predicted value	0.095 ± 0.005	6.414 ± 0.93	11.73 ± 0.46	12.54 ± 0.24	59.82 ± 3.92	2.28 ± 0.033	1.65 ± 0.037	85.84 ± 2.21	3.87 ± 0.066	149.83 ± 2.68	0.028 ± 0.00	8.72 ± 0.26

Fig. 7 FTIR spectrum of optimal film (gluten/CMC/indicator 3:1.5:10 wt%), gluten film, CMC film



FT-IR spectroscopy analysis (FT-IR)

According to Fig. 7, the broad absorption bands in the range of 3100–3500 cm^{-1} were related to the stretching vibration of O–H (hydroxyl) groups. The asymmetric and symmetric stretching vibrations of the N–H bonds in the amino group can be observed within the wavenumber range of 3500 to 3700 cm^{-1} . This range is significant for characterizing the presence of amino groups in a substance, as it reflects the molecular interactions and bond characteristics of the nitrogen and hydrogen atoms involved [23, 26, 50]. The peak at 2926 cm^{-1} is also related to the stretching vibration of C–H groups [39]. In the gluten film, the main functional groups of gluten i.e. amide I (C=O stretching vibration) at 1650 cm^{-1} and amide II (C–N stretching vibration) at 11540 cm^{-1} were shown, while it disappeared in the second peak, which indicates the integration of gluten with other components in the film [10, 48].

The band at 1640 cm^{-1} is related to the C=O stretching and interaction between C=O of aglycone-pyranoside groups and the C=C stretching vibration of the aromatic ring of anthocyanins [10, 39, 40, 50]. The peaks at 1400 cm^{-1} indicate CH_2 vibration and the peaks at 1008 cm^{-1} correspond to the stretching vibration of the C–O group of the anhydroglucose ring of anthocyanin [10, 40].

The FTIR spectra of the optimal film were similar to control films. Some obvious changes in the FT-IR spectrum occurred after the incorporation of anthocyanin into the film. The important changes in the disappearance of absorption bands in the range of 622–899 cm^{-1} and 1323–1433 cm^{-1} are related to CH_2 bending vibrations and C–C vibrations, respectively. Double bonds of anthocyanins (C=C) appear at 1600–1700 cm^{-1} . Therefore, the increase in intensity of the band at 1640 cm^{-1} can be attributed to the stretching of C=C aromatic bonds in the anthocyanins. These changes confirm the formation of new intermolecular

hydrogen bonds between gluten, CMC, and anthocyanin and the successful stabilization of anthocyanin in the film [60]. Electrostatic interactions and hydrogen bonds are the main interactions between anthocyanins with gluten and CMC [39]. The polysaccharides increased the helical structure of gluten, because of the hydrogen bond between amino groups of gluten and hydroxyl groups of polysaccharides [23, 26].

SEM imaging results (SEM)

SEM images (Fig. 8) show a regular, smooth, compact, and homogeneous structure with good dispersion for optimal film, due to the good interaction between gluten, CMC, and anthocyanin and cross-linking interactions of polysaccharide-protein [39]. On the other hand, the diffusion of compounds in the film was uniform and without accumulation and separation, which shows that anthocyanins were successfully incorporated in the film. No bubbles were observed in the optimal films.

The CMC-indicator film was relatively uniform and smooth with very small cracks, because of the electrostatic repulsion between the compounds [27, 63]. The gluten-indicator film has a porous surface correlated to the destruction of the gluten structure in an acidic environment [23, 71]. FTIR spectra confirmed SEM results. Zhang et al., (2019) showed that the compatibility of starch-polyvinyl alcohol-chitosan films was improved by anthocyanin. Evidence of crystal structure disorder and change in morphology of bacterial cellulose film after stabilization of anthocyanins of *Echium amoenum* was observed [60]. The red cabbage anthocyanins reduce the crystallization of polyvinyl alcohol/sodium carboxymethyl cellulose films [68]. The structural characteristics of polyvinyl alcohol/hydroxypropyl methylcellulose film showed that Roselle anthocyanin is stabilized in the film matrix by hydrogen bonds [62].

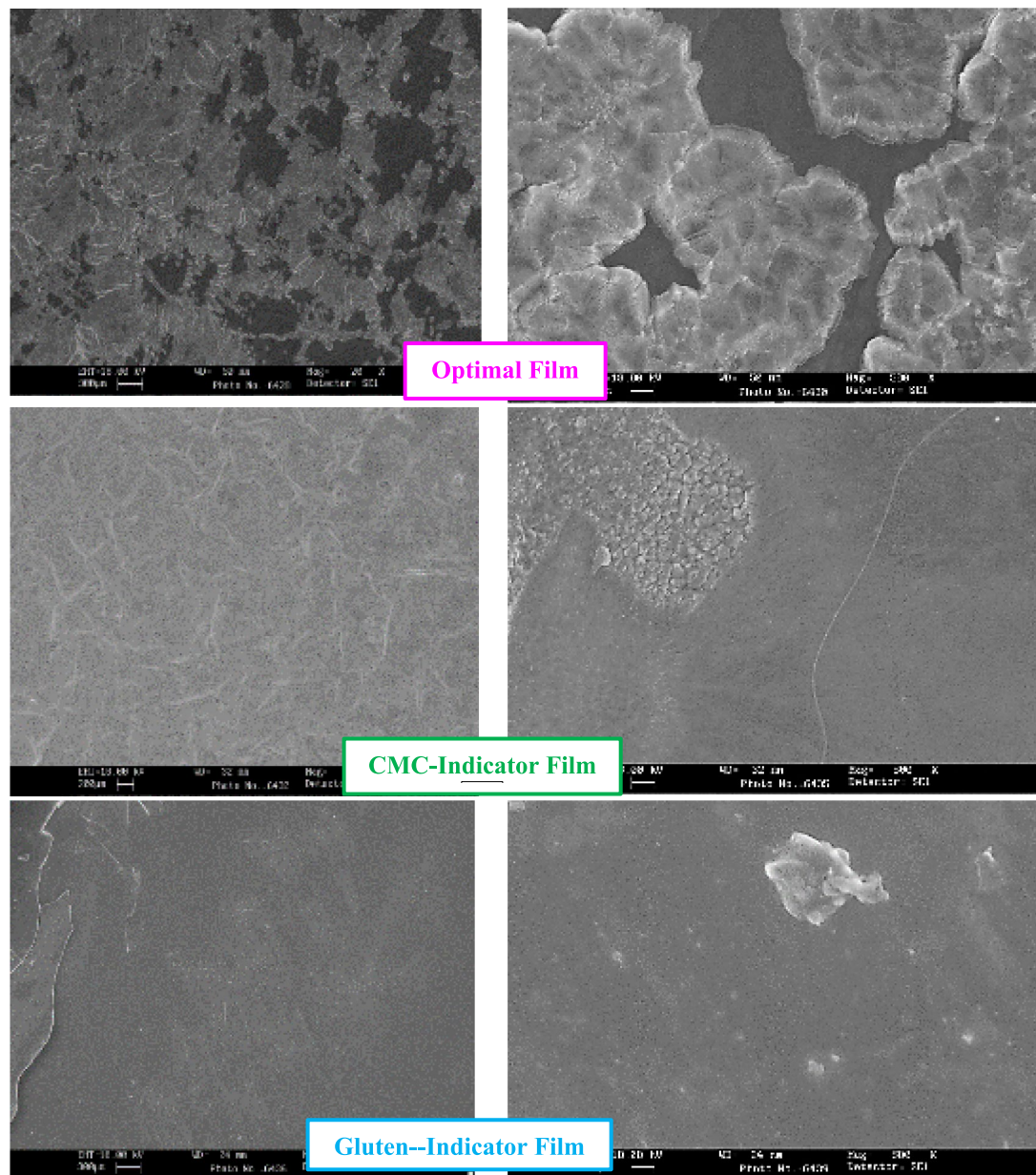


Fig. 8 SEM images of the optimal film, gluten film, and CMC film

X-ray analysis (XRD)

In the optimal film spectrum, the peaks at $2\theta = 22.6^\circ$, 31.8° , 45.5° , 57.3° , and 75.3° were related to anthocyanin. In the CMC-indicator film spectrum, the peaks were at $2\theta = 21.5^\circ$, 31.8° , 45.5° , 58.6° , and 75.3° and in the gluten-indicator film spectrum, the peaks were around $2\theta = 21.4^\circ$, 30.3° , 51.3° , and 1.78° (Fig. 9).

The XRD pattern of the optimal film was similar to the spectrum of CMC-indicator film, which shows that anthocyanin does not have much effect on the crystallinity of the

CMC film structure. The anthocyanin was physically combined with the polymer and there was no chemical interaction between them, so the anthocyanin did not change the physical properties of the film. The gluten matrix exhibits semi-crystalline characteristics due to the presence of intramolecular hydrogen bonds. The optimal films displayed a greater number of diffraction peaks compared to the gluten-indicator film, showed that the enhancement of anthocyanin increased the degree of crystallinity in the gluten film. An increase in the crystallinity of these films can have an impact on their mechanical properties [40].

Fig. 9 XRD patterns of the optimal film, gluten film, and CMC film

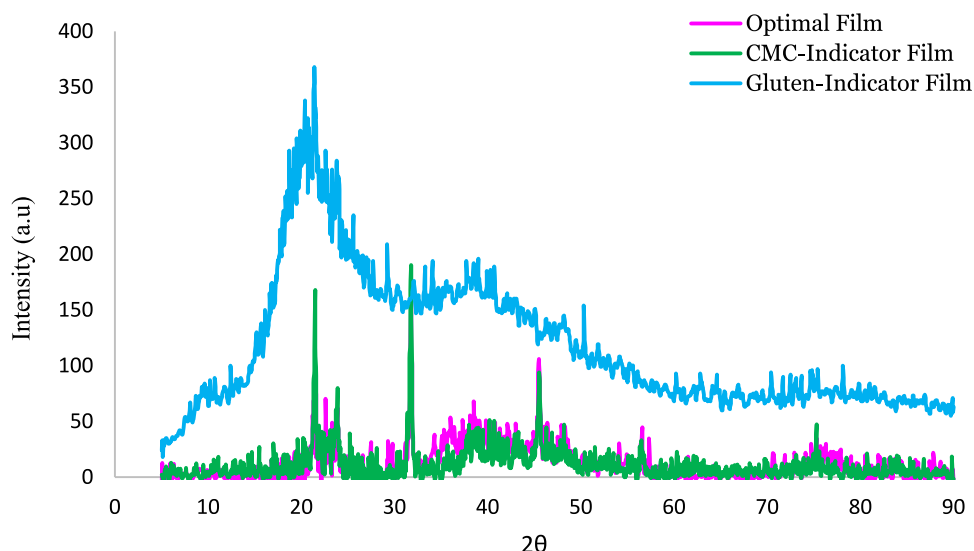
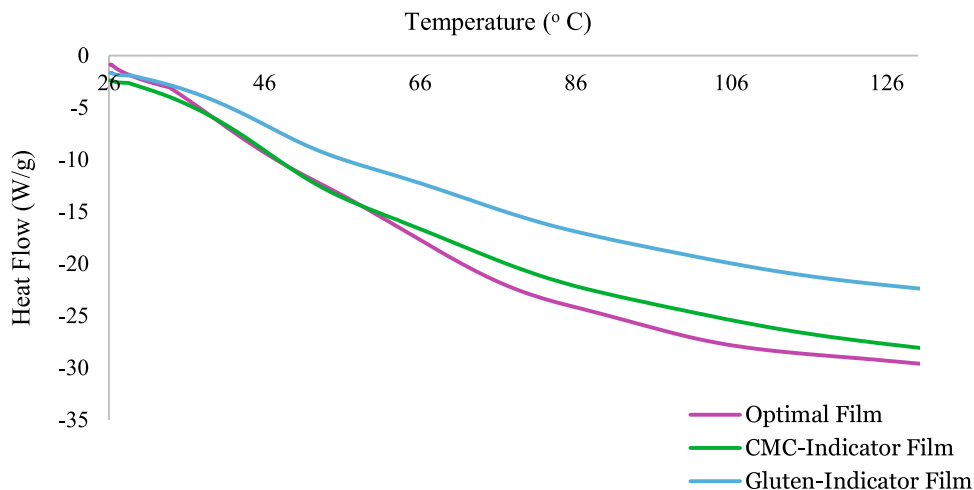


Fig. 10 DSC thermograms of the optimal film, gluten film, and CMC film



In the report of Wu et al. (2020) [50], the incorporation of *Clitoria ternatea* anthocyanins in gellan films did not change the XRD patterns. However, *E. amoenum* anthocyanins [60] and red cabbage anthocyanins [10] reduced the crystallinity and destroyed the original structures of cellulose film and chitosan films, respectively.

DSC analysis

In DSC thermograms, the melting peak is observed as an endothermic peak and is directly linked to the crystalline content of the polymer (Fig. 10). The peak's maximum point, known as the melting temperature (T_m), serves as a critical indicator of the polymer's thermal properties. The optimal film demonstrated a higher T_m , signifying superior thermal stability compared to other films. This increase in T_m , attributed to the incorporation of anthocyanins, underscores a significant enhancement in the film's thermal resilience. Anthocyanins, specifically those extracted from *H. sabdariffa*,

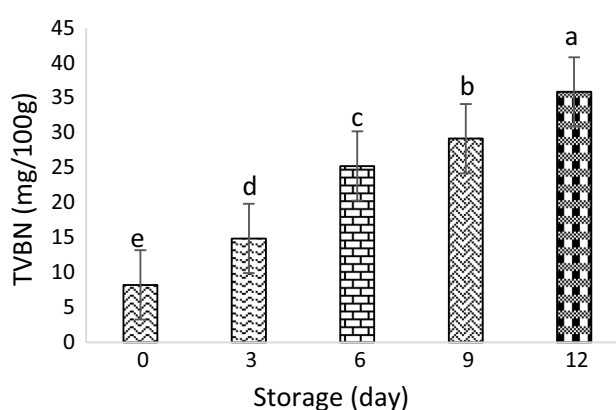


Fig. 11 TVB-N of fish fillets during storage at 4°C

effectively elevate the T_m of nanocomposite films, thereby boosting their thermal properties. This enhancement presents a substantial advantage for the thermal applications

of bio-composites, positioning anthocyanin-infused films as a superior choice for high-temperature environments [56].

TVN-B results and spoilage response films

At the beginning of the experiments, the total volatile base nitrogen (TVB-N) content in the fish was 8.24 mg N/100 g, indicative of high freshness and quality (Chen et al., 2020) (Fig. 11). over 12 days, TVB-N levels increased significantly to 35.87 mg N/100 g, signaling notable spoilage. TVB-N is a critical parameter in assessing fish spoilage, as it includes a range of volatile nitrogenous compounds such as ammonia, methylamine, dimethylamine (DMA), and trimethylamine (TMA). These compounds are produced through microbial activity and the enzymatic degradation of proteins. The established permissible TVB-N limit for fish is 35–40 mg N/100 g. Generally, TVB-N levels below 12 mg N/100 g indicate fresh fish, levels between 12–25 mg N/100 g suggest slight spoilage but are still edible, and values above 25 mg N/100 g denote significant spoilage and inedibility [8]. In the present study, the TVB-N level exceeded the acceptable

limit after 9 days of refrigeration at 4 °C, confirming that the fish was spoiled beyond the safe consumption period. Therefore, the fish could be stored safely for a maximum of 6 days at this temperature before spoilage occurs. Microbial proteolytic enzymes break down both protein and non-protein nitrogen compounds in the fish, leading to the formation of these volatile nitrogenous compounds. The colorimetric films developed for this study effectively detected increases in these volatile bases by exhibiting color changes. These changes correlate with elevated microbial contamination and spoilage, providing a visual indication of spoilage progression. Thus, the films offer a practical and effective method for monitoring fish freshness and ensuring food safety.

The gluten/CMC/*Hibiscus sabdariffa* indicators effectively monitored the freshness of fish fillets, with color changes directly correlating to increasing total volatile base nitrogen (TVB-N) levels (Fig. 12). This supports findings from Aghaei et al., who demonstrated that a polyaniline-based colorimetric indicator also responded to TVB-N with visible color shifts linked to microbial growth. Their research also included a curcumin/bacterial

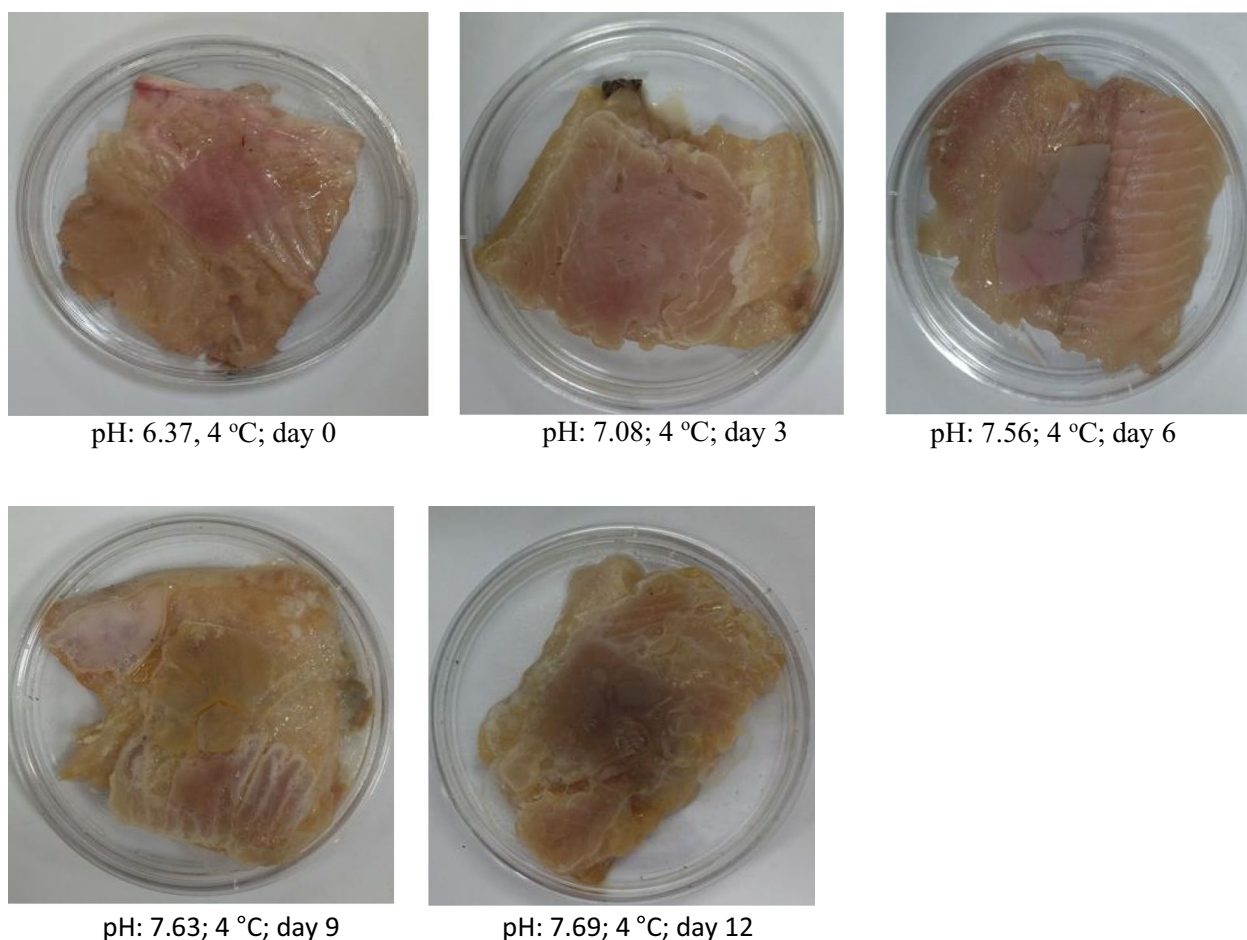


Fig. 12 Visual color changes of composite film as indicator during fish fillets storage at 4 °C

cellulose sensor that detected TVB-N in shrimp, with color changes reflecting pH shifts due to spoilage. Further advancements in spoilage detection include films based on Rose anthocyanins, which changed from purple to dark green at critical TVB-N levels, signaling shrimp spoilage.

Mohammadalinejhad et al. (2020) developed a pH-sensitive detector using anthocyanins from *Echium Amoenum* in bacterial cellulose films. These films displayed a color gradient from purple (fresh) to gray (early spoilage) to yellow (spoiled), corresponding with TVC, TVB-N, and pH changes. Additionally, *Clitoria ternatea* extract incorporated into gellan gum films was used as a pH indicator for shrimp freshness, demonstrating effective color changes in response to spoilage. Resazurin films with gelatin indicators also showed significant color transitions that correlated with changes in TVB-N and pH, providing a reliable method for monitoring seafood quality. These developments highlight the efficacy of various colorimetric and pH-sensitive indicators in detecting spoilage and ensuring food safety.

Conclusion

Anthocyanin in *Hibiscus sabdariffa* had indicator properties due to its color change in acidic and alkaline pH conditions. The addition of anthocyanins increased film thickness, tensile strength, and modulus of elasticity while decreasing elongation at break. Higher anthocyanin levels (30%v/w) reduced moisture content and increased solubility. Models with a high coefficient of determination (R^2) demonstrated strong alignment between experimental and model data, confirming that the concentration of all independent variables significantly impacted the results. Run 6, containing 3% gluten, 1.5% CMC, and 10% anthocyanin, was the optimal treatment. This formulation exhibited the lowest yellowness index (YI), water vapor permeability (WVP), swelling (SW), and modulus of elasticity (ME), while achieving the highest tensile strength (TS), redness (a^*), and thermal stability. The successful integration of *Hibiscus sabdariffa* anthocyanins into gluten/CMC composites highlights their effectiveness in bio-composite films and colorimetric indicators. These films showed pronounced color changes in fish fillets corresponding to variations in total volatile base nitrogen (TVB-N) levels, making them a reliable tool for detecting fish spoilage and enhancing food safety.

Author contributions Neda Fallah: Conceptualization, Methodology, Formal analysis, and Investigation, Writing- original draft preparation, Writing- review and editing, Resources.

Aazam Aarabi: design experiment, Validation, writing manuscript, Supervision.

Hassan Zaki Dizaji: Methodology, Validation.

Majid Ghashang: Methodology of XRD and analysis of results, Writing- review and editing,

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Data availability Data openly available in a public repository that issues datasets with DOIs.

Declarations

Competing interest The authors have no competing interests to be declared.

References

1. P. Shao, L. Liu, J. Yu, Y. Lin, H. Gao, H. Chen, P. Sun, Trends Food Sci. Technol. **118**, 285–296 (2021)
2. Z. Teymouri, H. Shekarchizadeh, Food Packag. Shelf Life **33**, 100884 (2022)
3. X. Luo, A. Zaitoon, L.T. Lim, Comprehensive reviews in food science and food safety. **21**, 2489–2519 (2022)
4. Y. Zou, Y. Sun, W. Shi, B. Wan, H. Zhang, Food Chem. **399**, 133962 (2023)
5. X. Zhai, J. Shi, X. Zou, S. Wang, C. Jiang, J. Zhang, X. Huang, W. Zhang, M. Holmes, Food Hydrocolloid **69**, 308–317 (2017)
6. C. Shi, J. Zhang, Z. Jia, X. Yang, Z. Zhou, J. Sci. Food Agric. **101**, 1800–1811 (2021)
7. M. Ghaani, C.A. Cozzolino, G. Castelli, S. Farris, Trends Food Sci. Technol. **51**, 1–11 (2016)
8. P. Müller, M. Schmid, Foods **8**, 16 (2019)
9. M.E. Genovese, J. Zia, D. Fragouli, in: Sustainable food packaging technology. 369–393 (2021)
10. M. Chen, T. Yan, J. Huang, Y. Zhou, Y. Hu, Int. J. Biol. Macromol. **179**, 90–100 (2021)
11. M.A. Sani, M. Tavassoli, H. Hamishehkar, D.J. McClements, Carbohydr. Polym. **255**, 117488 (2021)
12. I. Ahmed, H. Lin, L. Zou, Z. Li, A.L. Brody, I.M. Qazi, L. Lv, T.R. Pavase, M.U. Khan, S. Khan, L. Sun, Packag. Technol. Sci. **31**, 449–471 (2018)
13. G. Jiang, X. Hou, X. Zeng, C. Zhang, H. Wu, G. Shen, S. Li, Q. Luo, M. Li, X. Liu, A. Chen, Z. Wang, Z. Zhang, Int. J. Biol. Macromol. **143**, 359–372 (2020)
14. A.G. da Silva Carvalho, M.T. da Costa Machado, V.M. da Silva, A. Sartoratto, R.A.F. Rodrigues, M.D. Hubinger, Powder Technol. **294**, 421–428 (2016)
15. C.A. Morais, V.V. de Rosso, D. Estadella, L.P. Pisani, J. Nutr. Biochem. **33**, 1–7 (2016)
16. T. Tsuda, Mol. Nutr. Food Res. **56**, 159–170 (2012)
17. A. Sinela, N. Rawat, C. Mertz, N. Achir, H. Fulcrand, M. Dornier, Food Chem. **214**, 234–241 (2017)
18. D.-L. Aurelio, R.G. Edgardo, S. Navarro-Galindo, Int. J. Food Sci. Technol. **43**, 322–325 (2008)
19. S. Cichosz, A. Masek, M. Zaborski, Polym. Test. **67**, 342–348 (2018)
20. F. Silva, N. Gracia, B.H. McDonagh, F.C. Domingues, C. Nerin, G. Chinga-Carrasco, J. Mater. Sci. **54**, 12159–12170 (2019)
21. F. Zubeldía, M.R. Ansorena, N.E. Marcovich, Polym. Testing **43**, 68–77 (2015)
22. R. Liu, X. Cong, Y. Song, T. Wu, M. Zhang, J. Food Sci. **83**, 1622–1630 (2018)
23. S. Khashayary, A. Aarabi, Food Bioprocess Tech **14**, 665–678 (2021)

24. N. Singh, D.M.R. Georget, P.S. Belton, S.A. Barker, J. Cereal Sci. **52**, 282–287 (2010)
25. J. Duan, Q. Zhou, M. Fu, M. Cao, M. Jiang, L. Zhang, X. Duan, Food Bioprocess Tech **16**, 2443–2454 (2023)
26. N. Najafian, A. Aarabi, A. Nezamzadeh-Ejhieh, Int. J. Biol. Macromol. **223**, 1257–1267 (2022)
27. M. Dong, L. Tian, J. Li, J. Jia, Y. Dong, Y. Tu, X. Liu, C. Tan, X. Duan, LWT **154**, 112868 (2022)
28. J. Zink, T. Wyrobnik, T. Prinz, M. Schmid, Int. J. Mol. Sci. **17**, (2016)
29. M.S.A. Rani, S. Rudhzhiah, A. Ahmad, N.S. Mohamed, Polymers **6**, 2371–2385 (2014)
30. M. Tabari. Investigation of carboxymethyl cellulose (CMC) on mechanical properties of cold water fish gelatin biodegradable edible films. Foods **6**, (2017)
31. S. Van Haute, K. Raes, P. Van der Meeren, I. Sampers, Food Control **68**, 30–39 (2016)
32. J. Das, H.N. Mishra, J Food Meas Charact **17**, 5462–5462 (2023)
33. D.P. Crane, C.C. Killourhy, M.D. Clapsadl, Fish. Res. **175**, 142–147 (2016)
34. E. Prasetyo, N. Suciati, C. Fatchah, Aminin, E. Pardede, Ecological Inform. **80**, 102533 (2024)
35. S. Raeisi, M. Sharifi-Rad, S.Y. Quek, B. Shabanpour, J. Sharifi-Rad, Food Sci. Tech-LWT **65**, 112–121 (2016)
36. L. Gram, P. Dalgaard, Curr. Opin. Biotechnol. **13**, 262–266 (2002)
37. S. Sampels, J Food Process Pres **39**, 1206–1215 (2015)
38. V. Bagheri, B. Ghanbarzadeh, A. Ayaseh, A. Ostadrahimi, A. Ehsani, M. Alizadeh-Sani, P.A. Adun, Polym. Testing **78**, 105989 (2019)
39. B. Merz, C. Capello, G.C. Leandro, D.E. Moritz, A.R. Monteiro, G.A. Valencia, Int. J. Biol. Macromol. **153**, 625–632 (2020)
40. F.N. Eze, T.J. Jayeoye, S. Singh, Food Chem. **366**, 130574 (2022)
41. F. Kiani Aliabadi, A. Ahmadi Dastgerdi, J. Tabatabaeian Nima-vard, J. Food Qual. **2023**, 6263692 (2023)
42. J. Zhang, X. Zou, X. Zhai, X. Huang, C. Jiang, M. Holmes, Food Chem. **272**, 306–312 (2019)
43. M.M. Giusti, R.E. Wrolstad, Current protocols in food analytical chemistry. 00, F1.2.1-F1.2.13 (2001)
44. S. Omid, A. Aarabi, H. Zaki Dizaji, F. Shahdadi, J. Food Meas. Charact. **18**, 2505–2525 (2024)
45. M. Farsi, A. Nezamzadeh-Ejhieh, Surf. Interfaces **32**, 102148 (2022)
46. G.A. Valencia, C.G. Luciano, R.V. Lourenço, A.M.Q.B. Bittante, P.J. do Amaral Sobral, Food Pack. Shelf Life. **19**, 24–30 (2019)
47. N. Fallah, M.M. Marvizadeh, R. Jahangiri, A. Zeinalzadeh, A. Mohammadi Nafchi, J.f Chem. Health Risks **13**, 299–304 (2023)
48. N. Najafian, A. Aarabi, A. Nezamzadeh-Ejhieh, Iran J. Chem. Chem. Eng. 601–617 (2022)
49. H. Derikvand, A. Nezamzadeh-Ejhieh, J. Colloid Interf Sci. **490**, 652–664 (2017)
50. L.-T. Wu, I.L. Tsai, Y.-C. Ho, Y.-H. Hang, C. Lin, M.-L. Tsai, F.-L. Mi, Carbohydr. Polym. **254**, 117410 (2021)
51. N. Fallah, A. Aarabi, H. Zaki Dizaji, M.Ghashang. Food Bio-process Technol. **18**, 4632–4648 (2025)
52. I. Da-Costa-Rocha, B. Bonnlaender, H. Sievers, I. Pischel, M. Heinrich, Food Chem. **165**, 424–443 (2014)
53. S.C.S.R. de Moura, C.L. Berling, S.P.M. Germer, I.D. Alvim, M.D. Hubinger, Food Chem. **241**, 317–327 (2018)
54. C. Grajeda-Iglesias, E. Salas, N. Barouh, B. Baréa, A. Panya, M.C. Figueroa-Espinoza, Food Chem. **194**, 749–757 (2016)
55. P.-J. Tsai, J. McIntosh, P. Pearce, B. Camden, B.R. Jordan, Food Res. Int. **35**, 351–356 (2002)
56. E. Prenesti, S. Berto, P.G. Daniele, S. Toso, Food Chem. **100**, 433–438 (2007)
57. P.K. Wong, S. Yusof, H.M. Ghazali, Y.B. Che Man, Nutr. Food Sci. **32**, 68–73 (2002)
58. A. Castañeda-Ovando, M.d.L. Pacheco-Hernández, M.E. Pérez-Hernández, J.A. Rodríguez, C.A. Galán-Vidal, Food Chem. **113**, 859–871 (2009)
59. L.A. Toro-Márquez, D. Merino, T.J. Gutiérrez, Food Bioprocess Tech **11**, 1955–1973 (2018)
60. S. Mohammadalinejad, H. Almasi, M. Moradi, Food Control **113**, 107169 (2020)
61. C.-C. Chen, F.-P. Chou, Y.-C. Ho, W.-L. Lin, C.-P. Wang, E.-S. Kao, A.-C. Huang, C.-J. Wang, J. Sci. Food Agric. **84**, 1989–1996 (2004)
62. J. Huang, J. Liu, M. Chen, Q. Yao, Y. Hu, Int. J. Biol. Macromol. **184**, 666–677 (2021)
63. D. Liu, Z. Cui, M. Shang, Y. Zhong, Food Packag. Shelf Life **28**, 100641 (2021)
64. S. Asadi, S. Pirs, J. Polym. Environ. **28**, 433–444 (2020)
65. A. Aydogdu, C.J. Radke, S. Bezci, E. Kirtil, Int. J. Biol. Macromol. (2020)
66. R.B.V. Micard, M.-H. Morel, S. Guilbert, J. Agric. Food Chem. **48**, 2948–2953 (2000)
67. N. Fallah, N. Nabeghvatan, T. Sadeghi, A. Etemadi Razlighi, M.M. Marvizadeh, A. Mohammadi Nafchi, J. Chem. Health Risks (2023)
68. J. Liu, K. Li, Y. Chen, H. Ding, H. Wu, Y. Gao, S. Huang, H. Wu, D. Kong, Z. Yang, Y. Hu, Food Hydrocolloid **133**, 107979 (2022)
69. A. Aarabi, H. Ebad Idehaghani, S. Saiedi, J. Food Hygiene **5**, 1–12 (2015)
70. T. Tamiji, A. Nezamzadeh-Ejhieh, J. Electroanal. Chem. **829**, 95–105 (2018)
71. P. Thivya, Y.K. Bhosale, S. Anandakumar, V. Hema, V.R. Sinija, Food Chem. **390**, 133221 (2022)

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