Chapter 4

To develop the dye-based sensor for monitoring the freshness of fish during storage

4.1. Introduction

Fish are aquatic animals with gills and no limbs, providing a valuable source of high-quality protein, omega-3 fatty acids, vitamin D, and essential minerals like calcium, phosphorus, iron, zinc, iodine, magnesium, and potassium. Fish spoilage occurs due to enzymatic, chemical, and microbial actions present in the fish. When fish are freshly caught, they are relatively free from microorganisms, but bacteria can be found in the surface slime, gills, and intestines. The spoilage process involves rigor mortis, autolysis, bacterial invasion, and putrefaction (Cai et al., 2015; Yu et al., 2020). Bacterial activity leads to the breakdown of compounds like trimethylamine oxide (TMAO) into trimethylamine (TMA), resulting in offensive odors. Amino acids are broken down into primary amines, and urea is converted to ammonia which are collectively known as total volatile basic nitrogen (TVBN) and used as spoilage indicators (Hao et al., 2021). Spoiled fish exhibit changes in physical characteristics such as color, odor, texture, eye color, gill color, and muscle softness. These indicators signal the deterioration of fish quality.

Synthetic indicator dyes can exhibit different colors under acidic and alkaline conditions, making them valuable tools for determining pH levels. In recent years, the use of colorimetric sensors in assessing food freshness for safety and quality control has gained popularity due to their simplicity, affordability, and ability to non-destructively detect volatile compounds in food (Perez-Cacho et al., 2008; Huang et al., 2011; Chen et al., 2014; Chen et al., 2016; Chen et al., 2017; Chen et al., 2018). Examples of synthetic pH indicator dyes like bromophenol blue and bromocresol purple have been employed to detect amines, demonstrating high sensitivity to acid-base reactions within specific pH ranges (Koxmak et al., 2019; Chen et al., 2020; Yan et al., 2021). A colorimetric sensor array is constructed based on two fundamental principles. First, each chemical-responsive dye in the array should have a functional group that can interact strongly with specific analytes. Secondly, this interaction center should be closely related to a highly visible chromophore, which is the component of the molecule that absorbs light and produces color. The classes of dyes used in such design include Lewis's acid/base dyes that interact with metal ions through electronic properties, while Brønsted acidic or basic dyes, like pH indicators that change color based on acidity or alkalinity, and dyes with large permanent dipoles sensitive to solvent polarity changes (Janzen et al., 2006; Suslick et al., 2010; Feng et al., 2010). This method ensures that the sensor array can effectively

detect and differentiate analytes based on their specific chemical interactions with the responsive dyes.

Several studies have explored the development of colorimetric sensor arrays for monitoring food deterioration, reflecting the increasing demand for non-destructive methods to assess food quality. Morsy et al. (2016) investigated 16 chemo-sensitive compounds integrated into an array for colorimetric detection of spoilage compounds. Chen et al. (2017) introduced a low-cost smart diagnostic tool for food using a colorimetric sensor array. Researchers have contributed to advancements in this area, highlighting the diverse approaches and applications of colorimetric sensor arrays for food quality assessment and spoilage detection (Zaragozá et al., 2015; Meng et al., 2017; Majdinasab et al., 2018); Wells et al., 2019; and Koxmak et al., 2019). These efforts collectively demonstrate the potential of colorimetric sensors as effective tools for non-destructive monitoring of food freshness and quality. Evaluating fish freshness is challenging due to its perishable nature, and current conventional techniques are lengthy, sophisticated, and require trained personnel. Therefore, the present study developed a bromocresol purple dye-based label for the determination of fish spoilage or freshness during storage. The efficiency of the label as a colorimetric sensor was also investigated. The rohu (*Labeo rohita*) fish was chosen for this study due to its local availability. Belonging to the carp family, rohu has a large, silver-colored, typical cyprinid shape with a noticeably arched head. It is a significant freshwater species in South Asian aquaculture. Rohu is found in the rivers of northern, central, and eastern India, as well as in Pakistan, Bangladesh, Nepal, and Myanmar.

4.2. Material and Methods

All chemicals of AR grade, including bromocresol purple dye and ethanol, were obtained from Merck-Sigma, India. Fresh fish samples were procured from Tezpur's local market near Tezpur University.

4.2.1. *Preparation of bromocresol purple dye (BPD) label*

A 0.1 % solution of bromocresol purple in an aqueous ethanol solution (3:1) was prepared. Using a micropipette, varying amounts of the dye solution (0.2 mL, 0.5 mL, and 1 mL) were deposited onto an optically inactive polystyrene sheet to determine the optimal quantity of a UV-visible spectrophotometer (Cary 60 UV–Vis

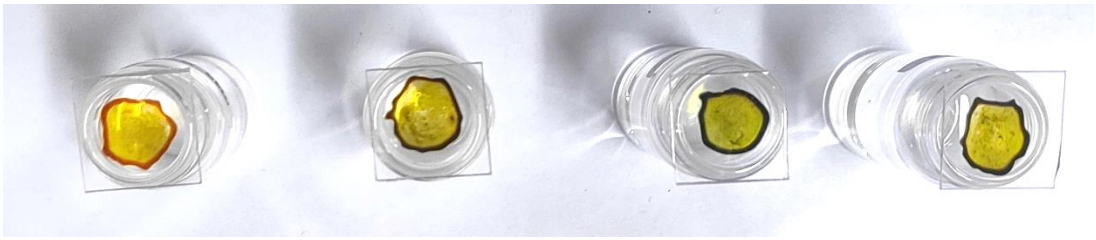
Spectrophotometer, Agilent Technologies, Germany) by scanning through the spectrophotometric spectrum (400-800nm) and observing the response. The sheets were then left overnight in the dark before analysis.

4.2.2. *BPD label response time*

The response time of the developed BPD label was assessed by exposing it to ammonia vapor (400 ppm solution) for varying durations (0 to 5 min). The response of the BPD label was evaluated by measuring the percentage transmittance spectra using a UV-Vis spectrophotometer (Cary 60 UV–Vis Spectrophotometer, Agilent Technologies, Germany) in the visible region spanning 400 to 800 nm.

4.2.3. *Sensor calibration*

The BPD labels were subjected to standard ammonia solutions ranging from 0 to 400 ppm for a duration of 5 minutes (**Fig. 4.1**). The peak wavelengths of the response were determined using the UV-Vis spectrophotometer. A calibration curve correlating percent transmittance with ammonia concentration was constructed. The linear equation obtained was used for monitoring the volatile amines released from the stored fish sample.



100ppm **200ppm** **300ppm** **400ppm**

Figure 4.1.BPD label exposed to different concentrations of ammonia vapor.

4.2.3. *Sample preparation and performance evaluation of the developed BPD label*

Fresh live rohu fish (*Labeo rohita*) (**Fig. 4.2a**) were obtained from the local market of Tezpur, Assam, India, and the experiment was conducted on the same day of harvesting. The fish were cleaned by removing the head, gut, scales, and middle bones, followed by washing under tap water. Uniform-sized fish fillets were prepared in a sterile environment. Each fresh fillet (30 g) was then stored in a sterile glass container at ambient temperature. The dye label was affixed to the inner side of the lid, leaving a headspace

of 2 cm (**Fig. 4.2b**). The label's response was evaluated at 2h intervals until 10h of storage at room temperature.

(a)

Figure 4.2. (a) Rohu (*Labeo rohita*), and **(b)** BDP label exposed to fish fillets.

4.2.4. *Measurement of TVB-N and pH*

The Total Volatile Basic Nitrogen (TVB-N), representing the volatile amines was quantified using an ion-selective electrode for ammonia (NH_4^+ ISE). 5 g fish sample was partially dissolved and dissociated in an aqueous phase, allowing measurement of the ammonia (NH_4^+) ion content in the water, which was then converted to mg/100 g (Kuswandi et al., 2012). Simultaneously, the pH of the fish was measured using a pH electrode.

4.2.5. *Microbial analysis of fish*

Microbial analysis of the fillet was conducted following the method outlined by Giarratana et al. (2016). 5 g of fish flesh was homogenized with 45 ml of 0.1 % peptone water. Serial ten-fold dilutions were prepared in 0.1 % peptone water, and aliquots of 0.1 mL of the diluted sample were spread onto nutrient agar plates with controls for microbial enumeration. Following incubation of the plates at 37 °C for 48 h, the total viable count (TVC) was calculated, and all counts were reported as log CFU/g.

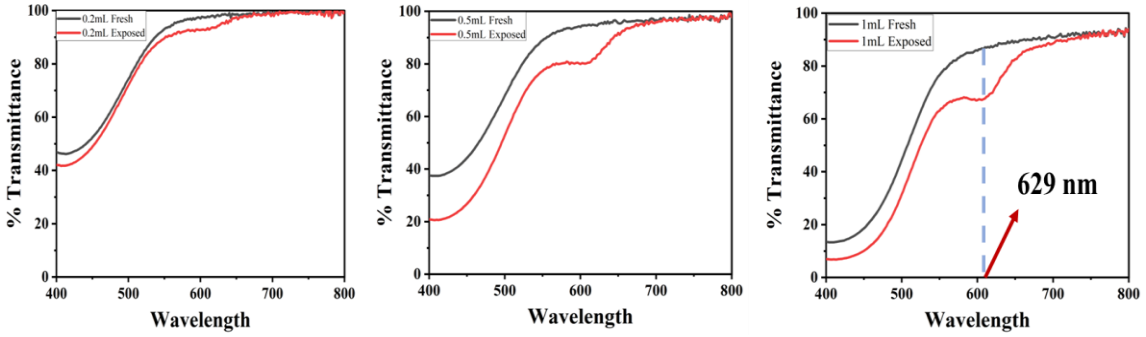
4.2.6. *Statistical analysis*

All experimental data were reported as mean ± standard deviation (SD) and statistical analysis were carried out using the IBM SPSS Statistics 21 (IBM, Armonk, NY, USA) software application for Windows through Duncan's multiple range test ($p \leq 0.05$). Experiments were conducted in triplicates (n=3), and data visualization was performed using Origin 2021.

4.3. Results and discussion

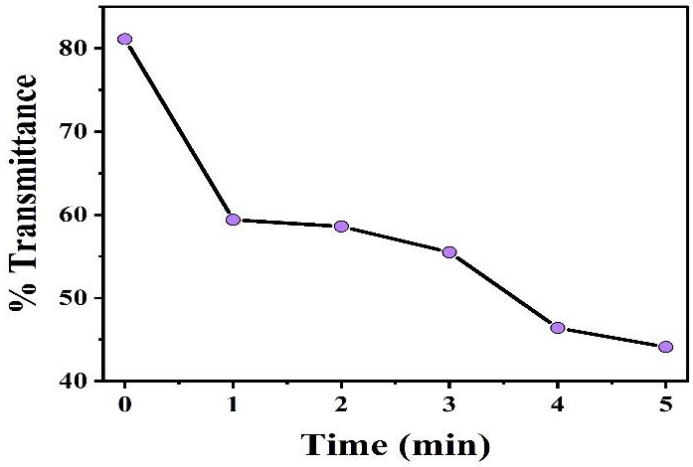
4.3.1. *Response time and calibration of the BPD label*

The deposition of 1 mL of dye solution proved to have superior outcomes in its reaction to ammonia vapor (**Fig. 4.3**). This optimal deposition amount was chosen for further analysis based on its performance in detecting and responding to ammonia. In response to ammonia vapor within 0-1 minute indicates a rapid and highly sensitive reaction of the developed dye label to ammonia. This means that the dye quickly changes color or exhibits another observable response when exposed to ammonia vapor, making it suitable for fast detection purposes. The label exhibited a response peak at 629 nm and the response time of the BPD label was observed in the range from 0-1 min, showcasing a remarkably rapid reaction to ammonia vapor (**Fig. 4.4a**). This quick response time is essential for real-time monitoring applications, enabling timely detection of ammonia presence in the fish samples. Furthermore, the spectrophotometric response of the BPD label to different ammonia concentrations obtained a linear equation with a regression coefficient (R^2) of 0.96 (**Fig. 4.4b**). A regression coefficient close to 1 indicates that changes in the label's color or other optical properties correlate closely with changes in ammonia concentration, demonstrating the effectiveness and precision of the BPD label for quantifying ammonia levels.

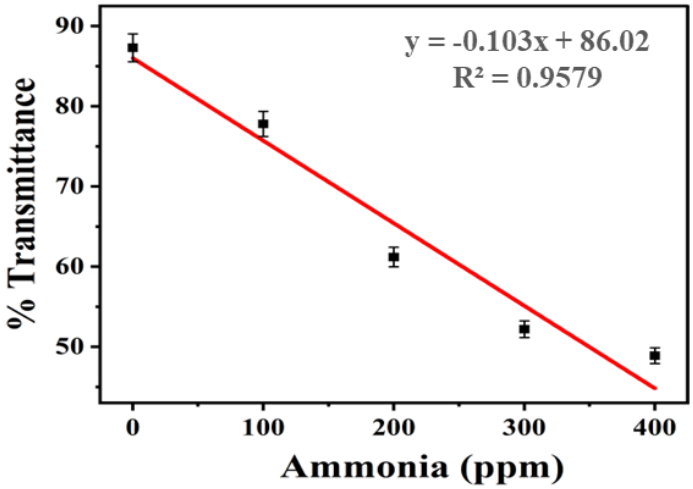


(a) **(b)** **(c)**

Figure 4.3. Response label toward ammonia vapor (0-400 ppm) using UV-vis spectrophotometer **(a)** 0.2 mL dye deposition, **(b)** 0.5 mL dye deposition, and **(c)** 1 mL dye deposition.



(a)



(b)

Figure 4.4. (a) Response time of dye label, and **(b)** Calibration curve of dye label with ammonia vapor using UV-vis spectrophotometer.

4.3.2. *Performance evaluation of the developed BPD label*

The freshness of the rohu fish fillets during storage at ambient temperature was tested against the developed BPD label using a UV-vis spectrophotometer at 629 nm. It was observed that as the storage time increased, the response in terms of percent intensity reduction increased (**Fig. 4.4.**). The changes in signal intensity observed on the label could be attributed to various volatile organic components, particularly amines, which contribute to an increase in pH within the headspace of the container storing fish fillets. Amines like trimethylamine (TMA), dimethylamine (DMA), and ammonia are produced through protein decomposition facilitated by microbial activity, leading to spoilage (Kuswandi et al., 2012; Pacquit et al., 2004). The accumulation of these amines during spoilage serves as an important indicator of fish freshness, enabling the colorimetric BPD label to detect and quantify the degree of deterioration in the stored fish sample. The fundamental reaction involved in understanding the colorimetric pH indicator dye response to ammonia and volatile amines is presented by equilibrium reaction (Wells et al., 2019):

$$RNH_2 + PH = RNH_3{}^+P^- \qquad (4.1)$$

Where R represents the chemical group (usually hydrogen or an alkyl group), PH is the protonated form and P^- is the deprotonated form of the pH indicator dye.

This reaction leads to a color change due to the transformation between the protonated and deprotonated forms of the dye. By observing the color change exhibited by the indicator, it becomes possible to identify specific thresholds of Total Volatile Basic Nitrogen (TVB-N) or Total Viable Count (TVC) levels, which are basic indicators of fish spoilage. A significant increase in percent intensity reduction at 6 h was observed that crossed 30 % and reached 42 % intensity reduction at 10h (**Fig. 4.5**). The change in the transmission spectrum with exposure time is due to the volatile amines and the change in colors is due to the reaction mechanisms of **Eq. 4.1.** The response characteristic of such dye-based indicators toward the volatile nitrogen compounds is discussed in the previous study (Wells et al., 2019; Cichero et al., 2021) which is in line with the present study. The change in the color of the BPD label from yellow to purple is proved by the intensity reduction when observed using the UV-vis spectrophotometer. However, the condensation of the vapor on the dye-based label could be a limiting factor in its use.

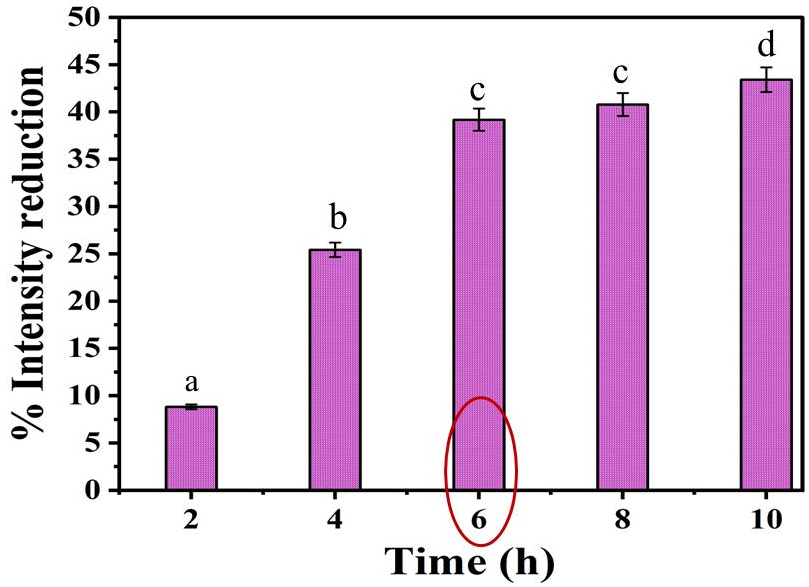


Figure 4.5. Percent (%) intensity reduction for spectrophotometer response of rohu fish at ambient room temperature. Different letters indicate significant differences ($p < 0.05$) in % intensity reduction with storage time.

The TVB-N value of the stored rohu fish fillets is illustrated in **Fig. 4.6.** Amines like trimethylamine (TMA) are produced through protein decomposition facilitated by microbial activity after the death of the fish, leading to spoilage. Studies have demonstrated a direct correlation between the increase in Total Viable Count (TVC) and the accumulation of amines such as TMA during spoilage processes (Mosry et al., 2016; Hao et al., 2021). In the early stage of storage, the TVB-N values at 0- 4 h were observed to be 10 mg/100 g, 19 mg/100 g, and 28 mg/100 g, respectively. At 6 h, it reaches 35 mg/100 g, crossing the acceptable limit in which the fillets are spoiled, and reaching 60 mg/100 g at 10 h, the fillets were observed to be spoiled. According to European Union Regulations, the limit of acceptability of fish was reported to be 30-35 mg/100 g, and fish with 35 mg/100 g TVB-N or above are considered spoiled (European Union Regulation (EC) no. 2074/2005). The response of the developed BPD label towards stored fish fillets correlates with the TVB-N value observed.

The correlation between pH value and fish freshness is shown in **Fig. 4.7**. Gradual and slow increases in the pH were observed during storage. This observation aligns with the findings of Kuswandi et al. (2012), who documented a gradual increase in pH with extended storage duration. As degradation progresses, alkaline substances such as amines accumulate in the fish flesh, leading to a pH rise. This observation aligns with the

findings of Kuswandi et al. (2012), who documented a gradual increase in pH with extended storage duration. As degradation progresses, alkaline substances such as amines accumulate in the fish flesh, leading to a pH rise. The change in pH of the fish is directly related to the change in the color of the label (**Eq. 4.1**). This pH increase is likely due to alkaline compounds produced by microbial activity during fish degradation, which correlates with the rise in TVB-N levels and the BPD response upon exposure to the fish sample.

The total viable count (TVC) of rohu fish fillet at ambient temperature is illustrated in Fig**. 4.7**. The microbial load of the fresh fillets was found to be 5 log CFU/g. However, the spoilage organisms multiply and increase over the storage time, reaching 6 log CFU/g, 6.6 log CFU/g, and 7.3 log CFU/g at 2 h, 4 h, and 6 h respectively, and subsequently increasing up to 7.5 log CFU/g at 10 h. Fish is considered spoiled when the microbial count of specific spoilage organisms reaches levels between 10^6-10^7 CFU/g. As per the International Commission on Microbiological Specifications for Food (ICMS), the limit for total aerobic count in fresh and frozen fish is set at 10^7 CFU/g. In this study, the TVC sharply increased to 10^7 CFU/g at 6 h in fillets stored at ambient temperature which corresponds with the BPD response observed using the UV-vis spectrophotometer. The growth of the microbial population is highly influenced by the temperature and composition of the fish. At a favorable temperature, the spoilage organisms grow faster which was reflected by the TVC count of the fillets and the response of the BPD label. Correlating the response of the BPD label with the TVB-N, pH value, and microbial count, a method could be established where a reduction in the intensity of 0-15 % indicates freshness, 15-25 % indicates intermediate freshness, and >25 % indicates spoilage. The decrease in transmission peak at 629 nm over storage time corresponds to the degree of fish spoilage, with a significant decline observed after 4-6 h at ambient temperature.

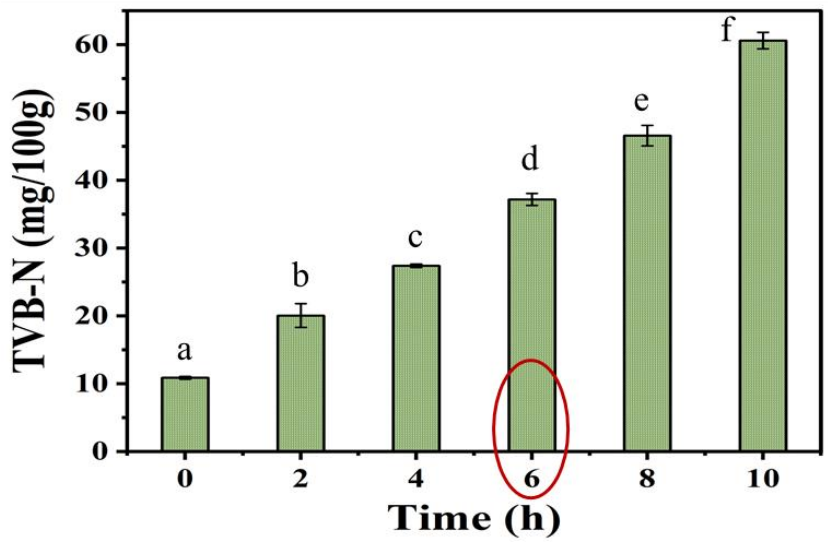


Figure 4.6. (a) TVB-N value of rohu fish sample at ambient temperature. Different letters indicate significant differences ($p < 0.05$) in TVB-N of fish fillet during storage.

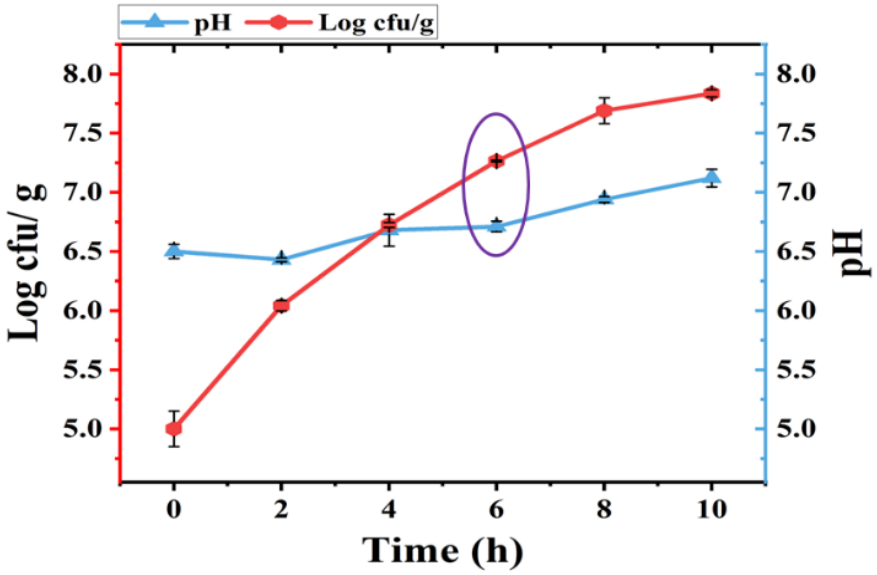


Figure 4.7.TVC (total viable count) and pH value of rohu fish sample at ambient temperature.

4.3.4. Leaching test of the developed labels

The developed BPD and PANI labels were tested by dipping in 10 mL distilled water for 5 min. The BPD label started leaching out into the water within a few seconds and completely leached out in 5 min whereas no change was found in the PANI label (**Fig.4.8**). The PANI label was selected for further experimental analysis. The organic pH indicator dyes are often water-soluble or soluble in certain organic solvents. If the dye is not sufficiently bound or encapsulated within the matrix, it can dissolve or leach

out into the surrounding environment, especially when exposed to water or solvent. This could be one of the reasons why the bromocresol purple dye leaches out in the present study. To prevent or minimize such dye leaching, the formulation and preparation of sensing materials must be optimized. This can include a proper selection of the binding matrix and increasing the binding affinity between the dye and the matrix, selecting appropriate matrices or encapsulation methods that provide strong dye containment, and considering the environmental conditions in which the sensor will be used to ensure long-term stability and reliability. Future work can be done to improve the property of the dye label using an appropriate matrix for sensing.

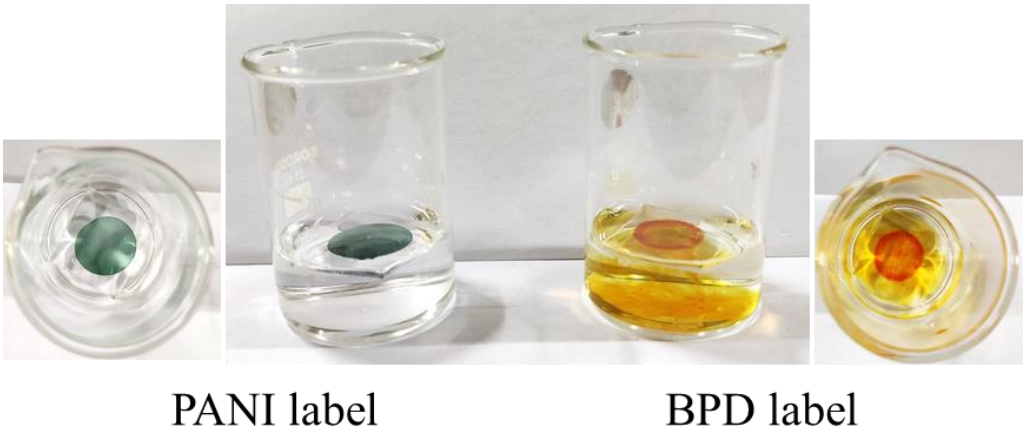


Figure 4.8. Leaching test of the developed labels.

4.4. Conclusion

The bromocresol purple dye (BPD) based label was successfully developed with a response time ranging from 0-1 min. The fast response of the BPD label makes it suitable for the determination of the chemical spoilage of fish. However, a significant drawback emerged due to dye-leaching problems, which compromised the label's efficiency and reliability over time. The leaching of the dye from the sensor affected its performance and restricted the application for one cycle, ultimately rendering the developed BPD label unsuitable for consistent and accurate detection purposes. In conclusion, while the BPD-based sensor exhibited favorable response characteristics to ammonia vapor and volatile amines, the issue of dye leaching poses a critical challenge that needs to be addressed to enhance the sensor's stability, reliability, and reusability for practical applications in detecting fish spoilage. Further research and development efforts are required to overcome this limitation and optimize the performance of dye-based sensors for effective food quality monitoring.

4.5. References

95/149/EC: Commission Decision of 8 March 1995 fixing the total volatile basic nitrogen (TVB-N) limit values for certain categories of fishery products and specifying the analysis methods to be used. (1995). *Official Journal*, L 97, 84-87 ELI: http://data.europa.eu/eli/dec/1995/149/oj [legislation]

Cai, L., Cao, A., Li, Y., Song, Z., Leng, L., & Li, J. (2015). The effects of essential oil treatment on the biogenic amines inhibition and quality preservation of red drum (*Sciaenops ocellatus*) fillets. *Food Control*, 56, 1-8.

Chen, H. Z., Zhang, M., Bhandari, B., & Yang, C. H. (2020). Novel pH-sensitive films containing curcumin and anthocyanins to monitor fish freshness. *Food Hydrocolloids*, *100*, 105438.

Chen, H., Zhou, K., & Zhao, G. (2018). Gold nanoparticles: From synthesis, properties to their potential application as colorimetric sensors in food safety screening. *Trends in Food Science & Technology*, *78*, 83-94.

Chen, Q., Hu, Z., Yao, F. Y. D., & Liang, H. (2016). Study of two-stage microwave extraction of essential oil and pectin from pomelo peels. *LWT-Food Science and Technology*, *66*, 538-545.

Chen, Q., Hui, Z., Zhao, J., & Ouyang, Q. (2014). Evaluation of chicken freshness using a low-cost colorimetric sensor array with AdaBoost–OLDA classification algorithm. *LWT-Food Science and Technology*, *57*(2), 502-507.

Chen, Y., Fu, G., Zilberman, Y., Ruan, W., Ameri, S. K., Zhang, Y. S., Miller, E., & Sonkusale, S. R. (2017). Low-cost smartphone diagnostics for food using paper-based colorimetric sensor arrays. *Food Control*, *82*, 227-232.

Cichero, M. C., & dos Santos, J. H. Z. (2021). Encapsulated bromocresol purple-based sensitive materials: The role of the nature and distribution of matrix layers on ammonia sensing performance. *Applied Surface Science Advances*, *4*, 100078.

Feng, L., Musto, C. J., Kemling, J. W., Lim, S. H., & Suslick, K. S. (2010). A colorimetric sensor array for identification of toxic gases below permissible exposure limits. *Chemical Communications*, *46*(12), 2037-2039.

Giarratana, F., Muscolino, D., Beninati, C., Ziino, G., Giuffrida, A., & Panebianco, A. (2016). Activity of R (+) limonene on the maximum growth rate of fish spoilage organisms and related effects on shelf-life prolongation of fresh gilthead sea bream fillets. *International Journal of Food Microbiology*, *237*, 109-113.

Hao, R., Roy, K., Pan, J., Shah, B. R., & Mraz, J. (2021). Critical review on the use of essential oils against spoilage in chilled stored fish: A quantitative meta-analysis. *Trends in Food Science & Technology*, 111, 175–190.

Huang, X., Xin, J., & Zhao, J. (2011). A novel technique for rapid evaluation of fish freshness using colorimetric sensor array. *Journal of Food Engineering*, *105*(4), 632-637.

Janzen, M. C., Ponder, J. B., Bailey, D. P., Ingison, C. K., & Suslick, K. S. (2006). Colorimetric sensor arrays for volatile organic compounds. *Analytical Chemistry*, *78*(11), 3591-3600.

Koxmak, S., Yimamumaimaiti, T., Abdukeremu, H., Nizamidin, P., & Yimit, A. (2019). Detection of amines in lamb spoilage by optical waveguide sensor based on bromophenol blue-silicon composite film. *Chemical Research in Chinese Universities*, 35, 193-199.

Kuswandi, B., Jayus, A. Restyana, A. Abdullah, Heng, L.Y., Ahmad, M., (2012). A novel Colorimetric Food Package label for fish spoilage based on polyaniline film. *Food Control*, 21, 184–189.

Majdinasab, M., Hosseini, S. M. H., Sepidname, M., Negahdarifar, M., & Li, P. (2018). Development of a novel colorimetric sensor based on alginate beads for monitoring rainbow trout spoilage. *Journal of Food Science and Technology*, *55*(5), 1695-1704.

Meng, X., Huang, H., Yan, K., Tian, X., Yu, W., Cui, H., Kong, Y., Xue, L., Liu, C., & Wang, S. (2017). Smartphone based hand-held quantitative phase microscope using the transport of intensity equation method. *Lab on a Chip*, 17 (1), 104-109.

Morsy, M. K., Zór, K., Kostesha, N., Alstrøm, T. S., Heiskanen, A., El-Tanahi, Sharoba, A., Papkovsky, D., Larsen, J., Khalaf, H. and Jakobsen, M.H. (2016). Development and validation of a colorimetric sensor array for fish spoilage monitoring. *Food Control*, 60, 346-352.

Pacquit, A., Lau, K. T., & Diamond, D. (2004). Development of a colorimetric sensor for monitoring of fish spoilage amines in packaging headspace. *SENSORS IEEE* (pp. 365-367).

Perez-Cacho, P., R. & Rouseff, R., L. (2008) Fresh squeezed orange juice odor: a review. Critical Reviews in Food Science and Nutrition *48*(7) 681-695

Suslick, B. A., Feng, L., & Suslick, K. S. (2010). Discrimination of complex mixtures by a colorimetric sensor array: coffee aromas. *Analytical chemistry*, *82*(5), 2067-2073.

Wells, N., Yusufu, D., & Mills, A. (2019). Colorimetric plastic film indicator for the detection of the volatile basic nitrogen compounds associated with fish spoilage. *Talanta*, 194, 830-836.

Yan, J., Cui, R., Qin, Y., Li, L., & Yuan, M. (2021). A pH indicator film based on chitosan and butterfly pudding extract for monitoring fish freshness. *International Journal of Biological Macromolecules*, *177*, 328-336.

Yu, D., Wu, L., Regenstein, J. M., Jiang, Q., Yang, F., Xu, Y., & Xia, W. (2020). Recent advances in quality retention of non-frozen fish and fishery products: A review. *Critical Reviews in Food Science and Nutrition*, 60 (10), 1747-1759.

Zaragozá, P., Fuentes, A., Ruiz-Rico, M., Vivancos, J. L., Fernández-Segovia, I., Ros-Lis, J. V., Barat, J., M., & Martínez-Máñez, R. (2015). Development of a colorimetric sensor array for squid spoilage assessment. *Food Chemistry*, 175, 315-321.