Production of pH-sensitive natural decomposition indicator with whey protein isolate-based edible film to monitor fish freshness

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ABSTRACT Packaging and indicators are among the important references of information that give an idea about the quality and freshness of the food. In this study, the freshness of the fish was monitored by producing a pH-sensitive decomposition indicator with edible films based on whey protein isolate with the addition of hibiscus tea. Significant differences were detected between normal (N) films and Hibiscus-added films (H3, H6). Hibiscus tea (HT) was added to the film solutions after heat treatment due to its high acidity. All films have become structurally not-resistant by absorbing moisture from the fish. It was observed that H6 was more successful in terms of a color indicator than other films, and later followed by H3 films.

KEYWORDS Hibiscus; pH indicator; Color; Edible film

1. Introduction

Along with parameters such as label information and product image, the freshness and quality of the food can also determine the consumers purchasing preferences (Balbinot-Alfaro et al., 2019; Roy and Rhim, 2021). In this direction, lately the use of technologies such as edible film packaging and natural indicators notice consumers by giving an idea about the freshness and quality of the food before consumption. Color indicators are used to show the color changes that occur as a result of microbial deterioration during the shelf life of the food (Pereira et al., 2015; Fang et al., 2017; Choi et al., 2017; Huang et al., 2019).

Indicators can be of synthetic or natural origin. In recent years, especially the preference for vegetables and fruits containing anthocyanins (hibiscus, blueberry, blackberry, red cabbage, black grape, red beet, purple carrot, purple onion, etc.) which have a natural coloring feature, as an indicator in the food sector has gained great momentum (Huang et al., 2019; Roy and Rhim, 2021). Thanks to they contain the phenolic components, films obtained from these products appear in different colors by undergoing structural changes at different pH values (Choi et al., 2017).

Fresh fish can deteriorate by undergoing many changes (physical, chemical, microbiological) from the moment it is taken out of the water. Especially, changes due to microbial contamination and enzymatic reactions further increase the level of deterioration (Ertaş, 1981; Huang et al., 2019). In this context, it is necessary to ensure the traceability of the fish in the storage and logistics and take the necessary precautions. As the fish begin to deteriorate (microbial), volatile nitrogen compounds (ammonia, amines, etc.) are released in the environment, increasing the pH of the food. At the same time a noticeable color change occurs in the natural color indicator used (pH sensitive) (Byrne et al., 2002; Pektaş, 2013).

The main purpose of this study is to contribute to the studies on the production of natural deterioration indicators sensitive to pH change. Hibiscus exhibits different color combinations at different pH values due to its high anthocyanin content. Whey protein isolates polymer material and hibiscus herbal tea were used to prepare natural, non-toxic pH-sensitive calorimetric color indicator films. With the sensitive color indicators developed in this direction, fish deterioration can be visually predicted in real-time.

2. Methods

2.1 Materials

Hibiscus (*Hibiscus sabdariffa* L.) plant was obtained from Bagdat Spice Company and anchovy (Engraulis encrasicolus) from a local fisherman in Iğdir. Whey protein isolate (Davisco Foods International Inc.-USA Hardline Nutrition 96 %), glycerol (Sigma Aldrich-15524) as plasticizer, and NaOH (Merck-106462) components were used to adjust pH values for edible film production.

2.2 Processes

2.2.1 Preparation of herbal tea

Hibiscus tea (HT) is brewed with hot water infusion. For this, the plant was crushed in a mortar and turned into powder. Then, 5 g of sample was weighed and transferred to a beaker. 50 mL (1:10 w/v) hot water was added and mixed in a magnetic stirrer for 10 minutes. The filtrate was then passed through filter paper, allowed to cool, and then used in the production of edible films (Sağdıç and Özcan, 2003). HT was prepared fresh on each analysis day.

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Table 1: Formulations of edible film groups.

Group	WPI (g)	G (mL)	pH	H (mL)	T (°C)	Time (min)
N	5	5	7-8	-	85	25
H3	5	5	8-8.5	3	85	25
H6	5	5	8-8.5	6	85	25

N: normal film (without addition of hibiscus)
H3: 3 % hibiscus added
H6: 6 % hibiscus added
G: glycerol, H: hibiscus, T: temperature

2.2.2 Edible film preparation

Whey protein isolate (WPI), 5 % (w/v) aqueous solution was prepared. 5 mL of glycerol was added to the solution and homogenized on a magnetic stirrer. The solution, which was brought to the pH range of 7-8 with 4 N NaOH, was then applied to a double boiler at 85 °C for 25 minutes. Then, the solution cooled to room temperature was poured into plastic petri dishes (25 mL) and left to dry for 36-48 hours (Kim and Ustunol, 2001).

2.2.3 Preparation of indicator edible film with added hibiscus tea

The pH was adjusted to the 8-8.5 band with 4 N NaOH by preparing a 5 % (w/v) aqueous solution of WPI. Then, glycerol (5 mL) was added and mixed. The solution, which was heat treated at 85 °C for 25 minutes, was cooled to room temperature. Then, HT was added little by little at different concentrations (3 %, 6 %) and centrifuged at 5000 rpm for 5 minutes. The resulting supernatant was poured (25 mL) into plastic petri dishes and left to dry for 36-48 hours (Kim and Ustunol, 2001; Kavrut, 2022).

Edible films were prepared in 3 different formulations and applied to food (Table 1).

2.2.4 Application of edible films for fish freshness

The prepared films were divided into 4 equal parts and glued to the middle of plastic-lidded transparent bowls containing approximately 50 g of fish (Figure 1). The bowls were then stored in a controlled room at 25 °C for 3 days. In this way the color changes of edible films applied to fish in different conditions (storage, logistics, etc.) will be seen quickly. The whole study was performed in 3 replications.

2.3 Color measurements of films

Color variations between films (N, H3, H6) were calculated with the aid of a Konica Minolta (CR-400, Osaka, Japan) meter combined with an 8 mm scaling zone and D65 illuminator in the CIE L^* a^* b^* color range. Color measurements were performed by instrument calibration. L^*, a^*, b^* values were recorded by taking the average of 3 different measurements of each film (Dadali et al., 2007).

2.4 Moisture measurements of films

Moisture values were measured with a rapid moisture meter (M0C63u Shimadzu). Moisture values were recorded by taking about 100 g from each film sample.

2.5 Microbiological analysis

25 g of each group sample was weighed and homogenized in 225 mL of 0.1 % peptone water. Then, inoculations were made on Plate Count Agar (PCA, Oxoid CM 325) medium using the cast plate method. Petri dishes were incubated at 37 °C for 18-24 hours. After then, microbial counts were reported as log (cfu/g) (Harrigan, 1998).

2.6 pH analysis of fish

The pH measurements of the fish were made with a pH meter (C3040 Consort). Accordingly, 10 g samples were weighed and homogenized in 100 ml distilled water. Then, the values measured with a pH meter were recorded (Gökalp et al., 1999).

2.7 Statistical analysis

The Multivariate procedure of General Linear Models analysis was applied to the data of the groups. The data were tested by Duncan multiple comparison method. SPSS 20 program was used in statistical analysis.

3. Results

3.1 Processes

While preparing edible film solutions, different formulations were applied for normal and hibiscus- added films (HAF) (Figure 2). It was not added to the solutions immediately, especially due to the acidic character of the hibiscus (pH: 2-3) and the problems of being exposed to structural deterioration due to heat treatment. The prepared solutions were dried in petri dishes to have a smooth structure. Anker et al. (2000), emphasized that drying materials are important for the films to have a ho-

Figure 1: Application of indicator films to food.

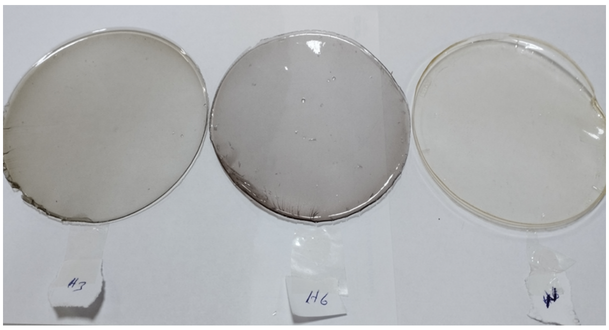

Figure 2: Images of the films (respectively H3, H6 and N).

mogeneous structure. WPI and plasticizing agents were used at a ratio of 1:1 in film making. Proportional differences (increase-decrease) between protein concentration and glycerol cause changes in the structure of the films. Indeed, Anker et al. (2000) stated that the change in protein amount can affect the critical gel concentration of proteins and change the mechanical, barrier and microstructure properties of the films. To achieve maximum performance in film production, the normal film was prepared in the pH range of 7-8, and hibiscus in the range of 8-8.5. With the effect of the applied heat treatment, the serum proteins in the WPI are rapidly denatured and precipitated by the effect of the acid. Akgül and Karaman (2017), reported that serum proteins contain many macro and micro proteins in themselves and undergo structural changes with the effect of temperature.

Hibiscus is a product with a bright red color. The abundant anthocyanins it contains are responsible for the coloration of the plant (Jabeur et al., 2017). The plant appears in different colors at different pH values. The H3 and H6 groups of edible films produced have different pH and colors due to the different concentrations of HT. Zhang et al. (2019) and Liu et al. (2021), determined that hibiscus has a color indicator that goes from red to green at different pH values.

3.2 Color measurements of films

Colors of foods come first among the parameters that affect the product purchasing criteria of consumers. Color values of edible films are given in Table 2. It was observed that the L^* value was on the 3rd day according to the days, and the H6 group made a statistically significant difference in terms of the groups $p < 0.05$. It was observed that the L^* value decreased the most in the H6 group (59.84) and the color gradually turned black Figure 4a. H6 was followed by H3 (63.43). Visually, the hibiscus groups are opaque, while the control is clear. According to whether the films contain hibiscus or not all storage days are important on a^* values, Figure 4b, In b^* value, the first day was found to be significant ($p < 0.05$) Figure 4c. This is particularly evident in Figure 3. As the deterioration levels of the fish increased, normal films retained their original color, while films containing hibiscus changed color (green). While the a^* value was less green (4.14) on the first day of storage, it decreased faster than the other groups on the last day of storage (1.15). b^* is bluer than the other groups on the last day of storage with a value of 5.83. This is due to the behavior of anthocyanins in hibiscus against pH change. Kang et al. (2018) stated that anthocyanin-containing films react to volatile ammonia formed as a result of degradation. Similarly, Zhang et al. (2019) stated that many volatile organic amines are released as a result of the deterioration of protein-containing foods. The released nitrogenous compounds cause color change in the films used as color indicators.

3.3 Moisture measurement results of films

The moisture values of the groups are given in Table 3. All groups absorbed moisture from the fish (68.31 %) during the storage days. This situation created a statistically significant difference ($p < 0.05$). As the moisture value

Table 2: Color parameters of the groups (L^* a^* b^*).

Feature	Group	1. Day	2. Day	3.Day
L^*	N	88.29$\pm$0.08^{Aa}	72.20$\pm$0.90^{Ba}	71.09$\pm$0.29^{Ca}
	H3	78.17$\pm$0.51^{Ab}	64.85$\pm$1.50^{Bb}	63.43$\pm$1.12^{Bb}
	H6	77.81$\pm$0.22^{Ab}	62.18$\pm$1.43^{Bc}	59.84$\pm$1.18C^{c}
a^*	N	-0.58$\pm$0.01^{Ac}	-0.59$\pm$0.06^{Ac}	-0.69$\pm$0.10^{Ac}
	H3	1.42$\pm$0.04^{Ab}	0.92$\pm$0.23^{Bb}	0.37$\pm$0.31^{Cb}
	H6	4.14$\pm$0.04^{Aa}	2.78$\pm$0.76^{Ba}	1.15$\pm$0.28^{Ca}
b^*	N	4.65$\pm$0.06^{Bb}	5.66$\pm$0.32^{Aa}	6.24$\pm$0.39^{Aa}
	H3	5.18$\pm$0.04^{Aa}	6.53$\pm$0.72^{Aa}	5.99$\pm$0.99^{Aa}
	H6	1.60$\pm$0.06^{Bc}	6.07$\pm$0.27^{Aa}	5.83$\pm$0.12^{Aa}

L^* denotes whiteness/blackness, a^* redness/greenness and b^* yellowness/blueness levels of films.
N: normal film (without addition of hibiscus)
H3: 3 % hibiscus added
H6: 6 % hibiscus added
The averages shown with the same capital letter in the same column are statistically indistinguishable from each other (according to storage days).
The averages shown with the same lowercase letter in the same row are statistically indistinguishable (according to group type).

increased, the films decomposed. H6 was observed as the most damaged group and N as the least damaged group. Plant extracts and water included in the film structure interact with polymers (whey protein isolate) and change the mechanical properties of the films (Mahcene et al., 2020). The addition of hibiscus caused the films to absorb more moisture. The results are consistent with the data of Aydin and Yildiz (2022) study in which *Hibiscus sabdariffa* L. extract was added to composite films.

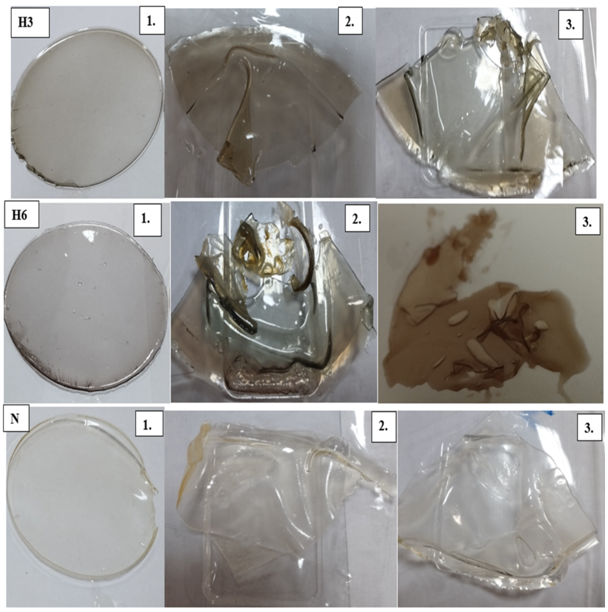

Figure 3: Images of the storage days (1,2,3) of the films (respectively H3, H6 and N).
N: normal film (without addition of hibiscus)
H3: 3 % hibiscus added
H6: 6 % hibiscus added

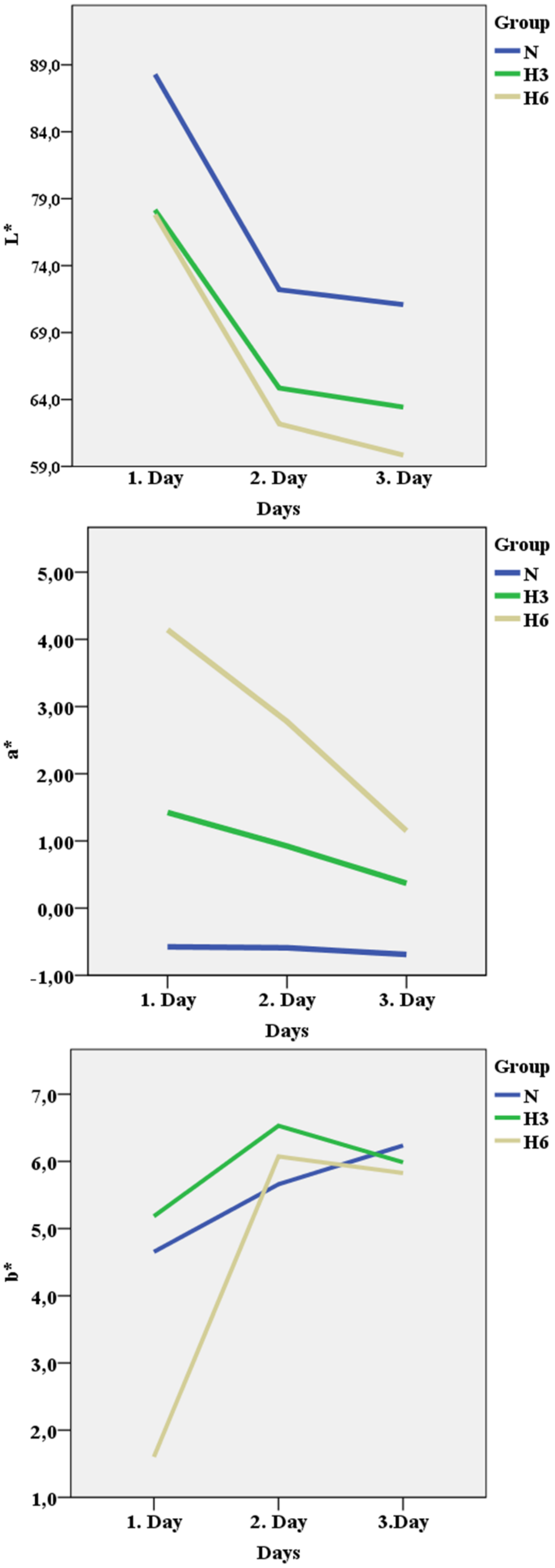

Figure 4: Graph of L^* values of the groups (a). Graph of the a^* values of the groups (b). Graph of the b^* values of the groups (c)

The texture and appearance of edible films show their harmony with organoleptic properties (Han, 2002). Films

Table 3: Moisture values of the groups.

Feature	Group	1. Day	2. Day	3. Day
Moisture	N	10.13 ± 0.35^{Ca}	53.73 ± 1.30^{Ba}	66.46 ± 1.07^{Ab}
	H3	6.42 ± 0.32^{Cb}	52.26 ± 4.03^{Ba}	66.93 ± 1.16^{Ab}
	H6	5.37 ± 0.11^{Cc}	56.79 ± 1.83^{Ba}	74.12 ± 2.09^{Aa}

N: normal film (without addition of hibiscus)
H3: 3 % hibiscus added
H6: 6 % hibiscus added
The averages shown with the same capital letter in the same column are statistically indistinguishable from each other (according to storage days). The averages shown with the same lowercase letter in the same row are statistically indistinguishable (according to group type).

Table 4: The mesophyll microorganism counts of the groups (log cfu/g).

Feature	Group	1. Day	2. Day	3. Day
Mo	N	4.79 ± 0.08^{Ca}	7.49 ± 0.52^{Ba}	8.78 ± 0.78^{Aa}
	H3	4.79 ± 0.08^{Ca}	7.27 ± 0.41^{Ba}	8.63 ± 0.75^{Aa}
	H6	4.79 ± 0.08^{Ca}	7.21 ± 0.88^{Ba}	8.73 ± 0.75^{Aa}

N: normal film (without addition of hibiscus)
H3: 3 % hibiscus added
H6: 6 % hibiscus added
The averages shown with the same capital letter in the same column are statistically indistinguishable from each other (according to storage days).
The averages shown with the same lowercase letter in the same row are statistically indistinguishable (according to group type).

produced from whey proteins have poor moisture barrier properties (Ramos et al., 2012). It has been observed that the films applied to the fish absorb moisture from the food during storage and their hard structure softens (Table 3). However, plasticizers added to the film solution have an effect on the moisture barrier of the films (Kokoszka et al., 2010). It was preferred to use glycerol in equal amounts with protein in terms of its hydrophilic property and its negative effect on the moisture barrier of the films.

3.4 Microbiological analysis

The number of microorganisms increased day by day and this was statistically significant ($p < 0.05$) (Table 4). While all groups were at 4 logarithm levels at the beginning, they increased (doubled) to 8 logarithms on the 3rd day. In terms of groups, the presence of hibiscus was ineffective on microbial activity ($p > 0.05$). But it is effective in terms of usability as a color indicator. The antimicrobial effect of hibiscus may be due to contact with the product to which it is applied. Kavrut (2022), determined that the edible hibiscus films used as an interlayer in hamburger meatballs have high antimicrobial activity at the contact points.

3.5 Fish pH analysis results

The pH values of edible films are given in Table 5. The pH values of the films containing hibiscus increased more than the normal film. This situation; As a result of micro-

Table 5: The mesophyll microorganism counts of the groups (log cfu/g).

Feature	Group	1. Day	2. Day	3. Day
	N	6.38 ± 0.03^{Ca}	6.54 ± 0.12^{Bb}	7.23 ± 0.04^{Aa}
pH	H3	6.38 ± 0.03^{Ca}	6.79 ± 0.07^{Ba}	7.30 ± 0.08^{Aa}
	H6	6.38 ± 0.03^{Ca}	6.71 ± 0.07^{Bab}	7.28 ± 0.02^{Aa}

N: normal film (without addition of hibiscus)
H3: 3 % hibiscus added
H6: 6 % hibiscus added
The averages shown with the same capital letter in the same column are statistically indistinguishable from each other (according to storage days).
The averages shown with the same lowercase letter in the same row are statistically indistinguishable (according to group type).

bial activity, nitrogenous compounds accumulated in the environment caused the pH to increase and the color of the indicator films (with hibiscus) to change. Kang et al. (2018) and Zhang et al. (2019), reported that components such as ammonia and amine accumulated in the environment are effective in increasing the pH of edible films. The pH change was found to be statistically significant according to the days ($p < 0.05$). Foods can show a rapid deterioration reaction with the change of pH values (Morris, 2000; Aksan, 2017). Fish are among the perishable products. Proteolytic microorganisms and protease enzymes are effective on pH values during storage (Mokhtar et al., 2014).

4. Conclusions

In this study natural, biodegradable edible films sensitive to pH were prepared in different formulations. It was concluded that films with the addition of hibiscus can be used as a color indicator in perishable foods. It was observed that the color change in the films was faster and more visible, especially due to the increase in hibiscus concentration. Due to the acidic character of whey protein isolate and hibiscus components, it is very difficult to use them together in the same environment. In this context, the structural properties of the indicator films to be prepared should be known. However, it has been observed that films with hydrophilic character can absorb moisture from the food and cause structural deterioration. It should be considered that especially its use on foods with high moisture surfaces will cause fragmentation in the films. It has been determined that the microbial inhibitory properties of hibiscus films that do not come into contact with food are poor. As a result, edible films with hibiscus can be a guide for their use in the food industry as a natural, non-toxic color indicator.

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