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Monitoring the Freshness of Rainbow Trout Using Intelligent PH-sensitive Indicator During Storage

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ABSTRACT

Background: The rainbow trout fish is susceptible to spoilage due to its high content of unsaturated fatty acids. It should be kept at low temperature to reduce microbial, enzymatic, and oxidation reactions. The purpose of this study was to design a packaging that contains a pH indicator for monitoring freshness of the rainbow trout fish during storage at refrigerator. **Methods:** The indicator contained agarose as the carrier, bromocresol green as pH indicator, and silica as surface provider. It was covered by polypropylene film and attached inside the package. Freshness of the trout stored in the refrigerator was assessed by chemical (total volatile basic nitrogen and pH) and microbiological (total viable count) methods. **Results:** The pH of fish gradually decreased after the third day since color of the indicator changed from yellow to green on day 3 and then to blue on day 6. The indicator's response was correlated with changes in the microbial population and also with levels of total volatile basic nitrogen and pH. The results showed that the designed indicator was sensitive to different pH levels and could be applied as part of the intelligent packaging system. **Conclusion:** The freshness indicator worked well before the expiry date of fish, which makes it suitable for food quality assessment. So, this indicator can be used for real-time monitoring of packaged fish freshness.

Keywords: *Rainbow trout; pH freshness indicator; Intelligent packaging; Silica nanoparticles*

Introduction

The fish genus Oncorhynchus (trout) has been farmed for hundreds of years. Rainbow trout (Oncorhynchus mykiss) is the most widely farmed trout in the world, which belongs to the salmonidae family (Hardy, 2002) and is related to pacific salmon. In recent years, the consumers and manufacturers' demands have increased for development of technologies intended to ensure the

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safety and to evaluate the real-time shelf life of the food products (Kerry *et al.*, 2006). Intelligent packaging is able to give some information about the food quality before or at the point of consumption (Mohebi and Marquez, 2015, Otles and Yalcin, 2008). Intelligent packaging is defined as a system that monitors the condition of packaged foods and gives information about quality of the packaged food during transport and storage (Kerry, 2012, Kerry *et al.*, 2006, Otles and Yalcin, 2008). Functions of intelligent packaging include detecting, sensing, recording, tracing, communicating information, and warning consumers or food manufacturers about possible problems (Raymana *et al.*, 2016). In this system, sensors, indicators (including integrity, freshness, and time-temperature indicators, TTI), and radio frequency identification (RFID) modules directly measure quality of the product inside the package (Otles and Yalcin, 2008). Freshness indicators were described for evaluating the fish quality (Chun *et al.*, 2014). Freshness is the most important quality attribute of fish and is the key element to be implemented in quality control (Chun *et al.*, 2014). Freshness indicators provide direct product quality information as a result of reactions between indicators within the package and microbial growth metabolites (Kerry, 2012, Vanderroost *et al.*, 2014). The majority of freshness indicators are based on indicator color change in response to microbial metabolites produced during spoilage (Kerry, 2012).

Spoilage of fish is due to microbiological activity, chemical oxidation of lipids, and autolysis. However, microbial activity is the main mechanism affecting the fresh fish quality and thereby determines the product's shelf life (Ghaly *et al.*, 2010). During fish spoilage, various compounds with characteristic emerge, such as trimethylamine (TMA), total volatile basic nitrogen (TVBN), sulphuric compounds, aldehydes, ketones, esters, etc., by various microorganisms and emit to the headspace of packaging (Dalgaard *et al.*, 2006, Ghaly *et al.*, 2010). Generally, after death, a consortium of bacteria, known as specific spoilage organisms (SSOs) such as *Shewanella putrifaciens*, *Aeromonas spp*., *psychrotolerant Enterobacteriaceae*, *P. phosphoreum*, and *Vibrio spp*. produce metabolites (chemical spoilage indices, CSIs), which are able to reduce trimethylamine N-oxide (TMAO) to TMA (Gram and Dalgaard, 2002).

Various methods including chemical, microbiological, physical methods, and sensory evaluation were used to determine fish and seafood freshness (Olafsdottir *et al.*, 2004). Traditional methods for monitoring fish freshness such as sensory methods require trained assessors, while microbiological methods are retrospective, expensive, destructive, and time-consuming (Shukla *et al.*, 2016). Consumers and manufacturers are eager to develop that is small, simple, low-cost, fast, and non-destructive device to evaluate real-time freshness of food products, which can be applied at any stage of the supply chain (Pons-Sánchez-Cascado *et al.*, 2006). The number of studies on package indicators for spoilage or freshness of food is still limited. However, some attempts have been conducted to construct indicators for volatile compounds produced in microbial spoilage by using a pH indicator (Kuswandi *et al.*, 2014, Zhang *et al.*, 2014) including methyl red (Kuswandi *et al.*, 2014) and curcumin (Kuswandi *et al.*, 2012). Based on a similar principle, indicator dyes such as bromocrosol green could also be used as a freshness indicator in fish. As the basic volatile amines are gradually piled up in the package headspace, the pH is increased and color of the indicator changes so that it is easily visible by the naked eye (Kuswandi *et al.*, 2014, Kuswandi *et al.*, 2012). In the current study, our aim was to develop a low-cost freshness indicator based on the volatile amines in the packaged fish that works best before its expiry date.

Materials and Methods

Materials: Bromocrosol green, sodium hydroxide, magnesium oxide, boric acid 99.5% were obtained from SAMCHUN in South Korea. Methyl red indicator (Lobachemie, India), filter paper No. 41 (CHM, Spain), plate count agar

(Liofilchem, Italy), sulfuric acid 0.1 N, agarose (Merck, Germany), hydrochloric acid, and paraffin oil (Carlo ERBA, France) were also purchased.

Preparation of the indicator: The indicator matrix was prepared from silica nanoparticles mixed with brmocromosol green (0.1%) and agarose solution (1%) in a ratio of 1:5. This preparation was made on flame and its pH was adjusted to 4-5. The solution was poured into glass plates and kept for 24 hours at room temperature for casting. The cast was put in the oven until complete drying, eventually to a thickness of 1 mm. The indicator was cut as 1.5 × 1.5 cm squares, placed inside polypropylene sheets, and then sealed by plastic sewing machine. An opening was also made on the indicator, so that it can contact with gasses in the package headspace since the indicator should react to volatile amines. Silica nanoparticles acted by increasing the surface area for better reactivity of the molecules and increased sensitivity of the indicator to volatile amines.

Scanning electron microscopy (SEM): The scanning electron microscopy was used to observe the surface morphology of the prepared indicator (Ezati *et al.*, 2019).

Determination of color code of the indicator: The color code (number) was used to distinguish color changes of the indicator during the storage. In order to determine the exact color, images were obtained from the indicator at different time intervals with a 13 megapixel digital camera. Then, the specific color number in the biosensor was read using Photoshop software.

Preparation of the fish sample: Fresh rainbow trout was purchased from a local market in Yazd, placed in an ice box, and immediately transferred to the laboratory. The fish were rinsed and filleted using a sterile scalpel followed by division into pieces of 100 ± 5 g. Samples were placed into a sterile suitable container with the indicator attached inside the package top (**Figure1**). The fish samples were stored at 4 °C in refrigerator. The TVBN value, pH, and microbiological test were determined at days 0, 3, 5, and 6.

Chemical analysis: The pH value was determined using a digital pH meter (AZ86502, Taiwan). The pH values were obtained after dissolving 5 g of trout sample in 45 ml of distilled water (ratio of 1:10 w/v), homogenization for 2 min with stomacher (model 3000, Mix Wel, France) (Kuswandi *et al.*, 2012), and filtered through whatman paper. The pH meter was calibrated using a pH buffer solution at pH values of 4, 7, and 10 at 25 °C (Baygar *et al.*, 2008).

In order to measure the total volatile basic nitrogen (TVBN) value (mg/100 g fish flesh), the micro diffusion method was used by distillation of 2 g magnesium oxide and 10 g of homogenized fish sample in 300 ml distilled water. The distillate was gathered in the flask containing 10 ml boric acid, methyl red, and methylene blue (Samchun, Korea) as the indicators. The distillation continued until the approximate solution volume reached 150 ml. The distillation product was titrated with sulfuric acid 0.1 N (Merck, Germany). The TVBN value was calculated by consuming sulfuric acid and expressed as mg 100/g fish muscle (Mirshekari *et al.*, 2016, Ojagh *et al.*, 2010).

Microbial analysis: For microbial analysis, an amount of 25 g fish sample was transferred into sterile stomacher bags containing 225 ml sterile phosphate buffer solution and homogenized by stomacher for 2 min. The decimal serial dilutions were carried out and then cultured using pour plate method in plate count agar (Italy, Liofilchem Italy). Later, PCA plates were incubated at 37 °C for 48 hours and colony forming units were counted as $\log_{10}$ CFU/g (Arashisar *et al.*, 2004, Sallam, 2007).

Data analysis: Results were analyzed using the SPSS (version 16, USA). Pearson correlation test was used to examine the correlation between variables. The significant difference between the means was tested at a level of 5%.

Results

Scanning electron microscopy (SEM): As seen in the SEM image, coating of silica nanoparticles with indicator was performed successfully. The nanoparticles of silica are apparent in the size of 100 nm (**Figure 1**).

Color changes in the indicator due to fish spoilage: The indicator color changes in the packaged fish are shown in **Figures 2** and **3**. Over the time, by producing volatile gases and increasing pH in the package headspace, the indicator color changed from yellow (at day 1) to green (at day 3) and finally to blue (at day 6). Analyses of microbial status, pH value, and TVBN were carried out to assess the accuracy performance of the indicator.

Indicator color number: In **Table 1**, the color codes obtained by photography software are shown. This number showed the apparent change in the indicators' colors within the color spectrum. However, no significant positive effect was observed between the color number and other variables of the study. Therefore, number of the indicator's color is not a good marker for measuring other variables; therefore, it can be independently used for determination of indicator-based fish freshness.

Chemical analysis: The pH changes during storage at refrigerator are shown in **Figure 4**. The pH values increased during storage, but no significant difference was observed in the mean values at different days. The pH values of rainbow trout were estimated as 6.61, 6.72, 6.92, and 7.20 on days 0, 3, 5, and 6, respectively (**Table 1**). According to **figure 4**, all P-values were > 0.05, in other words, no significant difference was observed between the means.

Total volatile basic nitrogen levels are shown in **Figure 5**. During the storage at 4 °C, TVBN values increased due to the decrease in freshness of fish samples (**Table 2**). The amount of TVBN values were determined as 13.49, 21.43, 26.06, and 30.93 (mg/100 g fish flesh) on days 0, 3, 5 and 6, respectively. There was a significant difference between the TVBN values in different days (P <0.05).

Microbiologic analysis: Major changes in fresh fish are largely due to microbial growth and activity. The total bacterial count in all samples significantly increased from 3.23 to 6.45 $\log_{10}$ CFU/g during the storage for 6 days at 4 °C (P < 0.05). A direct relationship was observed between color changes of the indicator (from yellow to green and then to blue on days 0, 3, and 6, respectively) and total viable count (TVC). The TVBN had a direct correlation with TVC and pH. No correlation was observed between the two variables of TVC and pH.

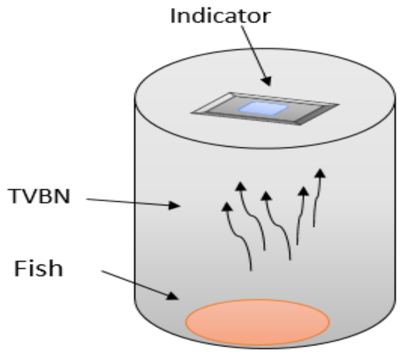

Figure 1. Experimental design of pH indicator for detection of fish freshness

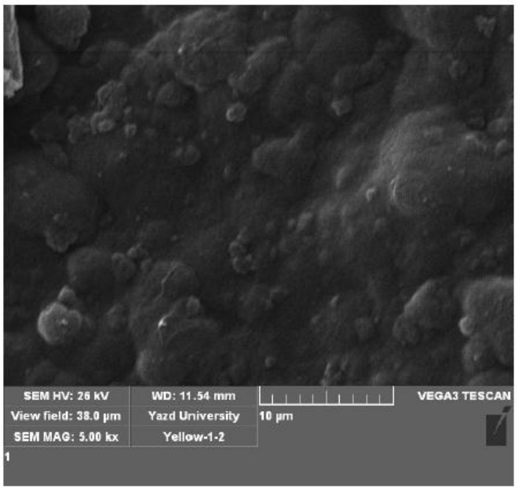

Figure 1. Nanoparticles of silica

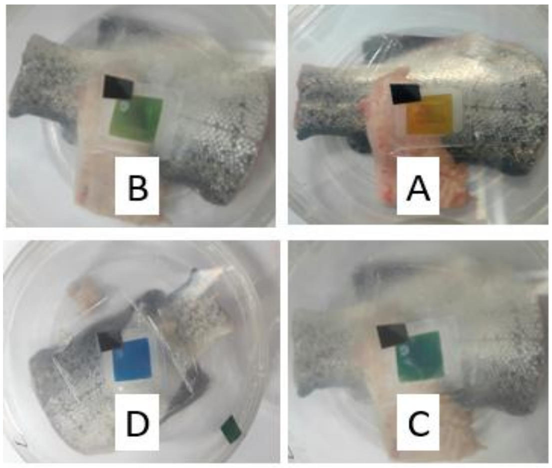

Figure 2. Changes in the color of freshness indicator during storage. (A) Fresh, (B) acceptable, (C) warning, and (D) unacceptable

Figure 3. Range of the color change in the indicator at different days: Yellow, fresh; yellowish green, acceptable; blue, unacceptable.

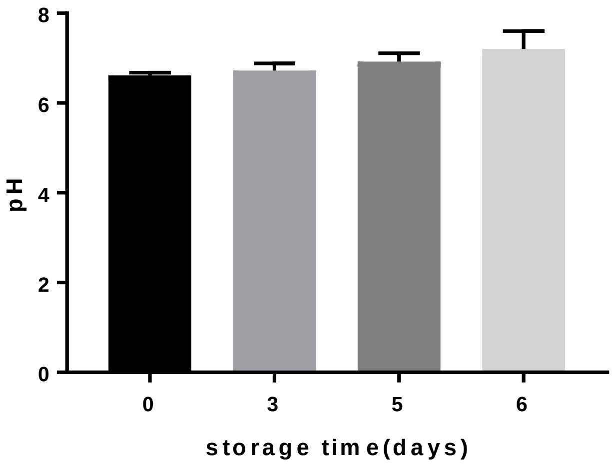

Figure 4. Changes in pH of rainbow trout stored in 4 ◦C.

Table 1. Color codes of the indicator at different days of fish storage according to red (R), green (G), and blue (B) colors

Days	**0**	**3**	**5**	**6**
R	154	98	66	43
G	152	110	84	98
B	53	70	66	133
R^2+G^2+B^2	49629	26604	15768	29142
Total color	222/77567	163/10733	125/5707	170/71028

Table 2. Evaluation of the pH, total volatile basic nitrogen (TVBN), and total viable count (TVC) in trout during cold storage (mean ± SD, n = 3)

Index	**Day 0**	**Day 3**	**Day 5**	Day 6
pH	6.61 ± 0.06	6.72 ± 0.15	6.92 ± 0.18	7.20 ± 0.39
TVBN (mg N/100 g)	13.49 ± 2.00	21.43 ± 2.05	26.06 ± 3.06	30.93 ± 2.00
TVC (1000 CFU/g)	3.23 ± 0.23	4.16 ± 0.66	5.15 ± 0.54	6.45 ± 0.26

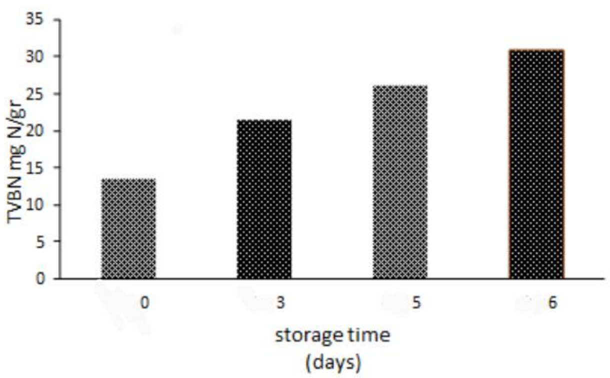

Figure 5. Changes in total volatile basic nitrogen (TVBN) of rainbow trout stored in 4 °C. *P* < 0.05 was defined as significant difference between the means

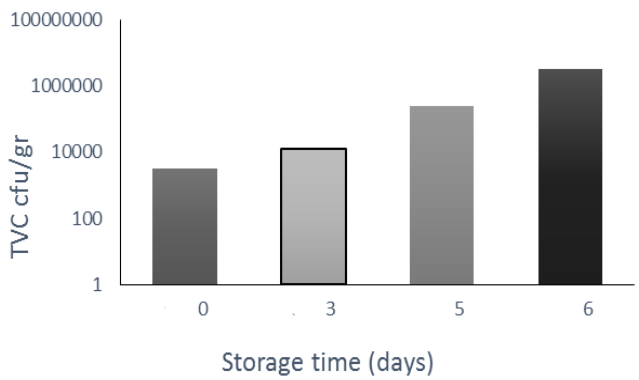

Figure 6. Changes in total viable count (TVC) of rainbow trout stored at 4 ◦C. The $P < 0.05$ showed significant difference between the means

Discussion

Sea foods with high proteins, fat-soluble vitamins, and omega-3 fatty acids are of great importance in human diet. Sea foods are considered as highly perishable foods that deteriorate faster than other animal meats. However, the presence of spoilage and pathogenic microorganisms cause changes in sensory characteristics such as off-odor, off-taste, gas production, and slime formation (Moini *et al.*, 2009). In the present study, the new pH-sensitive indicator was used to determine the rainbow trout freshness during storage at refrigerator. During the first three days, no drastic color change was observed in pH-sensitive indicator. When the indicator turns to blue, it means that fish is not fresh and should not be consumed. So, in the current study, yellow indicates freshness of the product, green indicates restriction or caution in consumption, and blue shows fish spoilage associated with sensory rejection. In the study conducted by Silva-Pereira, as a result of reaction between indicator and volatile bases of fish, the color changed to light blue after three days of storage in the refrigerator, indicating an increase in fish pH and initial spoilage. After 7 days, the color changed to yellow, which was indicative of fish spoilage (Silva-Pereira *et al.*, 2015). It is known that factors such as fish species, feeding habits, temperature, and general storage conditions (atmosphere, activity water, microbial cross-contamination etc.) may affect freshness and spoilage of fish (Kerry, 2012).

In the current study, the pH values of samples gradually changed from 6.61 on day 0 to 7.20 on day 6 at 4 °C. The pH value of live fish muscle is close to 7.0. However, the post-mortem pH can vary from 6.0 to 7.0 depending on season, species, and other factors (Simeonidou *et al.*, 1997). However, changes in pH values of rainbow trout samples were not statistically significant during storage in present study ($P > 0.05$), which is similar to the results found for other fish species during the storage (Kyrana and Lougovois, 2002, Rodríguez *et al.*, 1999). Castro et al. reported that pH value of approximately 7.0 indicated the onset of fish deterioration and suggested that the increase in pH level was due to the accumulation of alkaline compounds, such as TMA and other TVBN derivatives mainly produced by microbial activity during fish muscle spoilage (Castro *et al.*, 2006). Kyrana and Lougovois attributed low TVBN to relatively low pH and observed microbial flora composition during the tests (Kyrana and Lougovois, 2002). In Kuswandi et al.'s research (Kuswandi *et al.*, 2015), litmus reagent color changed from red to blue due to spoilage with change in pH of beef tissue from 5.61 to 6.24 in ambient condition and from 5.67 to 6.02 in chiller conditions. This result was consistent with other studies (Kuswandi *et al.*, 2012, Yoshida *et al.*, 2014, Zaragozá *et al.*, 2012). Niknam et al. observed no color change of bromocresol green and phenol red in the first 56 hours. However, the reagent started to change color after 56 hours with increase in pH. The authors concluded that pH increased due to the chicken meat spoilage, which caused the sensors' color change from yellow to blue for bromocresol green and from yellow to red for phenol red (Niknam, 2015). The results of the present study are similar to the findings presented by Kuswandi et al. (Kuswandi *et al.*, 2012), who used curcumin as an indicator of shrimp freshness. They investigated freshness of the shrimp in ambient and chiller conditions; so, the sensor changed color from yellow to dark orange due to the change of pH from 6.88 in day one to 7.53 after 10 days of storage. Furthermore, the correlation between changes in pH and indicator color in our study was similar to the reports by Bahmani et al., who used bromocresol green and phenol red to determine the freshness of rainbow trout (Bahmani *et al.*, 2016).

Volatile amines such as TMA, ammonia (NH_3), and dimethylamine comprise of the TVBN, which is commonly used as an estimate of spoilage and as a freshness index of fish (Wu and Bechtel, 2008). In the current research, the TVBN of days 5 and 6 were significantly ($P < 0.05$) higher than the first days. In this experiment, TVBN values were 13.49 and 30.93 mg N/100 g on days 0 and 6, respectively. A study suggested that the TVBN limit in muscles of processed fish products was about 25 to 35 mg N/100 g (Dalgaard, 2000). Similarly, a study recommended the TVBN levels of 20-30 mg/100 g, which indicated the beginning of spoilage and values more than 30 mg/100 g showed spoiled fish (Kimura and Kiamukura, 1934). When the concentration of TVBN exceeds 30 mg/100 g, the fish should be considered dangerous for consumption (Özoğul and Özoğul, 2000). Huss suggested that the recommended level of TVBN for rejection was 20 mg N/100 g flesh in fatty fish similar to the rainbow trout fish (Huss, 1988). According to the storage time and subsequent increase in TVBN, the quality of fish products is classified as high quality (up to 25 mg N/100 g), good quality (up to 30 mg N/100 g), limited acceptability (up to 35 mg N/100 g), and spoilt (above 35 mg N/100 g) (Amegovu *et al.*, 2012, Goulas and Kontominas, 2007, Kykkidou *et al.*, 2009, Özyurt *et al.*, 2009). As TVBN approached maximum levels in our study, a direct relationship was observed with color change of indicator, TVC, and pH, which is in line with the results reported by Morsy et al. (Morsy *et al.*, 2016), who used colored reagents as a marker of fish spoilage, and also with other studies (Chun *et al.*, 2014, Shukla *et al.*, 2016). Pacquit et al. used bromocresol green as a marker to detect spoilage in fish and showed color change as a result of increased TVBN (Pacquit *et al.*, 2007). Kuswandy et al. used polyaniline film containing indicator with various types of primary volatile amines to detect fish spoilage and reported similar results (Kuswandi *et al.*, 2012). The increase in TVBN from 13.49 in first day to 30.93 after 6 days in our study is consistent with that of Zaragoza et al., who showed color change of sensor due to increase in TVBN from 14 to 30 (Zaragozá *et al.*, 2012).

As different articles showed, measuring microbiological quality of fish by bacterial counts to define exact spoilage thresholds is difficult since they can vary depending on the catch season, geographical location, and above all the fish species. Koutsoumanis and Olafsdottir reported that TVC values of 10^7 CFU/g in fish samples were considered as the end of their shelf life (Koutsoumanis, 2001, Olafsdottir *et al.*, 2004). Gram and Huss suggested that spoilage of iced fish reached the levels of 10^8–10^9 CFU/g of specific spoilage organism (Gram and Huss, 1996, Huss *et al.*, 1997). Nevertheless, standards often use much lower TVC (10^6 CFU/g) as index of acceptability (Olafsdóttir *et al.*, 1997). According to Ozogul, the shelf life of trout is approximately 5-6 days in 4 °C and 10^6 microorganisms/g is considered as the TVC limit of acceptability (Özogul *et al.*, 2005). In present study, the increased bacterial number to more than 10^6 in the 6^{th} day was associated with color change of the indicator to blue. This is in line with a study conducted by Pacquit et al. (Pacquit *et al.*, 2007). We observed a considerable association between the microbial quality of the fish tissue and TVBN concentration. Rokka et al. similarly found a direct relation between microbial quality and the amount of volatile amines (Rokka *et al.*, 2004). However, Bahmani et al. reported that the spoilage of rainbow trouts stored in refrigerator occurred after 13 and 14 days of storage (Bahmani *et al.*, 2016). Furthermore, Zaragoza et al. (Zaragozá *et al.*, 2012) stated that the fish was spoiled 7 days after storage.

Conclusion

Results of the current study showed that a pH-sensitive indicator could be used as a visual means of detecting deterioration of the fish. The developed indicator can be part of a system of intelligent packaging with visual change in color. We found that the indicator response is associated with microbial growth in fish samples. Results of the microbial and chemical analysis showed that fish started to spoil after 6 days in cold storage. The advantages of this packaging system include simple and inexpensive production, not complicated chemical formula, "best before" date estimation, and reduced unnecessary meat waste. Intelligent packaging has the potential to ensure safety and quality of the seafood. The proposed system is advantageous due to its simple manufacturing process and visual change in color. Indicators incorporated in intelligent packaging show great potential for assuring the safety and quality of the food products.

Authors' contribution

Akrami Mohajeri F, Hekmatimoghaddam SH, Jebali A, Khalili Sadrabad E and Dehghani-Tafti A conceived and designed the experiments. Rastiani F carried out the experiment. All authors contributed to the final version of the manuscript.

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Conflict of interest

There is not any conflict of interest.

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