

**T.R.
SAKARYA UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES**

**DEVELOPMENT AND VALIDATION OF A pH SENSITIVE
COLORIMETRIC SENSING LABEL FOR MONITORING FISH
FRESHNESS**

MSc THESIS

Amal Samir Lutfi ALOBAIDI

Food Engineering Department

Food Engineering Program

JANUARY 2024

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Thesis Advisor: Prof. Dr. Zehra AYHAN

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The thesis work titled “DEVELOPMENT AND VALIDATION OF A pH SENSITIVE COLORIMETRIC SENSING LABEL FOR MONITORING FISH FRESHNESS” prepared by Amal Samir Lutfi ALOBAIDI was accepted by the following jury on 12/01/2024 by unanimously/majority of votes as a MSc THESIS in Sakarya University Graduate School of Natural and Applied Sciences, Food Engineering department, Food Engineering program.

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(17/01/2024)

Amal Samir Lutfi ALOBAIDI





To my father



ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my advisor, Prof. Zehra AYHAN, for all her guidance and encouragement. Her awe-inspiring determination, patience, and thoughtful insights were the fuel that kept our motivation from fading throughout this research.

I would also like to extend my sincere gratitude to Dr. Güliz HASKARACA, whose invaluable support inside and outside the laboratory made the completion of this research possible.

I could not have undertaken this journey without Irem Melda KARACA, with whom I had the pleasure of sharing thoughts and long laboratory days while facing every hindrance with great resilience until we reached our goal.

Special thanks to Eylem KARAKUŞ and Fatma AL, without whose support this scientific endeavor would not have been possible.

This acknowledgement will not be complete without thanking my friends and family, who showered me with love and support that kept me going tirelessly for the past years.

I would like to acknowledge Ispak Flexible Packaging Company for their financial support and the invaluable support of their R&D department team led by Mr. Emre GÜLTEKİN, as well as Sakarya University Scientific Research Projects Unit (BAP) for their financial support of this research project (with project number of 2020-7-24-111). I also thank A-Pack Packaging Company for providing PE/PA pouches for this study.

Many thanks should also go to everyone -people and institutions- who provided me with the support and assistance I needed to complete this thesis.

Finally, I would like to thank the jury members; Prof. Servet Gülüm ŞUMNU and Asst. Prof. Güliz HASKARACA for their valuable time and insights.

Amal Samir Lutfi ALOBAIDI



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ABBREVIATIONS

BTB	: Bromothymol blue
CA	: Cellulose acetate
CO₂	: Carbon dioxide
DMA	: Dimethylamine
HCl	: Hydrochloric acid
LDPE	: Low density polyethylene
MAP	: Modified atmosphere packaging
MC	: Methyl cellulose
NaOH	: Sodium hydroxide
PA	: Polyamide
PEG-400	: Polyethylene glycol-400
PEG-600	: Polyethylene glycol-600
PET	: Polyethylene terephthalate
RFID	: Radio frequency identification
TMA	: Trimethylamine
TTI	: Time-temperature indicators
TVB-Ns	: Total volatile basic amine



SYMBOLS

pKa	: Acid dissociation constant
m²	: Area measuring unit, meter square
L*	: Lightness
M	: Liquid concentration measuring unit, mol/liter
μl	: Liquid volume measuring unit, microliter
ml	: Liquid volume measuring unit, milliliter
g	: Mass measuring unit, gram
mg	: Mass measuring unit, milligram
a*	: Redness
rpm	: Round per minute
°C	: Temperature (Degree Celsius)
ΔE	: Total color difference
b*	: Yellowness



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DEVELOPMENT AND VALIDATION OF A pH SENSITIVE COLORIMETRIC SENSING LABEL FOR MONITORING FISH FRESHNESS

SUMMARY

With the increasing consumers' awareness of the importance of food quality, new packaging technologies emerged to cater to their needs. Intelligent packaging is a packaging technology that allows consumers to observe -in real-time- the quality of food products. The main component of intelligent packaging is a pH-sensitive, color-changing dye that responds to certain metabolites produced during the storage period, due to either microbial or chemical deterioration of the product. Thus, upon accumulation of a certain amount of the target metabolite, the color of the intelligent label changes, informing the consumers that the food product is not edible. This promising technology is not only expected to have a huge impact on the consumers' satisfaction, but it will also contribute to an overall reduction in food waste, which will lead to decreasing both economic loss and preserving resources. Real-time quality observation will eliminate the need to keep track of the best-before date, which means that no food product will be disposed of before actually reaching the spoilage threshold. Even though lots of research related to intelligent packaging is present, the implementation of this technology in real food systems and supply chains is still limited. However, in the coming years the application of intelligent packaging is expected to increase tremendously.

In this thesis, the aim was to fabricate and validate an intelligent label intended to be used in fish packaging. The labels produced have a three-layer structure: a color-changing layer that consists of a pH-sensitive dye and a binder, upper layer with hydrophobic properties to prevent moisture from the outer atmosphere entering the label, and lower layer that allows moisture and other metabolites to reach the color-changing layer.

For the color-changing layer, bromothymol blue (BTB) was used as the pH-sensitive dye, while both methyl cellulose (MC) and cellulose acetate (CA) were used as binders. For the outer layer, polyethylene terephthalate (PET) film and for the inner layer polyethylene (PE) were used. Two dye solutions were prepared: bromothymol blue and methyl cellulose (water base), and bromothymol blue and cellulose acetate (solvent based). To produce labels sensitive to different metabolites (i.e., CO₂ & total volatile basic amines (TVB-Ns)), either HCl or NaOH were added to decrease or increase the initial pH of the labels according to the target metabolite (HCl for TVB-N sensitive labels and NaOH for CO₂ sensitive labels). The four different solutions (MC-based with either HCl or NaOH, and CA-based with either HCl or NaOH) were cast in circular shapes over the PET film and dried at 40°C overnight for the MC-based labels, and at room temperature for the CA-based labels. The labels were then cut and laminated with PE polymer.

The labels were divided into two groups: CO₂ sensitive labels (BTB-MC-NaOH and BTB-CA-NaOH) and TVB-N sensitive labels (BTB-MC-HCl and BTB-CA-HCl).

Accordingly, two different simulations were conducted: CO₂ simulation with CO₂ concentrations of 0%, 5%, 10%, 15%, 20%, 25%, and 30%, and trimethyl amine (TMA) as a representative of TVB-Ns, with concentrations of 0%, 5%, 10%, 15%, 20%, 25%, and 30%. The CO₂ was flushed into polyethylene/polyamide (PE/PA) packages containing the CO₂ sensitive intelligent labels using a modified atmospheric packaging (MAP) machine. The packages containing the TVB-N sensitive intelligent labels TMA solutions of different concentrations were placed in closed vials that were reopened after flushing the packages with N₂ gas prevent the interference of other gases. The two groups were then stored at 4°C for 10 days and the color change was observed and measured using a portable colorimeter every 2 storage days to determine the total color change (ΔE) by measuring L*, a*, b* values.

To validate the intelligent labels in a real food system, atlantic bonito (*Sarda sarda*) fish was used. The fish were placed in PE/PA packages containing all four intelligent labels (BTB-MC-NaOH, BTB-CA-NaOH, BTB-MC-HCl and BTB-CA-HCl). To further imitate the commercially available fish packaging, two different MAP compositions usually used in fish packaging were used: 100% N₂ and 70% N₂ 30% CO₂. The packages were then stored at 4°C for 10 days.

In addition to monitoring the total color change of the labels, quality of fish was monitored by measuring headspace gas composition, total mesophilic aerobic bacteria, *Pseudomonas spp.*, pH, TVB-N, TMA concentration using gas chromatography (GC), and sensory evaluation.

In CO₂ simulation, BTB-MC-NaOH labels changed from blue to green at 15% CO₂, while BTB-CA-NaOH labels changed from green to yellow at 25% CO₂. The color change in both labels was visible to the naked eye within 24 hours and stayed constant through the storage period. For the TMA simulation, BTB-MC-HCl and BTB-CA-HCl labels changed color completely at 15% TMA (BTB-MC-HCl from yellow to navy and BTB-CA-HCl from yellow to green). For the CO₂ sensitive labels, the color change was also visible to the naked eye within 24 hours and stayed constant through the storage period. These results were promising since the color-changing range of the TVB-N sensitive labels is also considered the spoilage threshold of fish.

In the food validation trial, the headspace gas composition (CO₂ and O₂ concentrations) did not change significantly during the storage period (p<0.05), however, trace amounts of O₂ were found in both MAP groups' packages despite not having O₂ in the MAP composition, probably due to the release of O₂ from the pores of fish during the storage period.

Both total mesophilic aerobic bacteria and *Pseudomonas spp.* in both MAP groups increased significantly (p<0.05). In 70% N₂ 30% CO₂ group, total mesophilic aerobic bacteria increased from 5.22 log cfu/g to 7.07 log cfu/g and *Pseudomonas spp.* increased from 3.74 log cfu/g to 6.80 log cfu/g by the 10th storage day, while in 100% N₂ group, total mesophilic aerobic bacteria increased from 5.22 log cfu/g to 7.75 log cfu/g and *Pseudomonas spp.* increased from 3.74 log cfu/g to 7.94 log cfu/g by the 10th storage day. Since the spoilage threshold for fish under MAP is 7 log cfu/g, 70% N₂ 30% CO₂ group reached upper acceptable limit (6 log cfu/g) after 6th day (approximately on 7th day) and microbial spoilage threshold (7 log cfu/g) after the 8th day while 100% N₂ group reached upper acceptable limit after the 4th day (approximately on 5th day) and microbiological spoilage threshold at the 6th day. The difference between the two groups is thought to be the effect of CO₂ in the 70% N₂

30% CO₂ group, which may have contributed to the slightly longer shelf-life, since CO₂ is thought to have an inhibitory effect on bacterial growth.

Throughout the storage period (10 days at 4°C) no significant difference in pH values occurred in fish packaged under the two different MAP compositions ($p > 0.05$).

As part of the TVB-Ns, the TMA concentration in the headspace also showed a similar gradual increase over the storage period ($p < 0.05$), although the highest value did not exceed 6 ppm in both MAP groups. The TMA level in 100% N₂ group was slightly higher than 70% N₂ 30% CO₂ group, even though the final TVB-N levels are similar. This could be the result of CO₂ presence in 70% N₂ 30% CO₂ group, which has a more inhibitory effect on the specific spoilage organisms that contribute to the production of TMA.

Considering the score 3 as the acceptability level for all sensory attributes, both MAP groups (30% CO₂ 70% N₂ and 100% N₂) are considered unacceptable by the 6th day of storage for all the sensory attributes (color, odor, texture, and overall acceptability).

The CO₂ sensitive labels in 70% N₂ 30% CO₂ MAP group, changed color from blue to green in BTB-MC-NaOH labels and from green to yellow in BTB-CA-NaOH labels by the second day of storage since 30% CO₂ was used in the MAP composition, which color change is the same as the color change observed in these labels in the CO₂ simulation results. The total color change values (ΔE) for these labels stayed almost constant with no significant difference ($p > 0.05$) in both labels during the storage period (10 days at 4 °C). In 100% N₂ group, the CO₂ sensitive labels (BTB-MC-NaOH and BTB-CA-NaOH) did not change color, since the CO₂ level in the packages stayed in the range 0-5% during the storage period. The total color change values (ΔE) of these labels stayed almost constant for both labels with no significant difference throughout the storage period ($p > 0.05$). The results in both groups can be considered a further validation of the CO₂ simulation results.

TVB-N sensitive labels (BTB-MC-HCl and BTB-CA-HCl) did not show any color change during the storage period of fish in both MAP groups. The total color change values (ΔE) of these labels showed no significant differences, whether within the same group during the storage period or within the two different groups within the storage period ($p > 0.05$). In the TMA simulation, the color change in both TVB-N sensitive labels (BTB-MC-HCl and BTB-CA-HCl) changed color when the TMA level reached 10 ppm. Since the TMA values obtained for both MAP groups did not exceed 6 ppm, the result is in parallel with the TMA simulation results.

In conclusion, the results of this research show that the fabricated intelligent labels - regardless of the binder used- could successfully monitor the different concentrations of spoilage metabolites (i.e., CO₂ and TMA) under simulated conditions. However, since fish is usually packaged under MAP (usually without oxygen in the MAP gas compositions), it has a different spoilage mechanism than fish packaged under atmospheric conditions. Hence, these labels are not suitable to appropriately monitor the freshness of fish under MAP conditions, which is a factor often dismissed when conducting a validation experiment for intelligent labels.



BALIK TAZELİĞİNİN İZLENMESİ İÇİN pH DUYARLI KOLORİMETRİK AKILLI ETİKET GELİŞTİRİLMESİ VE VALIDASYONU

ÖZET

Tüketicilerin gıda kalitesinin önemi konusundaki farkındalıklarının artmasıyla birlikte, artan tüketici ihtiyaçlarını karşılamak için yeni ambalaj teknolojileri ortaya çıkmıştır. Akıllı ambalaj, tüketicilerin gıda ürünlerinin kalitesini gerçek zamanlı olarak izlemelerine olanak tanıyan bir ambalaj teknolojisidir. Akıllı ambalajın temel bileşeni, ürünün depolama süresi boyunca mikrobiyal veya kimyasal bozulmasına bağlı olarak belirli metabolitlere tepki veren pH'a duyarlı ve renk değiştiren bir boyadır. Bu sayede, hedef metabolitin birikmesi durumunda akıllı etiketin rengi değişerek tüketicilere gıda ürününün tüketilmemesi gerektiği konusunda bilgi verir. Bu umut verici teknoloji, tüketicilerin memnuniyetini artırmanın ötesinde, aynı zamanda gıda israfını genel olarak azaltarak ekonomik kayıpları en aza indirgeyecek ve kaynakların korunmasına katkı sağlayacaktır. Gıda kalitesinin gerçek zamanlı takibi, son kullanma tarihini izleme ihtiyacını ortadan kaldırarak, herhangi bir gıda ürününün bozulma aşamasına gelmeden veya tüketilmeden önce israf edilmesini önleme kapasitesine sahiptir. Ancak, bu teknoloji henüz yeni olduğu için çok sayıda araştırma bulunsa da gerçek gıda sistemlerinde ve tedarik zincirlerinde uygulanması hala sınırlıdır. Ancak bu teknolojinin uygulamada önümüzdeki yıllarda artacağı tahmin edilmektedir.

Bu tez çalışmasında, balık ambalajında kullanılabilecek akıllı bir etiketin tasarlanması, geliştirilmesi, simülasyon ve validasyon çalışmalarının gerçekleştirilmesi amaçlanmıştır. Geliştirilen etiketler üç katmanlı olarak tasarlanmıştır. İlk katman, pH'a duyarlı bir boya ve bir bağlayıcıdan oluşan bir renk değişim katmanıdır. İkinci katman, dış atmosferdeki nemin etiketle temasını önlemek için hidrofobik özelliklere sahip bir dış üst katmandır. Üçüncü katman ise nem ve diğer metabolitlerin renk değişim katmanına ulaşmasına izin veren bir alt katmandır.

Renk değişim katmanı için pH'a duyarlı bir boya olan bromotimol mavisi (BTB), bağlayıcı olarak metil selüloz (MC) ve selüloz asetat (CA) kullanılmıştır. Dış katman için polietilen tereftalat (PET) film, iç katman için ise polietilen (PE) kullanılmıştır. İki farklı boya çözeltisi hazırlanmıştır: Birincisi, bromotimol mavisi ve metil selüloz içeren bir su bazlı çözelti iken, diğeri bromotimol mavisi ve selüloz asetat içeren bir solvent bazlı çözeltidir. Bozulma metabolitlerinden CO₂ ve toplam uçucu bazik aminlere (TVB-N'ler)) duyarlı etiketler üretmek amacıyla, hazırlanan boya solüsyonlarının pH değerleri HCl veya NaOH kullanılarak ayarlanmıştır. TVB-N'ye duyarlı etiketler için HCl, CO₂'ye duyarlı etiketler için ise NaOH kullanılmıştır. Hazırlanan dört farklı boya çözeltisi (HCl veya NaOH içeren MC bazlı ve HCl veya NaOH içeren CA bazlı) PET film üzerine dairesel şekillerde dökülmüş ve MC bazlı etiketler 40°C'de bir gece boyunca, CA bazlı etiketler ise oda sıcaklığında kurutulmuştur. Sonrasında etiketler kesilmiş (2x2 cm) ve PE film ile lamine edilmiştir.

Etiketler iki farklı gruba ayrılmıştır: CO₂ metabolitine duyarlı etiketler (BTB-MC-NaOH ve BTB-CA-NaOH) ve TVB-N metabolitine duyarlı etiketler (BTB-MC-HCl ve BTB-CA-HCl). Bu doğrultuda, iki ayrı simülasyon çalışması gerçekleştirilmiştir:

İlk olarak, farklı CO₂ konsantrasyonlarını (%0, %5, %10, %15, %20, %25 ve %30) içeren CO₂ simülasyonu; ikinci olarak ise TVB-N metabolitini temsil etmek amacıyla farklı konsantrasyonlarda trimetil amin (TMA) içeren simülasyon çalışması gerçekleştirilmiştir.

CO₂ simülasyonu için CO₂'ye duyarlı akıllı etiketler polietilen/poliamid (PE/PA) torbalarına yerleştirilmiş ve modifiye atmosfer paketleme (MAP) makinesi kullanılarak belirlenen konsantrasyonlardaki CO₂ gazı torbalara enjekte edilmiştir. TVB-N simülasyonu için ise TVB-N duyarlı etiketleri içeren PE/PA torbalara farklı TMA çözeltilerini içeren kapaklı şişeler yerleştirilmiş, ardından paket ortamına N₂ gazı enjekte edildikten sonra şişelerin kapakları açılmıştır. Her iki grup da 4°C'de 10 gün boyunca depolanmış ve her 2 günde bir kolorimetre yardımıyla L*, a*, b* değerleri ölçülerek renk değişimi belirlenmiş ve toplam renk değişimi (ΔE) değeri hesaplanmıştır.

Validasyon çalışmasında ise Atlantik palamut (Sarda sarda) balığı, akıllı etiketlerin (BTB-MC-NaOH, BTB-CA-NaOH, BTB-MC-HCl ve BTB-CA-HCl) tamamını içeren PE/PA paketlere yerleştirilerek, akıllı etiketlerin gerçek bir gıda sisteminde doğrulanması için kullanılmıştır. Balık ambalajları, genellikle endüstriyel balık ambalajlarında kullanılan iki farklı MAP bileşimi olan 100% N₂ ve %70 N₂ %30 CO₂ ile gerçekleştirilmiştir. MAP uygulanan ambalajlar 4°C'de 10 gün boyunca depolanmıştır. Etiketlerin toplam renk değişiminin yanı sıra, balık kalitesi tepe boşluğu gaz konsantrasyonu, toplam mezofilik aerobik bakteri, *Pseudomonas* spp., pH, TVB-N, TMA konsantrasyonu ve duyuşal değerlendirme ile izlenmiştir.

CO₂ simülasyonu çalışmasında, BTB-MC-NaOH etiketleri %15 CO₂ konsantrasyonunda maviden yeşile dönerken, BTB-CA-NaOH etiketleri %25 CO₂ konsantrasyonunda yeşilden sarıya dönüşmüştür. Her iki etiketteki renk değişimi 24 saat içinde çıplak gözle tespit edilebilmiş ve depolama süresi boyunca renk değişimi sabit kalmıştır. TMA simülasyonunda, BTB-MC-HCl ve BTB-CA-HCl etiketleri %15 TMA seviyesinde tamamen renk değiştirmiştir (BTB-MC-HCl sarıdan laciverte ve BTB-CA-HCl sarıdan yeşile). CO₂'ye duyarlı etiketlerde olduğu gibi, renk değişimi 24 saat içinde çıplak gözle tespit edilmiş ve depolama süresince değişmeden kalmıştır. TVB-N'ye duyarlı etiketlerin renk değiştirme aralığı aynı zamanda balığın bozulma konsantrasyonlarında gözlemlendiği için TVB-N'ye duyarlı etiketler, balığın tazelik kaybını belirleme potansiyeli açısından umut vadedicidir.

Gıda validasyon denemesinde, tepe boşluğu gaz bileşimi (CO₂ ve O₂ konsantrasyonları) depolama süresince önemli ölçüde değişmemiştir (p<0.05), ancak her iki MAP grubunun ambalajlarında, O₂ bulunmamasına rağmen, iz miktarda O₂ tespit edilmiştir. Bu durumun muhtemel nedeni, depolama süresi boyunca balıkların gözeneklerinde kalan O₂'nin tepe boşluğuna salınmasıdır.

Her iki MAP grubunda da, hem toplam mezofilik aerobik bakteriler hem de *Pseudomonas* spp. önemli ölçüde artmıştır (p<0.05). %70 N₂ & %30 CO₂ MAP grubunda, toplam mezofilik aerobik bakteri sayısı 10. depolama gününde 5.22 log kob/g'dan 7.07 log kob/g'a artarken, *Pseudomonas* spp. sayısı 3.74 log kob/g'dan 6.80 log kob/g'a çıkmıştır. %100 N₂ MAP grubunda ise toplam mezofilik aerobik bakteriler 10. depolama gününde 5.22 log kob/g'dan 7.75 log kob/g'a, *Pseudomonas* spp. ise 3.74 log kob/g'dan 7.94 log kob/g'a artış göstermiştir. Her iki MAP grubundaki balıklar için mikrobiyolojik bozulma eşiği 7 log kob/g olduğu için %70 N₂ %30 CO₂ grubu 6. günden sonra (yaklaşık 7. günde) kabul edilebilir üst sınıra (6 log kob/g) ve 8. günden sonra mikrobiyolojik bozulma eşiğine (7 log kob/g) ulaşmıştır. Diğer yandan, %100

N₂ içeren balık 4. günden sonra (yaklaşık 5. günde) kabul edilebilir sınır değere ve 6. günden sonra mikrobiyolojik bozulma eşiğine ulaşmıştır. İki grup arasındaki fark, %70 N₂ %30 CO₂ grubunda CO₂'nin antibakteriyel etkisi ile açıklanabilir.

Depolama süresince iki farklı MAP altında ambalajlanan balıkların pH'larında istatistiki olarak nemli düzeyde bir fark tespit edilmemiştir (p>0.05).

MAP uygulanan balık ambalajlarının tepe boşluğundaki TMA konsantrasyonu depolama süresince kademeli artış göstermiştir (p<0.05), ancak her iki MAP grubunda da en yüksek değer 6 ppm'i aşmamıştır. Nihai TVB-N seviyeleri benzer olsa da, %100 N₂ grubundaki TMA seviyesinin %70 N₂ ve %30 CO₂ grubundan biraz daha yüksek olduğu tespit edilmiştir. Bu durum, TMA üretimine katkıda bulunan spesifik bozulma organizmaları üzerinde daha fazla inhibe edici etkiye sahip olan %70 N₂ & %30 CO₂ grubundaki CO₂'nin varlığına bağlanabilir.

Duyusal değerlendirmede her iki MAP grubu da (%30 CO₂ ve %70 N₂ grubu ve %100 N₂ grubu) depolamanın 6. gününde renk, koku, doku ve genel kabul edilebilirlik açısından kabul edilebilir bulunmamıştır..

MAP grubundaki (%30 CO₂ %70 N₂) CO₂'ye duyarlı etiketler, paketleme sırasında %30 CO₂ kullanıldığı için ikinci depolama gününden itibaren renk değiştirmeye başlamış; BTB-MC-NaOH etiketleri maviden yeşile, BTB-CA-NaOH etiketleri ise yeşilden sarıya dönmüştür, ve 10. depolama gününe kadar sabit kalmıştır.. Bu renk değişimi, CO₂ simülasyon sonuçlarında bu etiketlerde gözlemlenen renk değişimiyle aynıdır. Bu etiketler için toplam renk değişimi değerlerinde (ΔE), 10 günlük depolama süresince her iki etikette de önemli bir fark gözlenmemiş (p>0,05) ve sabit kalmıştır %100 N₂ içeren grupta depolama süresince ambalajlardaki CO₂ seviyesi %0-5 aralığında kaldığı için CO₂'ye duyarlı etiketlerde (BTB-MC-NaOH ve BTB-CA-NaOH) gözle görülebilir bir değişim ortaya çıkmamıştır. Bu etiketlerin toplam renk değişim değerlerinde (ΔE) her iki etiket için de depolama süresince anlamlı bir değişiklik tespit edilmemiştir (p>0.05). Her iki gruptaki sonuçlar, CO₂ simülasyon sonuçlarının doğrulaması olarak kabul edilebilir.

TVB-N'ye duyarlı etiketlerde (BTB-MC-HCl ve BTB-CA-HCl) her iki MAP grubunda da depolama süresince herhangi bir renk değişimi tespit edilmemiştir. Bu etiketlerin toplam renk değişim değerleri (ΔE) depolama süresince aynı grup içinde veya farklı gruplar arasında anlamlı bir farklılık göstermemiştir (p>0.05). TMA simülasyonunda, TMA seviyesi 10 ppm'e ulaştığında, TVB-N'e duyarlı her iki etikette de (BTB-MC-HCl ve BTB-CA-HCl) renk değişimi izlenmiştir. Ancak, her iki MAP grubu için elde edilen TMA değerleri 6 ppm'i aşmadığı için simülasyon sonuçları ile paralellik içindedir.

Sonuç olarak, bu araştırmada elde edilen bulgulara göre, üretilen akıllı etiketlerin kullanılan bağlayıcıya bakılmaksızın simüle edilmiş koşullar altında bozulma metabolitlerinin (CO₂ ve TMA) farklı konsantrasyonlarını başarılı bir şekilde izleyebildiğini göstermektedir. Ancak balıklar genellikle oksijensiz MAP ile paketlenmiş olduğundan atmosferik koşullar altında paketlenen balıklardan farklı bir bozulma mekanizmasına sahiptir. Dolayısıyla bu etiketler, MAP koşulları altında balığın tazeliğini uygun şekilde izlemek için uygun değildir; bu durum, akıllı etiketler için doğrulama çalışması yapılırken sıklıkla göz ardı edilen bir faktördür.



1. INTRODUCTION

Fish is considered an important part of the human diet. It gained this spot since it's packed with protein, essential fatty acids, minerals, and vitamins. Despite its high nutritional value, fish is one of the most perishable products in the food sector, owing this highly perishable nature to its high-water activity (a_w), neutral pH, and autolytic enzymes (Abelti et al., 2022; Milijasevic et al., 2019; Sivertstvik, et al., 2002; Wu et al., 2021). With consumers' increasing awareness of the importance of a balanced diet, the demand for fish is also increasing. To preserve the quality of fish for as long as possible, proper packaging should be used during handling and distribution. However, to be able to meet the expectations of the consumers of high-quality products, the packaging used in the supply chain should evolve in a way that exceeds the roles of conventional packaging (Holman et al., 2018; Muller & Schmid, 2019; Schaefer & Cheung, 2018; Verma et al., 2021). Over the last two decades, the term "smart packaging" started to surface to keep up with the consumers' demands. Smart packaging refers to both active packaging and intelligent packaging. Active packaging aims to extend the shelf life of the product thanks to active materials like antimicrobials, antioxidants, etc. On the other hand, intelligent packaging monitors the status of the product in real time and provides information accordingly (Cheng et al., 2022; Holman et al., 2018). One of the types of intelligent packaging is "freshness indicators", which can be used as non-destructive, real-time freshness monitoring method for different products (Kuswandi et al., 2011). A basic freshness indicator consists of a pH indicator (synthetic or natural dyes) entrapped within a polymer matrix. During the storage period microbiological and chemical spoilage occurs, leading to the production of different chemicals (e.g., CO_2 , amines, ethanol, H_2S etc.) which accumulate in the headspace of the packaging. In the presence of a freshness label, these chemicals infiltrate the inner layer of the label and react with the pH indicator dye, causing a visible color change (Drago et al., 2020; Ozcan, 2020). During the storage of fish, volatile amines (namely trimethylamine (TMA) and small amounts of dimethylamine (DMA)) are produced by the breakdown of trimethylamine oxide (TMAO) at chill temperatures, which are responsible for the development of the

'fishy' odor associated with fish spoilage (Summers et al., 2016). The basic volatile amines (TVB-Ns) resulting from fish spoilage interact with the pH-sensitive dye in the freshness indicators, causing an elevation in the pH of the dye; which in turn induces a visible color change, making the monitoring of the freshness of fish possible. Many research papers investigating the potential use of synthetic (Byrne et al., 2002; Chun et al., 2014; Ibrahim et al., 2021; Lee et al., 2016; Liu et al., 2020; Morsy et al., 2016; Paquit et al., 2006; Paquit et al. 2007; Sariningsih, et al., 2019; Wells et al., 2019) and natural (Agustianti et al., 2021; Chen et al., 2020; Ezati et al., 2019; Ezati & Rhim, 2020; Gasti et al., 2021; Qin et al., 2021; Sani et al., 2021; Vedove et al., 2021;) pH indicators in intelligent food packaging can be found in the literature. Nevertheless, the commercial application of freshness indicators is still relatively low, which comes as a surprise despite the high need of such a concept, since it will not just contribute to reducing unnecessary food waste by providing a true expiry date based on a real-time analysis of the spoilage metabolisms, but will also reduce potential food poisoning among customers and increase the food safety and product traceability (Balbinot-Alfaro et al., 2019; Kusuma et al., 2023; Muller & Schmid, 2019). Another important aspect of why it is necessary to conduct more research on intelligent packaging and commercializing it, is the expected growth in smart packaging sector worldwide, which is expected to reach \$44.39 billion in 2024. The demand for smart packaging is mainly driven by the US, Japan, Australia, UK, and Germany (Sobhan et al., 2021).

In recent years, there was a special focus on utilizing biopolymers natural dyes instead of synthetic dyes, which might have the benefit of abandoning some dyes that are considered toxic to obtain overall more "natural" freshness indicators. However, the commercializing of such indicators faces many problems, starting with the fact that natural polymers have high brittleness, and high sensitivity to water, and both natural polymers and dyes are known for their low stability, which means their application is considered limited (Becerril et al., 2021; Kusuma et al., 2023). Another problem regarding freshness indicator was reported by Biji et al. (2015), who stated that one of the disadvantages of freshness indicators is the false-positive incidents, since the presence of a certain target metabolite does not necessarily mean poor quality. A freshness indicator should be fabricated keeping in mind that the spoilage of a certain food product is a complex process, and sensing more than one metabolite

simultaneously is a must to provide a more precise assessment of food spoilage, hence: food quality (Zhang et al., 2020).

Unfortunately, intelligent packaging reported in most literature is not suitable for industrial applications (Obaidi et al., 2022). Some articles mention the use of filter paper as a white background (Chen et al., 2019; Rukchon et al., 2014) to increase the visibility of the color change, or the direct use of filter paper as the color changing layer without using any form of lamination (Hidayat et al., 2019; Morsy et al., 2016; Nopwinyuwong et al., 2010; Zhang & Lim, 2018), which poses health issues since the dye may leach into the food packaged. Another setback with is the use of polymers that are costly and not commonly used in the packaging industry (Baek et al, 2018; Lee et al., 2019a; Lee et al., 2019b), which adds an extra cost to the package. In addition, even though in commercial applications of fish packaging modified atmospheric packaging (MAP) without oxygen is used, no article mentions the use of such packaging conditions in the validation trials, which is considered a major issue when it comes to commercializing the fabricated intelligent labels. Most of the developed intelligent indicators were validated in petri dishes under air atmosphere which is far from the real packaging practices.

The aim of this study is to investigate the possibility of producing freshness indicators with a potential commercial use for fish packaging, using bromothymol blue (BTB) pH-sensitive dye and two different binders (methyl cellulose (MC) and cellulose acetate (CA)), in addition to using the commercially common PET/LDPE films as an outer and inner layer. The freshness indicators' performance was first evaluated by conducting simulations for both CO₂ and TVB-Ns (represented by trimethylamine (TMA)), then utilized in fish packaging to validate the commercial use potential by applying real packaging conditions.



2. LITERATURE REVIEW

According to the World Health Organization (WHO), food contamination in 2017 caused more than 600 million cases of foodborne diseases and 420,000 deaths (Kusuma et al., 2023). These alarming numbers call for serious solutions in order to increase food safety practices around the globe, especially for high-risk food (e.g. meat, fish, poultry, etc.).

Food packaging plays an important role in protecting food products from surrounding conditions during transport and storage (Balbinot-Alfaro et al., 2019), which may cause adverse effect on the quality and safety of the packaged food. However, during the last decades, developments in food packaging sector occurred due to the rapid increase in different food industrial capacities and the awareness of the consumers of food safety, resulting in new packaging technologies (Kuswandi et al., 2011), which mainly aims to increase the safety of packaged food and decrease foodborne diseases and unnecessary food waste.

2.1. Intelligent Packaging

Intelligent packaging has been introduced to the packaging industry a few decades ago, driven by the need for more innovative packaging materials that exceed the roles of traditional packaging (Kuswandi et al., 2011; Schafer & Cheung, 2018). Intelligent packaging includes multiple technologies used to monitor the packaged food products using different principles. Radio frequency identification (RFID), time-temperature indicators (TTI), and freshness indicators are the most widely used types of intelligent packaging.

2.1.1. Radio frequency identification (RFID)

RFID is a technology where tags with data storage property are used (Muller & Schmid, 2019). The tags, which act as a data carrier, store up to 1 MB of information, allowing the collection, storage, and transmission of real-time info about food products to a user's information system. These tags play a role in facilitating traceability

throughout the supply chain, as well as speeding stock rotation and tracking processes (Kuswandi et al., 2011). This technology is already in use in big retail chains like Walmart and Home Depot, who use RFID tags to improve the safety of food products by tracking food suppliers and retailers. A RFID tag is a wireless system consisting of a transponder and an antenna with a unique number or alphanumeric order (Figure 2.1 (Muller & Schmid, 2019)). RFID readers use radio waves to obtain the data from the RFID tags, which is then displayed using a host computer, which allows the data to be analyzed (Kusuma et al., 2023). Unfortunately, the high cost of the tags prevents the implementation of this technology commercially, increasing the need for more research for a more affordable method of real-time tracking of food products.

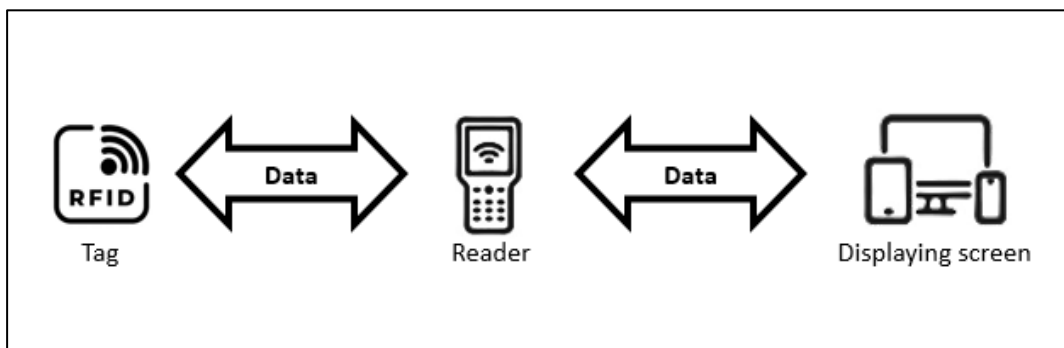


Figure 2.1. A basic RFID system.

2.1.2. Time-Temperature indicators (TTI)

Time temperature indicators are used when products are sensitive to the changes in storage conditions, namely temperature (Cheng et al., 2022; Drago et al., 2020). TTIs have different working principles, which can be divided mainly into chemical and physical indicators. Chemical TTIs are based on chemical reactions while physical TTIs are based on physical change according to the change in time and temperature. Good examples of chemical and physical based reactions are acid-base reaction, melting, and polymerization (Drago et al., 2020; Kusuma et al., 2023; Kuswandi et al., 2011; Muller & Schmid, 2019). The TTIs available in the market are based on the before mentioned working mechanisms. MonitorMarkTM, Timestrip[®], Fresh-Check[®], and Check point[®] are examples of the TTIs commercially available (Figure 2.2. (Upper left corner: MonitorMarkTM, Upper right corner: Timestrip[®], Lower left corner: Fresh-Check[®], Lower right corner: Check point[®])). Overall, TTIs are a simple and inexpensive method to monitor the change of temperature during storage of food

products; however, it cannot provide an indication of the condition of the food product itself, which is considered the major setback of this type of intelligent packaging.

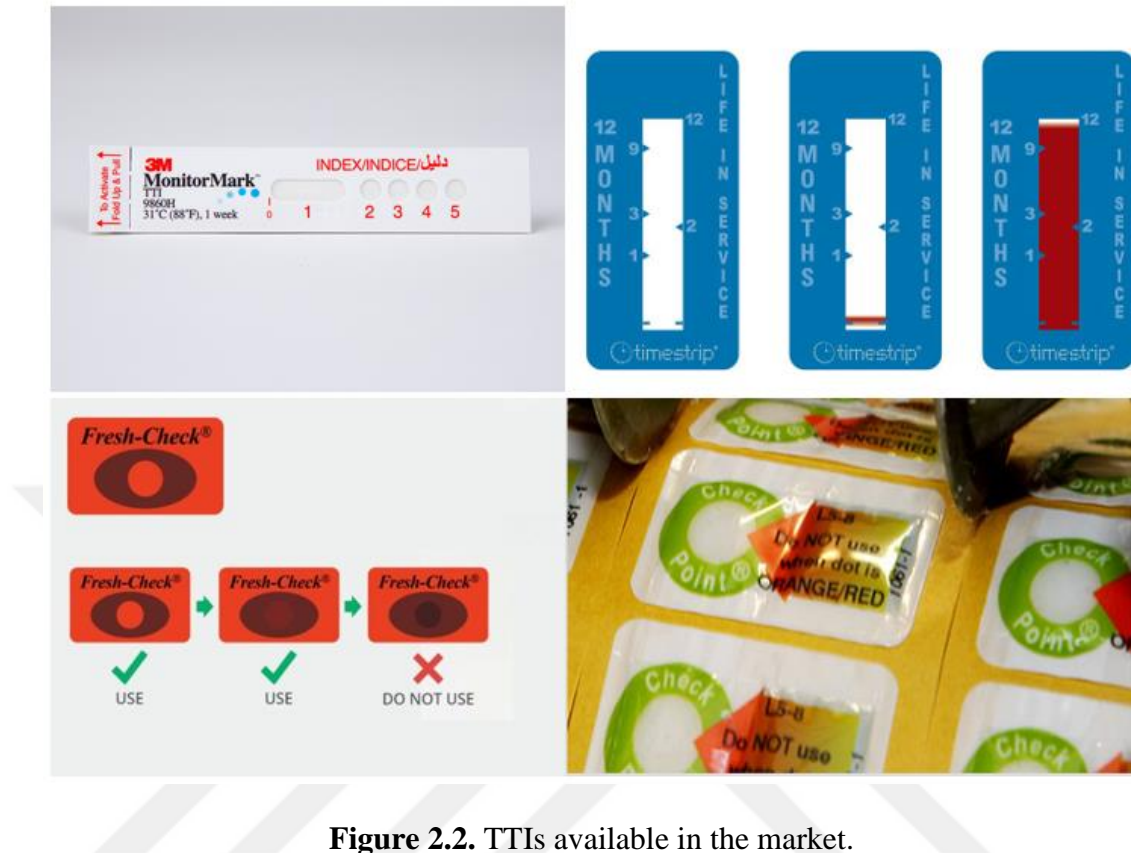


Figure 2.2. TTIs available in the market.

2.1.3. Freshness indicators

Freshness indicators are the most promising type of intelligent packaging, since it allows real-time, non-destructive monitoring of the products within the package, based on the changes in the product over the storage period (Kuswandi et al., 2011). Freshness indicators depend mainly on detecting the change in the metabolites produced during storage by either the chemical degradation of food itself or the microbial growth in the food product (e.g., CO₂, amines, organic acids, ethanol, H₂S, etc.). Thus, a clear understanding of the spoilage mechanism of the different food products is necessary to develop the freshness indicator that will successfully be able to detect the change in the target metabolite (Drago et al., 2020; Muller & Schmid, 2019).

Freshness indicators contain chemicals (dyes, enzymes, etc.) that react with the target metabolites, causing a color change that indicates the freshness state of food products. When it comes to research regarding freshness indicators, CO₂ and TVB-Ns are selected as the most dominant target metabolites. CO₂ is produced during the spoilage

of many food products like dessert (Nopwinyuwong et al., 2010), bell peppers (Chen et al., 2018), kimchi (Baek et al., 2018), and chicken (Lee et al., 2019; Rukchon et al., 2014). On the other hand, total volatile basic nitrogen (TVB-N) refers to a group of amines (e.g., trimethylamine (TMA), dimethylamine (DMA), and ammonia (NH₃)) that is often associated with the spoilage of fish, meat, and chicken since the spoilage microorganisms in these products produce them over the storage period. Many authors worked on developing freshness indicators that correspond to TVB-Ns, especially in fish (Byrne et al., 2002; Chun et al., 2014; Ezati et al., 2019; Paquit et al., 2006; Paquit et al., 2007; Morsy et al., 2016; Sariningsih et al., 2019; Wells et al., 2019), beef (Ezati et al., 2019; Hidayat et al., 2019, Kuswandi & Nurfawaidi, 2017; Lee & Shin, 2019), pork (Chen et al., 2019), and chicken (Kim et al., 2017; Lee et al., 2019; Rukchon et al., 2014). Although less common, freshness indicators focusing on hydrogen sulfide (H₂S) as the target metabolite were also developed to be used on chicken (Smolander et al., 2002). Some of the developed indicators even made it to the market, such as ripeSense™, which changes color according to show the ripeness condition of the packaged fruit by reacting to the aromatic compounds released during the storage period (Kuswandi et al., 2011). Another commercially available freshness indicator is SensorQ™, which detects the change in biogenic amines in food products by employing enzymes (amine oxidase or transglutamase) that change color upon reacting with biogenic amines (Muller & Schmid, 2019). Although many different structures of freshness indicators can be found throughout literature, a typical freshness indicator consists of 3 main components: a pH-sensitive dye, polymer matrix to entrap the pH-sensitive dye (binder), and polymers that engulf the color changing layer (pH-sensitive dye & binder) which are explained in detail below. Each component is crucial when it comes to developing a freshness indicator that corresponds to a certain metabolite in a certain food product (Kuswandi et al., 2011; Lee et al., 2019).

2.1.3.1. Dyes

pH sensitive dyes are the main component of freshness indicators since they cause visible color change that allows the real-time monitoring of the target spoilage metabolites (Kuswandi et al., 2011). The most used dyes for freshness indicators fabrication are synthetic and natural dyes. Synthetic dyes are generally weak protolytes which change color according to the pH (Barbosa, 2005). One important factor in determining the suitable dye to use in a freshness indicator is the dissociation value

(pKa) of the dye and the target metabolite. For example, if CO₂ is the target metabolite, the color changing mechanism in this case involves the dissociation of CO₂ in water to form carbonic acid (weak acid), which in turn dissociates and produces H⁺ ions that protonate dyes and consequently causes a color change. Since the acid dissociation value (pKa) of the resulting carbonic acid is 6.35, dyes with pKa value greater than 6.35 should be chosen to obtain an optimal color change (Zhang & Lim, 2018). Dyes are classified into four groups: azo dyes, phthaleins, sulfonphthaleins, and triphenylmethane dyes. Azo dyes like methyl red (MR) and methyl orange (MO) dyes, usually show a color change from red to yellow upon being subjected to basic mediums. The mechanism of the color change of an azo dye (methyl orange) is shown in Figure 2.3 (Barbosa, 2005).

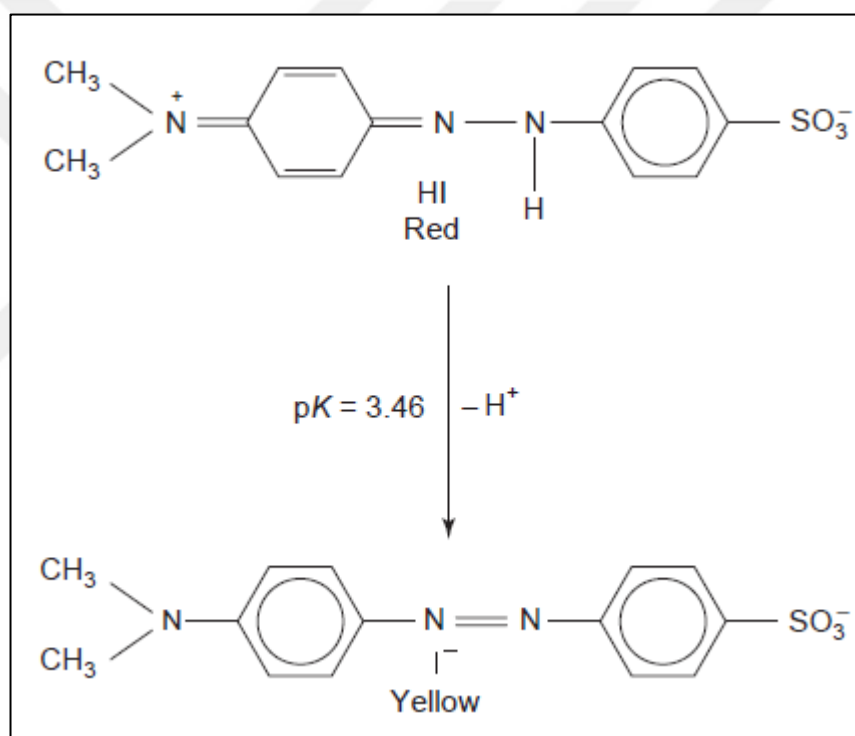


Figure 2.3. Methyl orange color change mechanism in basic solutions.

Azo dyes are widely used in freshness indicators, especially methyl red dye. Kuswandi et al. (2014) used a methyl red based freshness indicator to monitor the freshness of chicken and reported a color change from red to yellow following the increase in TVB-Ns in the headspace of the package during the storage period. Similar results were also reported by Kuswandi and Nurfawaidi (2017), Lee & Shin (2019), who also used methyl red to fabricate freshness indicators to monitor the freshness of beef, and Sariningsih et al. (2019), for monitoring the freshness of Tilapia Fillets. However,

when used in an array to monitor the freshness of Atlantic salmon by Morsy et al. (2016), methyl red based freshness indicators showed a slight change in color and did not turn to yellow completely, and the color change did not correlate with the spoilage metabolites. As it was mentioned before, azo dyes show a color change in basic mediums, which makes it a good choice for the detection of basic metabolites like TVB-Ns. In the case of acidic metabolites as CO₂, or to simply increase the sensitivity of methyl red, other authors used a mixture of methyl red with other dyes like bromothymol blue to assess the freshness of different products like pork (Chen et al., 2019), dessert (Nopwinyuwong et al., 2010), bell peppers (Chen et al., 2018), kimchi (Baek et al., 2018), and chicken (Rukchon et al., 2014).

Sulfonophthalein dyes can be considered the most used type of synthetic dyes since they exhibit a sharp color transition (Barbosa, 2005). Phenol red (PR), bromocresol green (BCG), bromocresol purple (BCP), bromothymol blue (BTB), cresol red (CR), *m*-cresol purple (CP) and thymol blue (TB) are examples of sulfonophthalein dyes. Some dyes in the sulfonophthalein group have two color change ranges, like phenol red (Figure 2.4 (Barbosa, 2005)). However, only the second color change is beneficial (pH 6.4-8.2).

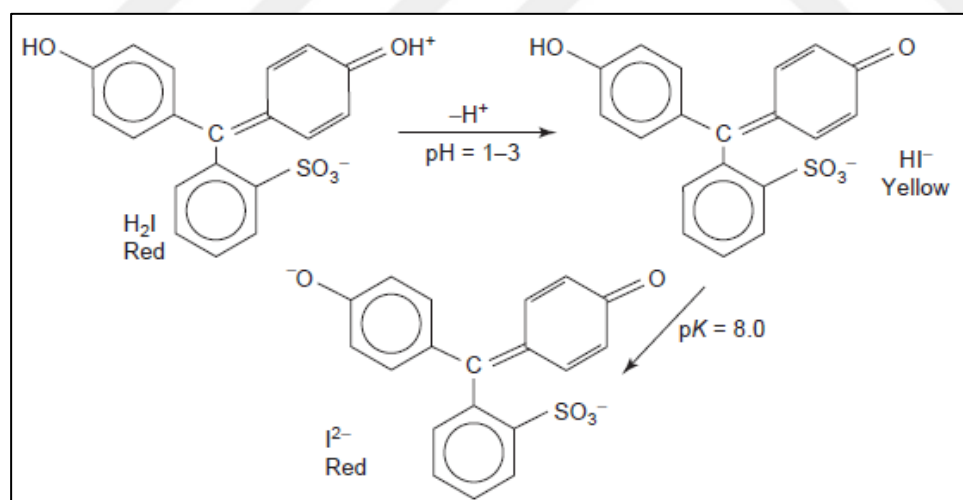


Figure 2.4. Phenol red color change mechanism.

For freshness indicators application, bromothymol blue (BTB), bromocresol green (BCG), and phenol red (PR) were used by many authors for different food applications, whether individually or as a mixture with other dyes as the case of methyl red, like fish (Chun et al., 2014; Paquit et al. 2006; Paquit et al., 2007), meat (Hidayat et al.,

2019), poultry (Lee et al., 2019; Ruckchon et al., 2014), bell peppers (Chen et al., 2018), kimchi (Baek et al., 2018), and dessert (Nopwinyuwong et al., 2010).

Although less common, cresol red (CR) and bromocresol purple (BCP) were used in the fabrication of freshness indicators for monitoring fish (Byrne et al., 2002), and beef (Kuswandi et al., 2017) freshness, respectively. In addition, Morsy et al. (2016), Zhang and Lim (2018), and Lee et al. (2019) developed arrays using different dyes including several of the beforementioned sulfonophthalein dyes.

On the other hand, natural dyes are being investigated by many authors lately to assess their use in freshness indicators, such as anthocyanins, alizarin, and curcumin (Priyadarshi et al., 2021).

Anthocyanins are among the most widely used natural dyes due to their wide range of pigments, which can exhibit red, orange, pink and blue colors (Zhao et al., 2022). They can be extracted from various natural resources, including vegetables (e.g., red cabbage, purple carrot, etc.), fruits (e.g., grape, pomegranate seed, berries etc.), and flowers (e.g., butterfly pea, red rose, etc.). The different colors of anthocyanins at different pH values can be seen in Figure 2.5 (Priyadarshi et al., 2021).

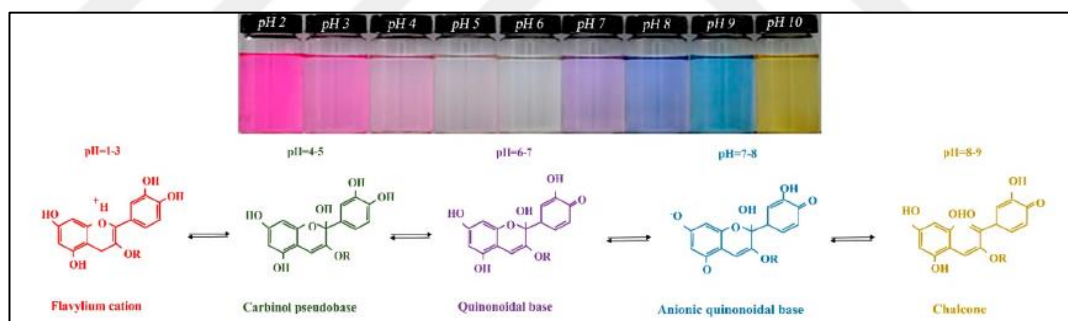


Figure 2.5. The different colors of anthocyanins at different pH values.

The potential of anthocyanins extracted from grape skins (Vedove et al., 2021), red barberry (Sani et al., 2021), *Lycium ruthenicum* flowers (Qin et al., 2021), *Phyllanthus reticulatus* fruit (Gasti et al., 2021), red cabbage (Agustianti et al., 2021; Freitas et al., 2020), and roselle flowers (Zhang et al., 2018) was investigated in order to be used in freshness indicators' fabrication for various food applications like pork and fish.

Alizarin dye is derived from the roots of madder plant (*Rubia tinctorum*) and has an orange-brown color (Priyadarshi et al., 2021). The color of alizarin dye at different pH values is shown in Figure 2.6 (Priyadarshi et al., 2021). It was reported in literature

that alizarin-based was successfully utilized in freshness indicators to monitor the freshness of rainbow trout fillet (Ezati et al., 2019a) and minced beef (2019b).



Figure 2.6. The different colors of alizarin at different pH values.

Although not as common as anthocyanins and alizarin, curcumin (Chen et al., 2020) and myoglobin (Smolander et al., 2002) were also utilized in freshness indicators to monitor the freshness of fish and chicken, respectively.

It is worth noting that although natural dyes are gaining attraction due to their sustainability and environmentally friendly nature, issues regarding the stability and sensitivity of freshness indicators based on natural dyes still arise (Priyadarshi et al., 2021; Zhao et al., 2022)

2.1.3.2. Binder polymers

The purpose of binders in a freshness indicator is to immobilize the pH-sensitive dye used and form the color-changing layer (Kim et al., 2017). The dye itself is not sufficient to form the color-changing layer, since it may leach to the food product without a proper encapsulation polymer (Wells et al., 2019). This encapsulation/binding process is achieved by one of three methods: physical adsorption on solid support, covalently attaching to hydrophilic or hydrophobic support, or to physically entrap the dye in a polymer matrix (Balbinot-Alfaro et al., 2019). The physical adsorption method was used by Morsy et al. (2016), who applied the dye solutions directly on silica gel plates. The second method is widely used especially with binders like methyl cellulose, which structure is shown in Figure 2.7

(Nasatto et al., 2015). Methyl cellulose is a water-soluble polymer which was used by Nopwinyuwong et al. (2010), Rukchon et al (2014), Chen et al. (2019), and Obaidi et al., 2022).

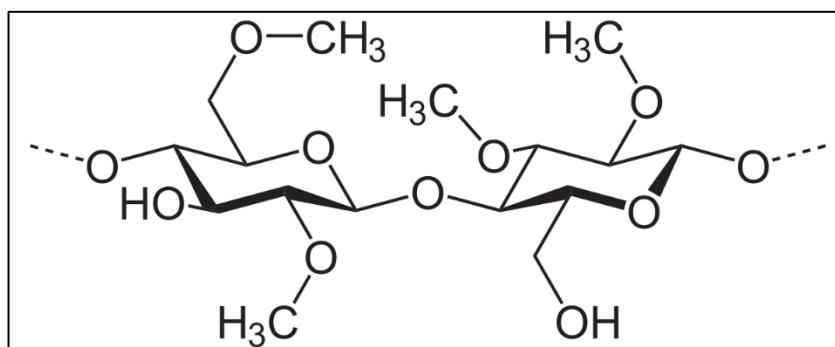


Figure 2.7. Chemical structure of methyl cellulose.

Cellulose acetate (Figure 2.8) (Vatanpour et al., 2022) on the other hand is solvent soluble and is also widely used in freshness indicators' fabrication (Byrne et al., 2002; Chun et al., 2014; Lee & Chen, 2019; Pacquit et al., 2006; Pacquit et al, 2007), which is due to its favorable properties, such as biodegradability, high chemical and mechanical stability, excellent film-forming property, and high sustainability (Vatanpour et al., 2022).

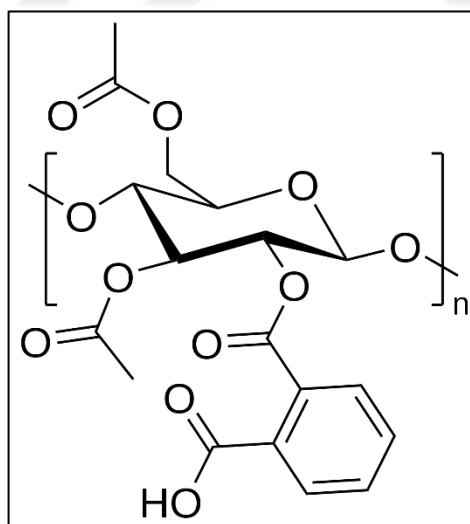


Figure 2.8. Chemical structure of cellulose acetate

Although less common, other binders like polyvinyl alcohol (PVA), ethyl cellulose (EC), poly(ether-block-amide) (PEBA), and ethylene vinyl acetate (EVA) were also used by Kim et al. (2017), Zhang and Lim (2018), Baek et al. (2018), and Lee et al. (2019a), respectively. When preparing binder-dye solutions using the covalent attaching method for the before mentioned binders, plasticizers (i.e., polyethylene

glycol 400 (PEG 400), polyethylene glycol 600 (PEG 600), dibutyl phthalate (DBP), etc.) are also added to prevent brittleness in the color-changing layer (Rukchon et al., 2014). The color-changing layer obtained for these binders are then often laminated with outer and inner polymers, which are mentioned in detail in the following lamination polymers part.

In some cases, polymers are embedded with dyes to obtain a one-layer structured freshness indicator film -especially in the case of natural polymers and natural dyes- in which the polymer plays the role of the binder and both the inner and outer layers. The films used are mostly obtained by the solution-casting method, since it is an inexpensive, simple, and rapid method. However, it is not suitable for large-scale commercial applications (Cheng et al., 2022). The most widely used biodegradable polymers for freshness indicators applications include chitosan, which is a high molecular natural cationic polysaccharide derived from chitin. However, chitosan has a brittle texture and poor mechanical properties, thus it requires mixing with other polymers to improve these properties (Zhao et al., 2022). Ezati et al. (2019) used chitosan and alizarin dye to coat cellulose paper using sol-gel method, then used the resulting freshness indicators to monitor the freshness of minced beef. Another application of chitosan using coating method was reported by Agustianti et al (2021), who coated chitosan films with anthocyanins extracted from red cabbage to produce freshness indicators that were reportedly successful in monitoring the freshness of tuna fillets. Gasti et al (2021) and Sani et al. (2020) used chitosan and methyl cellulose mixtures with anthocyanins to produce freshness indicators, while the prior used the indicators to monitor the freshness of fish fillets, the latter did not report a food validation trial.

Cellulose acetate was also used to obtain freshness indicators by casting method after mixing it with anthocyanin (Pyranoflavylum), however, no food application was conducted to validate the possibility of using these films as freshness indicators in real food systems (Gomes et al., 2022). A similar application was also reported by Freitas et al. (2020).

Another commonly used biodegradable polymer is starch, which has many advantages like sustainability, low cost, good biocompatibility, and film-forming properties. These qualities, however, are held back by some negative traits like hydrophilicity, easy degradation, and poor mechanical qualities (Zhao et al., 2022). Starch also needs

to be blended with other polymers to overcome these shortcomings, such as chitosan (Zhang et al. 2019), or amylose (Vedove et al., 2021).

2.1.3.3. Lamination polymers

Another important factor that determines the sensitivity of freshness indicators is the polymers used as the inner or outer layer for color changing layer (dye-binder). The outer layer should form a barrier that protects the freshness indicator, or more specifically, the color-changing layer, from being subjected to external atmosphere. Polymers like polyethylene terephthalate (PET) and low-density polyethylene (LDPE) are the most used polymers (Baek et al., 2018; Byrne et al., 2002; Kim et al., 2017). The inner layer is especially crucial since it should allow gas and vapor-type metabolites to reach the color changing layer to react with dye and cause a visible color change, eliminating the possibility of dye leaching from the color-changing layer into the food, and providing a white background for the color-changing layer so the color change can be more visible (Lee et al., 2019). Polymers like PEBA (Baek et al., 2018; Lee et al., 2019a), spun bound polyolefin sheet (Tyvek) (Lee et al., 2019b), polytetrafluoroethylene (PTFE) (Chun et al., 2014; Lee & Shin, 2019; Pacquit et al., 2006; Pacquit et al., 2007), and polyethylene (PE) with high water vapor transmission rate (WVTR) and high gas permeability rate (Chen et al., 2018) were utilized in the fabrication of freshness indicators as the inner layer. However, other authors used only filter paper (Byrne et al., 2002; Nopwinyuwong et al., 2010), or no inner layer (Hidayat et al., 2019; Kuswandi et al., 2013, Kuswandi et al., 2017; Morsy et al., 2016) to allow the metabolites to reach the color-changing layer, which may be acceptable to assess the performance of the color-changing layer, but is considered non-practical if the freshness indicator is to be commercialized considering the possible dye migration in direct contact with the food.

2.2. Fish

2.2.1. Fish composition & nutritional value

Fish is an important part of human nutrition, and an important factor in defining a balanced diet since it is packed with protein and essential fatty acids. Figure 2.9 (Balami et al., 2019) shows the macronutrient composition of fish meat.

Despite being packed with nutrients, the unit price of fish is lower than other protein sources like chicken, beef, pork, etc. (Balami et al., 2019). These reasons explain why fish consumption witnessed an average annual increase rate of 3.1% in the period between 1961-2017, which is twice the annual growth rate of the world population (1.6%) in the same period, while other animal-based protein foods average consumption rate (i.e., meat, milk, etc.) increased only by 2.6% (FAO, 2020).

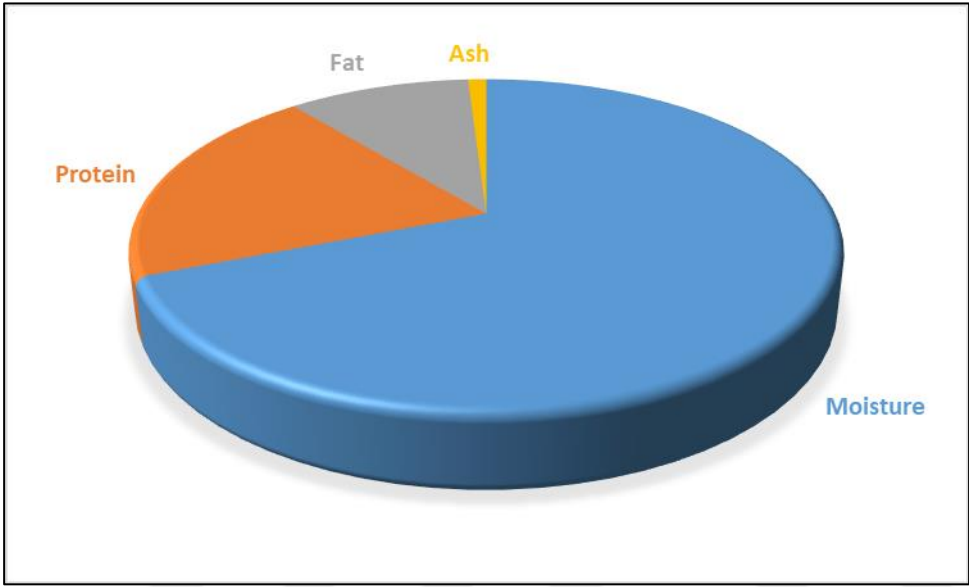


Figure 2.9. Macro nutrient composition of fish.

As can be seen in Figure 2.9 (Balami et al., 2019), the moisture content of fish is relatively high (65-80%). The protein content is between 15-20%, while fat content - according to fish species- is between 5-20% (Balami et al., 2019). In addition to being rich in protein, fish is packed with minerals, including iodine, phosphorus, sodium, calcium, and magnesium (Ashraf et al., 2020). Fish protein is of a high quality compared to other types of protein, since it contains all the essential amino acids, including sulphur-containing amino acids (i.e., cysteine and methionine) that are lacked in plant-based proteins. It also provides a higher sense of satiety compared to other animal proteins from sources like chicken, beef, etc. In addition, the lower level of connective tissues in fish makes fish muscle more digestible than other animal-based proteins. High-quality protein is crucial for building and repairing muscle tissues and boosting immunity and blood level.

The unsaturated fatty acids (omega 3) in fish are of great importance to human health, it has been proven that it has a positive effect in preventing cardiovascular diseases,

coronary heart diseases, as well as being crucial in the growth of children (Mohanti et al., 2019).

Unfortunately, the high water and protein contents of fish make it a target for microbial spoilage, which is mentioned in detail below (Balami et al., 2019; Zhuang et al., 2020). The understanding of the spoilage mechanism of fish is of great importance to increase the consumers' safety and developing counter measures during handling and storage stages.

2.2.2. Fish spoilage mechanism

Zhuang et al. (2020) stated that the spoilage that occurs in fish is often caused by microorganisms, since the protein is a perfect source of nutrition, especially for bacteria. However, the microflora of fish differs according to multiple factors (i.e., species, environment, storage conditions, processing etc.). Most common bacterial species in fish microflora includes *Pseudomonas*, *Alcaligenes*, *Vibrio*, *Serratia* and *Micrococcus* (Ghaly et al., 2010).

The bacterial metabolism is dependent on carbon and nitrogen containing compounds. Bacteria cause the breakdown of proteins -especially structural proteins- into peptides and amino acids by bacterial protease. This breakdown causes major changes in (i.e., texture, color, etc.). The peptides and amino acids undergo further microbial breakdowns like transamination, deamination and decarboxylation which result in biogenic amines (i.e., histamine, tyramine, cadaverine, putrescine). TVB-Ns are the result of the metabolism of sulfur-containing, branched, and aromatic amino acids, and are the main metabolites that cause the off-odors during the spoilage of fish. Thus, the spoilage of fish is mainly assessed by the content of TVB-Ns, biogenic amines, and volatile compounds. Figure 2.10 (Zhuang et al. 2020) shows a brief description of the spoilage mechanism in fish caused by bacteria.

These compounds play a huge role in developing new packaging technologies (i.e. intelligent packaging) which helps consumers to monitor the state of fish in real-time, increasing the safety and reducing the unnecessary food waste (Wells et al., 2019).

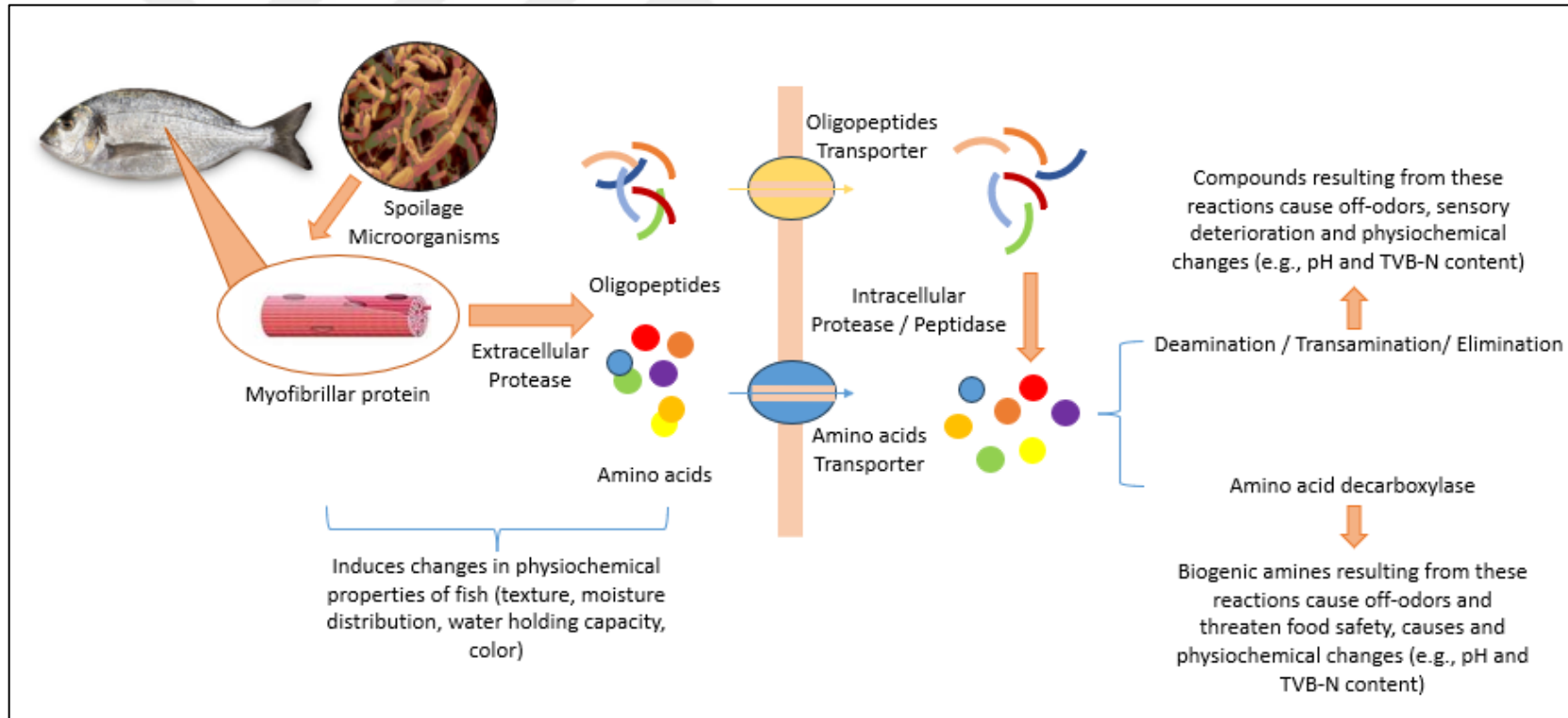


Figure 2.10. Microbial spoilage of fish.

2.2.3. Intelligent packaging for fish

FAO (2020) reported that 88% of total fish production was directly used for human consumption, with 44% of it being fresh or chilled, and 35% of it being frozen. These numbers show the importance of fish storage, handling, and preservation to provide customers with the best quality possible (Zhuang et al., 2020).

As mentioned before, in recent years intelligent packaging technology is expected to play a role in this endeavor, since it helps both retailers and customers alike to assess the quality of fish non-destructively. For the past two decades, scientists have been trying to develop intelligent packaging for fish using different dyes, binders, and polymers. The target metabolites in fish freshness indicators are usually the TVB-Ns. Since considerable amounts of TVB-Ns are produced as the result of microbial spoilage of fish, TVB-Ns increase the pH in the headspace of the package causing the pH-sensitive dyes in the freshness indicators to change color (Wells et al., 2019).

One of the first works in the field was by Byrne et al. (2002) who used cresol red (CR) and cellulose acetate (CA) binder as the color-changing solution. Droplets of the solution on PET polymer were then enclosed with filter paper to produce intelligent labels, and the labels were validated using cod and orange roughy fish samples. TVB-N levels and the color change of the labels were monitored during the storage period (ambient temperature for 48 hours), and although it was reported that a color change occurred, it was not shown in the article, not to mention that no use of MAP or conventional packaging methodology were followed. Following similar methodology, Pacquit et al. (2006), Pacquit et al. (2007), and Chun et al. (2014) used bromocresol green dye (BCG) and CA as binder using polymers like PET, polytetrafluoroethylene (PTFE), and filter paper. Although Pacquit et al. (2006) reported a lag of the indicator and the microbial population, Paquit et al. (2007) and Chun et al. (2014) reported a correlation between the microbial population and color-response of the intelligent labels, however, TVB-N levels were not measured in both works. Furthermore, since the authors conducted the validation trials in lab conditions that are not like real commercial packaging conditions, they all recommended a further study of the intelligent labels' response under different environmental conditions to further validate the use of the produced freshness indicators.

Other methods and synthetic dyes were used by other researchers, like Wells et al. (2019), who utilized bromophenol blue (BPB) dye in coating hydrophilic silica nanoparticles and blended it with low density polyethylene (LDPE) powder to produce TVB-N sensitive films. Another practice was described by Lie et al. (2020) who used sol-gel method to fixate BCG dye onto filter paper and used the resulting freshness indicators to monitor the quality of red drum (*Sciaenops ocellatus*) fish. Silica gel was also used by Morsy et al. (2016) to fixate different dyes (including methyl red (MR), phenol red (PR), bromothymol blue (BTB), bromocresol purple (BCP), and bromocresol green (BCG)) on and used the produced array in monitoring the freshness of atlantic salmon (*Salmo salar*). Methyl red (MR) dye was also used by Sariningsih et al. (2019) who used a mixture of chitosan and polyvinyl alcohol (PVA) for tilapia fish freshness monitoring, and Ibrahim et al. (2021) who used nanoencapsulation technique to encapsulate polylactic acid films with methyl red dye solution to produce freshness indicators suitable to be used in fish packaging. Nevertheless, the validation of the mentioned freshness indicators was solely conducted in non-commercial way, meaning that it eliminated the possibility of using different MAP gas compositions and their possible effect on the dynamics of the color-changing mechanism in the freshness indicators produced.

In a similar endeavor, natural dyes and polymers were also explored as possible alternatives to the synthetic ones. For the dyes, anthocyanins were the dominant type used due to their non-toxicity and the abundance of sources of extraction (Ezati et al., 2019). The natural polymers include -but not limited to- starch (Chen et al., 2020; Ezati et al., 2019; Qin et al., 2021; Vedove et al., 2021) and chitosan (Ezati et al., 2020; Gasti et al., 2021; Sani et al., 2020). As mentioned earlier, in addition to the hindrance with the packaging method used (not commercially suitable), natural dyes and polymers have other issues regarding the stability in real food-systems and need to be well studied to compete with the synthetic dyes and polymers in terms of stability. Table 2.1 shows a brief overview of the freshness indicators developed to monitor the freshness of different fish species.

It should also be mentioned that other metabolites (like CO₂) are not well studied and may have a potential in developing a freshness indicator for fish.

Table 2.1. Freshness indicators to monitor the freshness of different fish species using different dyes and polymers.

Dye(s) used	Binder	Inner matrix	Outer matrix	Fish species	Reference
CR	CA	Filter paper	PET	Cod, Orange Roughy	Byrne et al. (2002)
BCG	CA	PTFE	PET	Cod, Cardinal, Round nose Grenadier	Pacquit et al. (2006)
BCG	CA	PTFE	PET	Cod, Whiting	Pacquit et al. (2007)
BCG	CA	PTFE	PTFE	Mackerel	Chun et al. (2014)
MR, PR, BPB, BTB, BCP, BCG	Silica gel	Silica gel	Silica gel	Atlantic Salmon	Morsy et al. (2016)
BTB, PR	N/M	N/M	PET	Skate	Lee et al. (2016)
MR	PVA & Chitosan	PVA & Chitosan	PVA & Chitosan	Tilapia	Sariningsih et al. (2019)
Alizarin	Starch-Cellulose	Starch-Cellulose	Starch-Cellulose	Rainbow Trout	Ezati et al. (2019)
BPB	LDPE	LDPE	LDPE	Cod	Wells et al. (2019)
Curcumin / Anthocyanins	Starch / PVA	Starch / PVA	Starch / PVA	Big Head Carp	Chen et al. (2020)

CR: Cresol red, BCG: Bromocresol green, MR: Methyl red, PR: Phenol red, BPB: Bromophenol blue, BTB: Bromothymol blue, BCP: Bromocresol purple, CA: Cellulose acetate, PET: Polyethylene terephthalate, PTFE: Polytetrafluoroethylene, PVA: Polyvinyl alcohol, LDPE: Low density polyethylene, N/M: Not mentioned

Table 2.1. (Continued) Freshness indicators to monitor the freshness of different fish species using different dyes and polymers.

Dye(s) used	Binder	Inner matrix	Outer matrix	Fish species	Reference
Alizarin	Chitosan	Chitosan	Chitosan	Mackerel	Ezati et al. (2020)
BCG	Sol-gel matrix	Sol-gel matrix	Sol-gel matrix	Red drum (<i>Sciaenops ocellatus</i>)	Liu et al. (2020)
Anthocyanins	Chitosan	Chitosan	Chitosan	Tuna	Agustianti et al. (2021)
MR	PLA	PLA	PLA	Tuna	Ibrahim et al. (2021)
Anthocyanins	Chitosan / MC	Chitosan / MC	Chitosan / MC	N/M	Gasti et al. (2021)
Anthocyanins	Chitin / MC	Chitin / MC	Chitin / MC	N/M	Sani et al. (2021)
Anthocyanins	Starch	Starch	Starch	<i>Carcarhinus</i> spp.	Vedove et al. (2021)

BCG: Bromocresol green, MR: Methyl red, MC: Methyl cellulose, PLA: Polylactic acid, N/M: Not mentioned

2.3. Hypothesis

Freshness indicators can be used effectively to monitor the food freshness inside a package over the storage period in real-time. The color of the freshness indicators changes according to the spoilage metabolites (e.g. CO₂, TVB-Ns, etc.) released in the headspace of the package that cause a shift in the pH of the pH-sensitive dye. The fabricated three-layered freshness indicators consist of a PET as an outer layer, highly gas permeable white LDPE or PE as an inner layer, and in between them a color-changing layer containing BTB dye and either MC or CA as a binder. The freshness indicators can be utilized in the packaging of fish to assess the freshness over the storage period based on the color change, hence increase the safety and reduce both food waste and the resulting economic losses.



3. MATERIALS AND METHODS

3.1. Materials

Bromothymol blue (BTB), polyethylene glycol 400 g/mol (PEG 400), polyethylene glycol 600 (PEG 600), ethyl alcohol, and sodium hydroxide (NaOH) were purchased from Merck Chemicals Co. (Darmstadt, Germany); methyl cellulose (4000 cp viscosity, Sigma M0512) and cellulose acetate were purchased from Sigma Aldrich Chemical Co. Ltd. (St. Louis, USA). Polymers used as outer layer (Polyethylene terephthalate film (PET, 13 μm)), inner layer (white colored, low-density polyethylene (LDPE), 50 μm with carbon dioxide transmission rate (CO_2TR) of 17612 $\text{cm}^3/\text{m}^2\text{day}\cdot\text{atm}$, water vapor transmission rate (WVTR) of ~ 10 $\text{g}/\text{m}^2\text{day}$ or breathable polyethylene (PE), 16 μm with WVTR of 3000-5000 $\text{mg}/\text{m}^2\text{day}$), and the adhesive material used for lamination (obtained from organic compounds, two-components, solvent free, fast curing process, high chemical resistance, suitable for polymer lamination) were supplied by Ispak Flexible Packaging Company (Kocaeli, Turkey). Plate count agar (PCA) (Merck 70152), *Pseudomonas* selective agar base (Merck 1,07620), *Pseudomonas* CFC selective supplement (Merck 1,07627), propylamine hydrochloride (Sigma, 242543), TMA hydrochloride (Sigma, T72761), HCl (Sigma, %37), boric acid (Merck 1,00160), magnesium oxide (Merck, 1,05865), polydimethylsiloxane-divinylbenzene (PDMS-DVB) solid phase microextraction (SPME) fiber (Agilent, 5191-5874) were used in the food validation experiment. The PA/PE pouches used in both the simulation and the food validation experiments were supplied by Apack Packaging Company (Istanbul, Turkey).

3.2. Fabrication of the Intelligent Labels

Dye solutions (1%, w/v) were prepared by dissolving 0.2 g of bromothymol blue (BTB) in 2 mL of 50 % (v/v) ethanol at room temperature for 15 min with a magnetic stirrer. Methyl cellulose (MC) solutions were prepared by dissolving methyl cellulose (3 % w/v) and PEG-400 (1 % w/v) in 50 mL distilled water. Two milliliters of the dye solution were then added. Five hundred μL of 2M NaOH were added to obtain the CO_2 -

sensitive intelligent labels (BTB-MC-NaOH), while five hundred μl of 2M HCl were added to obtain the TVB-Ns sensitive intelligent labels (BTB-MC-HCl). The mixtures were homogenized for 10 min at 10,000 rpm using Ultra-Turrax (Model T18 digital, IKA®-Werke GmbH & Co. KG., Deutschland, Germany). The indicator solutions were degassed for 1 h using a vacuum pump. The obtained indicator solutions were then cast as circles with approximately 2 cm diameter on PET films and dried at 40°C overnight.

As one of the most commercially used polymers in food packaging applications, PET polymer was chosen as an outer layer due to its favorable properties, such as high optical clarity that allows the observing of the resulting color-change of the labels, as well as the low gas permeability property which prevents the gases and moisture from the packages' surrounding atmosphere to enter the color-changing layer, which may cause a false result (Nistico, 2020). Due to these reasons, PET was used as an outer layer by many authors for smart packaging applications (Lee & Shin, 2019; Lee et al., 2019; Baek et al., 2018; Kim et al., 2017; Paquit et al., 2007; Byrne et al., 2002). Freshness indicators were then laminated with white LDPE film (50 μm) with an adhesive material using the solvent-free adhesive material to prevent dye leaching from the color-changing layer (Obaidi et al., 2022). The inner layer choice is extremely crucial since the inner layer should serve as a hydrophobic membrane that protects the color-changing layer from moisture (which causes dye migration), while allowing the gaseous compounds to pass (Lee & Shin, 2019; Lee et al., 2019). An extra advantage of the polymers used as the inner layer in this paper is the white color of the polymers, which provides a background for a better color-change observation, without the need to use white filter paper for that purpose (Nopwinyuwong et al., 2010; Byrne et al., 2002), since it is considered impractical for commercial use.

The CA-based intelligent labels were fabricated with modifications to methods found in the literature (Lee & Shin, 2019; Chun et al., 2014; Paquit et al., 2006; Byrne et al., 2002). 0.5 g of CA were dissolved in 10 ml 1:1 acetone:2-butanone by mixing on a magnetic stirrer at 50°C for 4 hours. 0.2 g of the plasticizer (PEG-600) was then added, and the mixture was further stirred for 1 hour before adding 0.02 g of BTB pH-sensitive dye. To obtain the CO₂-sensitive (BTB-CA-NaOH) and the TVB-Ns sensitive (BTB-CA-HCl) intelligent labels, 80 μl of 2M NaOH or 80 μl of 2M HCl were added to the mixture; respectively. The solutions were then dispensed over the

outer layer polymer (PET) using a micropipette to form circular shapes, then dried at room temperature for 1 hour. The labels were then laminated using breathable PE (16 μm). After drying the laminated MC-based and CA-based intelligent labels at 30°C overnight, the labels were cut into 4 x 4 cm² squares (dye diameter of 2 cm) and placed inside PE/PA vacuum bags to be used in simulation and the food application. The fabrication of the intelligent labels is shown briefly in Figure 3.1.

It is worth mentioning that the fabrication method used is highly reproducible since the polymers used are widely used commercially polymers, and the application method (casting and lamination) can be used traditionally in packaging factories with some modifications to the machinery.

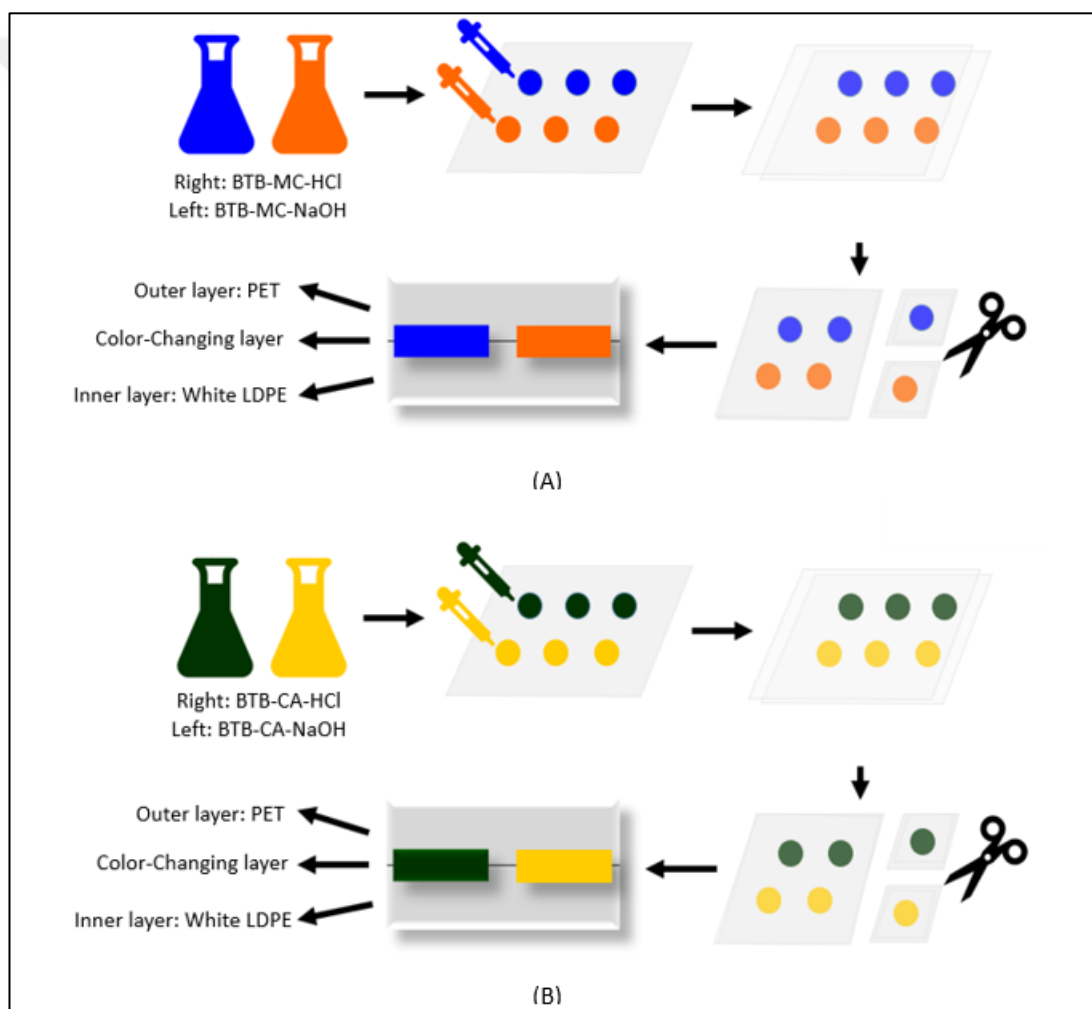


Figure 3.1. The fabrication of the MC-based (A) and CA-based (B) intelligent labels.

3.3. Simulations

3.3.1. CO₂ simulation

The simulation method used in the study differs from the methods reported in the literature (Zhang & Lim, 2018; Rukchon et al., 2014; Nopwinyuwong et al., 2010) and thought to be more realistic and closer to a real food packaging system. The CO₂-sensitive intelligent labels (BTB-MC-NaOH, BTB-CA-NaOH) were placed inside PE/PA bags, then the bags were filled with different concentrations of CO₂ (0, 5, 10, 15, 20, 25, & 30 %) using a MAP machine (Reepack, Italy) (N₂ gas was used to make up the concentration up to 100%). The sealed bags were stored at 4°C for 10 days to monitor the effect of different CO₂ concentrations on the color change of the intelligent labels.

3.3.2. TMA simulation

TMA was used as a representative of TVB-Ns as it is thought to be the most abundant species of TVB-Ns produced by fish (Chun et al., 2014). The simulation was designed to be more practical and as close as possible to a real food system, unlike other methods reported earlier in the literature (Lee et al., 2019a; Lee et al., 2019b; Wells et al., 2019; Morsy et al., 2016; Chun et al., 2014). Vials containing 10 ml of TMA solution with different concentrations (0, 5, 10, 15, 20, 25, & 30 %) were placed in the PE/PA bags containing the TVB-Ns sensitive intelligent labels (BTB-MC-HCl, BTB-CA-HCl), then the bags were sealed using a MAP machine after flushing the bags with 100% N₂ gas. The sealed bags were stored at 4°C for 10 days to monitor the effect of different TMA concentrations on the color change of the intelligent labels.

3.4. Verification of the Developed Indicators for Fish Packaging

3.4.1. Fish preparation & packaging

The simulations and validation trials scheme are shown in Figure 3.2. Fresh Atlantic bonito (*Sarda sarda*) fish was purchased from a local seller and kept cool until it reached the laboratory. A hundred grams of fish were aseptically placed in polypropylene (PP) trays and then inserted into PE/PA pouches (3:1 headspace to food product volume ratio) where the labels have already been placed. The intelligent labels were placed carefully attached to the top of the pouch not be in direct contact with

food, with the PET side of the labels facing upwards. The packages were then sealed with the help of a MAP machine. The package setup can be seen in Figure 3.3. Two different gas compositions were used to imitate the commercial packaging conditions: 30% CO₂ & 70% N₂ (Group 1), and 100% N₂ (Group 2). The packages were stored at 4 ± 0.5 °C for 10 days. The color analysis of the labels, headspace gas composition of the packages and pH, TVB-Ns, TMA concentration using headspace solid phase micro extraction coupled with gas chromatography (HS-SPME GC), microbial, and sensory analyses of the fish were carried out every two days (on the 0th, 2nd, 4th, 6th, 10th days). Four parallel packages were prepared under each MAP group and total of eight analyzes were conducted on each analysis day.



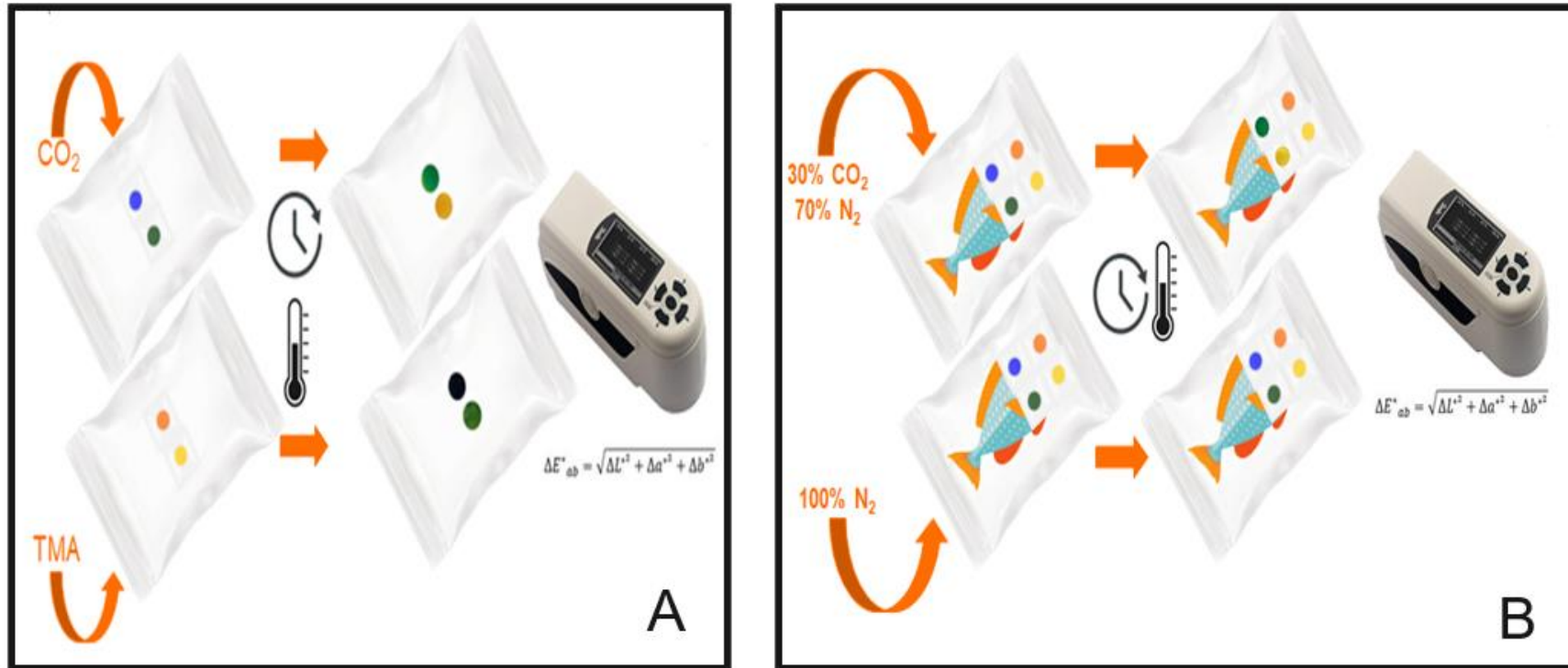


Figure 3.2. The application of freshness indicators in simulations (A) and food application (B).



Figure 3.3. Freshness indicators set-up in the package for the food application.

Upper left: BTB-MC-HCl, Upper right: BTB-CA-HCl, Lower left: BTB-MC NaOH, Lower right: BTB CA-NaOH

3.4.2. Headspace gas analysis

The headspace gas composition of the packages was measured by inserting the needle of a gas analyzer (Oxy baby, Germany) into the headspace through a rubber septum attached to the outer upper surface of the package. O₂ & CO₂ concentrations are expressed as % v/v (Obaidi et al., 2022; Lee et al., 2019).

3.4.3. Color analysis

Color values L* (lightness), a* (redness), and b* (yellowness) were measured using a portable colorimeter (PCE-CSM 7, Meschede, Germany) before and after the storage period for each label in each package. The obtained values were then used to calculate the total color difference (ΔE) value using the following equation 3.1:

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad (3.1)$$

Where L₀^{*}, a₀^{*}, b₀^{*}, are initial color values of the freshness indicators while Where L^{*}, a^{*}, b^{*}, are the color values after the designated storage period (Ezati et al., 2019).

3.4.4. pH

The pH of fish samples was measured by using a digital pH meter (WTW, Germany). A 10 g of homogenized fish sample was weighed in a beaker and 90 ml of distilled water were added, then the mixture was mixed with a magnetic stirrer. pH values were recorded by immersing the pH probe in the stirred mixture (Ezati et al, 2019).

3.4.5. TVB-N

Steam distillation method was used to determine the TVB-N content of the fish samples. Fish meat (10 g) was mixed with 100 ml distilled water then homogenized at 5,000 rpm for 5 min using an Ultra-Turrax (Model T18 digital, IKA®-Werke GmbH & Co. KG., Deutschland, Germany). The homogenized mixture was then filtered using N₂ free filter paper, and 10 ml of the resulting filtrate were transferred to a Kjeldahl digestion tube, and 10 ml of magnesium oxide (MgO) suspension (10 g/L) were added. Steam distillation was carried out for 5 minutes using a Kjeldahl distillation unit (Behr Labor Technik S4, Düsseldorf, Germany). The distillate was collected in an Erlenmeyer flask containing 20 ml of 20 g/L boric acid (H₃BO₃) and a few drops of methyl red & bromocresol green dyes solution. The mixture was then titrated with 0.01 M HCl, and the TVB-N content was determined using the following equation 3.2:

$$TVB - N \text{ content (mg/100g)} = \frac{(V1 - V2) \times C \times 14}{m \times 5/100} \times 100 \quad (3.2)$$

V1 (ml): Titration volume of the sample

V2 (ml): Titration volume of the blank

C (M): Actual concentration of HCl used for titration

m (g): Weight of the fish sample used

The TVB-N content is expressed as mg/100 g (Chen et al., 2019).

3.4.6. TMA analysis

The headspace solid phase microextraction (HS-SPME) method was used to extract TVB-Ns prior to GC injection. 10g of fish from each of the 8 packages were cut and homogenized, then placed in 50 ml centrifuge tubes. 20 ml of 0.5 N HCl were then added to each tube and the tubes were vortexed for 5 minutes followed by centrifuging at 4000 rpm for 10 minutes at 5 °C. One milliliter of the supernatant was transferred

to a 10 ml volumetric flask and made up to the mark with 0.5 N HCl. 0.5 ml of the resulting aliquot and 1 ml 15 M NaOH were placed in a 20 ml vial. Using magnetic stirrer, the components of the vial were stirred for 15 minutes. With the aid of SPME needle guide the needle was inserted into the headspace of the vial and the fiber was exposed for extraction. The polydimethylsiloxane-divinylbenzene (PDMS-DVB) fiber was carefully positioned to prevent any direct contact with the contents of the vial during the extraction period. After 15 minutes of extraction, the fiber was retracted into the needle and inserted into the injector port of the GC (Shimadzu, Model GC-2010, Japan) to initialize the gas chromatography flame ionization detector (GC-FID) analysis.

After a CP-Volamine column (30 m x 0.32 mm) was installed, the temperatures of the injector and detector were set to 200 and 250, respectively. The column temperature was maintained at 40 °C for 5 minutes then gradually increased by 10 °C/min to 120 °C and maintained at that temperature for 2 minutes (Karaca et al., 2023; Lee & Shin, 2019; Lee et al., 2016). The carrier gases consisted of nitrogen (40 ml/min), hydrogen (40 ml/min), and air (400 ml/min). The trimethylamine (TMA) content in samples was calculated according to the calibration curve obtained using five different TMA concentrations (0.0, 10.0, 20.0, 30.0 and 40.0 ppm). The average of three peak area measurements for each concentration were used to determine the calibration curve. TMA concentration was calculated as mg/100 g with the calibration equation in equation 3.3.

$$y = 1082.1 x \text{ (R}^2 = 0.9951\text{)} \quad (3.3)$$

3.4.7. Microbiology

Total plate count (TPC) and *Pseudomonas aeruginosa* counts were carried out to monitor microbial growth in fish samples. A 10 g fish sample is taken aseptically and homogenized with 100 ml 0.9 % w/v NaCl physiological solution (PS) to obtain the initial dilution of 10^{-1} . Serial dilutions were then prepared by adding 1 ml of the previous dilution to 9 ml PS and 10^{-2} , 10^{-3} , and 10^{-4} dilutions were obtained accordingly. The obtained dilutions were spread on the surface of either plate count agar (PCA) to determine the TPC, or *Pseudomonas* agar base with CFC (cetrimide Fucidin cephalosporin) selective supplement to determine *Pseudomonas aeruginosa*

count. PCA petri dishes were incubated in reverse position at 37 °C for 24 hours, while *Pseudomonas* agar petri dishes were incubated at 25°C for 48 hours (Agustianti et al., 2021; Chun et al., 2014). The resulting colonies are expressed as log cfu/g.

3.4.8. Sensory analysis

The color, odor, texture, and general acceptability attributes were evaluated by 6 experienced panelists. Equal amounts of fish samples were placed in coded plates and the beforementioned sensory attributes were evaluated using a 5-point scale, with 1 being the lowest score and 5 being the highest score. Three and above is considered acceptable. The scores for each attribute are represented by the following explanations:

- Color: 1 represents brown color, 3 represents a honey wax yellow / blurry / pale yellow, and 5 represents characteristic red / pink / shiny with no change in color.
- Smell: 1 represents bad / spoiled odor and the loss of seaweed smell, 3 represents the decrease in seaweed smell, and 5 represents a fresh / prominent seaweed smell.
- Texture: 1 represents a quite rough / loose surface, 3 represents a mat and slightly loose surface with decreased hardness and elasticity, and 5 represents a shiny, hard, and elastic surface.
- Overall liking: 1 represents dislike extremely, 3 represents neither like or dislike, and 5 represents like extremely.

3.4.9. Statistical analysis

The results were represented as mean \pm SD (standard deviation). The variance analyses of means were evaluated using Minitab (State College, Pennsylvania, USA) statistical program. The Tukey's pairwise test with 95% confidence level was used to determine the significant differences by using a general linear model of the program.

4. RESULTS & DISCUSSION

4.1. Simulation Results

4.1.1. CO₂ simulation

BTB-MC-NaOH & BTB-CA-NaOH labels were used in CO₂ simulation. The change in labels' color over the storage period can be seen in Figure 4.1 (A & B). Both labels showed a change in color related to the increase in CO₂ concentration, similar results were reported in literature for bromothymol blue dye (Baek et al., 2018; Chen et al., 2018; Rukchon et al., 2014; Nopwinyuyong et al., 2010). CO₂ forms carbonic acid in the color-changing layer in the presence of moisture, causing a shift towards a more acidic medium, and hence causing the pH-dye (BTB) to change color accordingly (Baek et al., 2018; Zhang & Lim, 2018; Nopwinyuyong et al., 2010). The methyl cellulose-based labels showed a sharp color change from blue to green between 10% and 15% CO₂ concentrations, while the cellulose acetate-based labels showed a sharp color change from green to yellow between 20% and 25% CO₂ concentrations. After the mentioned CO₂ concentrations, the color of the labels stabilizes, indicating that the labels reach a saturation phase after these concentrations. The different color of both labels depends on the binder solution used. In the case of methyl cellulose, the color displayed in the beginning is blue, which is the color exhibited by bromothymol dye in a basic medium with a pH value above 8. After being exposed to CO₂, the color turns green, which indicates that the pH of the medium is decreasing to become between 7 and 8. On the other hand, the CA-based labels have an initial green color (which indicates a medium with pH 7-8) that changes to a yellow color (which indicates a medium with pH 4-6) after exposure to CO₂ (Chen et al., 2018). In both labels, the change in color intensifies as the CO₂ increases and the color starts to resemble a more acidic medium than the initial medium. The MC-based labels showed the same result as in our previous work (Obaidi et al., 2022) which shows the reproducibility of the labels. The effect of CO₂ concentration on CA-based labels was never explored in the previous literature, even though the color change obtained is rather promising. The CO₂ concentration is often an overlooked freshness indicator in

case of fish, and the articles related to the use of freshness indicators to monitor fish spoilage are mostly focused on TVB-Ns as the main spoilage metabolite (Liu et al., 2020; Ezati et al., 2019; Sariningsih et al., 2019; Wells et al., 2019; Morsy et al., 2016). This is probably due to CO₂ which is often used in commercial fish MAP (Milijasevic et al., 2019, Sivertsvik et al., 2002). Nevertheless, if CO₂ is not used in MAP, the potential of using CO₂ sensitive labels should be further investigated.

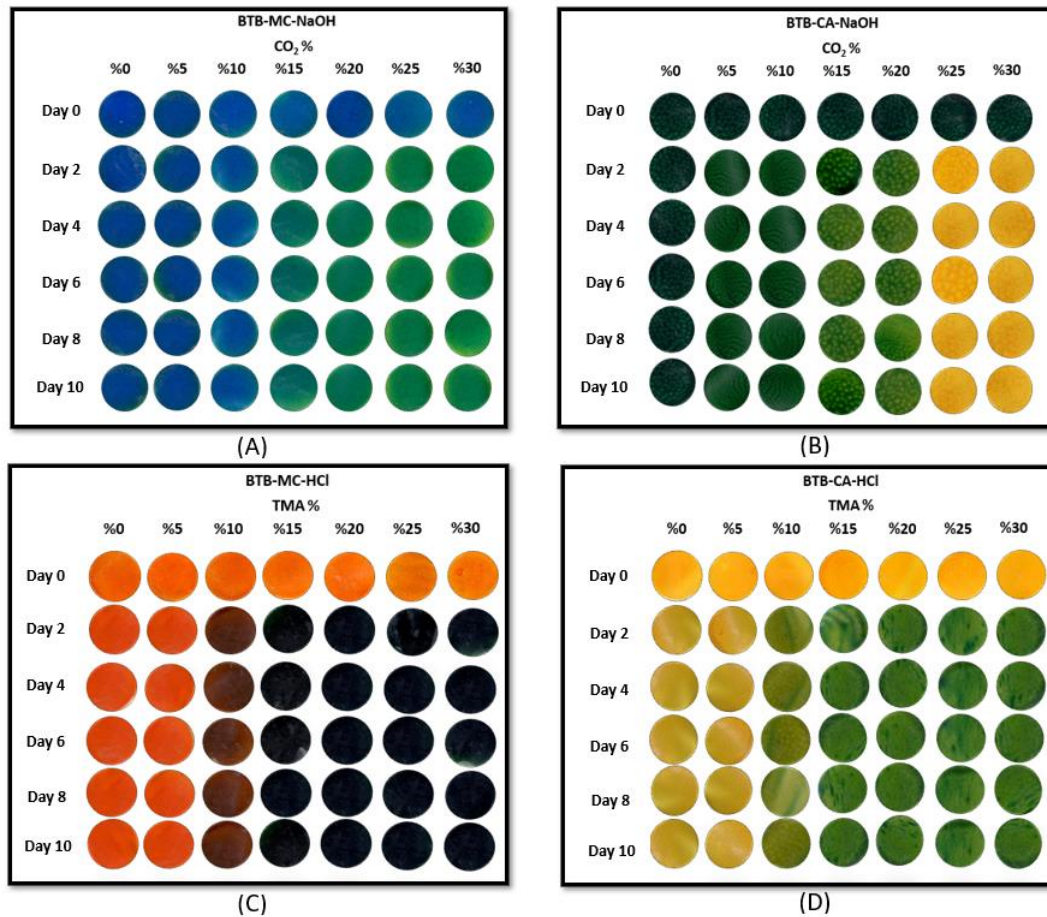


Figure 4.1. The effect of the CO₂ concentration on the color of the CO₂ sensitive labels (A & B) and the effect of the TMA concentration on the color of the TVB-N sensitive labels (C & D) during the storage at 4 °C for 10 days.

In addition, unlike the method used in this study, the CO₂ simulations mentioned in literature do not simulate the response of the intelligent labels to CO₂ in a real food packaging. For example, Zhang et al. (2018) subjected an array of different dyes to CO₂ by fixing the array on the inner surface of a glass bottle, and generated the CO₂ by a chemical reaction. Even though the authors reported a good reaction to CO₂ in alkaline-doped dyes, no food application was done to further validate these results. It is also worth mentioning that the dye array did not have an inner layer/polymer, which

is an important aspect of fabricating and testing intelligent labels intended for food packaging, since the leaching of dyes into foodstuff can pose serious food safety issues. Another method used for simulation was reported by Rukchon et al. (2014) and Nopwinyuwong et al. (2010), who simply placed the dye solution, and the fabricated labels in vials and injected these vials with CO₂.

4.1.2. TMA simulation

TVB-N compounds (NH₃, DMA, and TMA) are produced by microbial degradation by specific spoilage organisms (SSO) in fish, thus the level of TVB-Ns is considered an important freshness indicator in the assessment of fish (Paquit et al., 2007; Paquit et al., 2006). TMA is the dominant compound among the TVB-N compounds, since it is produced immediately after the death of fish and considered the compound responsible for the “fishy” odor (Summers et al., 2017; Chun et al., 2014). The color change in TVB-N sensitive labels is shown in Figure 4.1 (C & D). The MC-based labels show a complete color change from orange to navy blue when the TMA concentration is 15% (with a transitional brownish color at 10%), and continues to exhibit the same navy-blue color until 30% TMA. CA-based labels show a color change from yellow to green at 10% TMA concentration, after which the color stays stable even after reaching 30% TMA. TMA acceptability level in fresh fish has been reported to be 10-15 ppm (Nevigato et al., 2018). Taking these values into consideration, the color change obtained for both labels start at 10, which can be a good indication of the freshness of the fish.

Even though TVB-Ns are the often-targeted metabolite in the freshness indicators studies -especially for chicken, fish & meat- only a few numbers of studies conducted simulations. As in the case of CO₂ simulations, TVB-Ns simulations in literature are conducted under laboratory conditions that are far from a real food packaging system. Morsy et al. (2016), who fabricated a freshness indicator array using different dyes, conducted a TMA (as a TVB-N representative) simulation by taping the prepared array to the inner surface of a glass jar and then subjecting it to different TMA concentrations. The same procedure was also mentioned in studies for fabrication of intelligent labels for chicken freshness, where the labels were also placed in glass jars (Lee et al., 2019a; Lee et al., 2019b). Zhang et al. (2018) used the same method, however instead of TMA as a TVB-N representative, ammonia gas (NH₃) was used,

and however, no food validation experiment was mentioned. Other methods were mentioned by Chun et al. (2014) placed the freshness indicators inside a 24-well plate for a TMA simulation, and Pacquit et al. (2007) who reported using a sophisticated method using a mixture of nitrogen and ammonia gases, with the freshness indicators placed in an optical scanner that is in turn placed in a flow cell.

4.2. Verification of the Developed Indicators for Fish Packaging

4.2.1. Headspace gas composition

Both CO₂ and O₂ levels were measured during the storage period (Figures 4.2 & 4.3). CO₂ levels did not show any statistically significant difference during the storage periods for both MAP groups (30% CO₂ 70% N₂ (Group 1) and 100% N₂ (Group 2)) ($p>0.05$). However, a small decrease in the CO₂ level in group 1 (Figure 4.2) -which is thought to be the result of the solubility of the CO₂ in the flesh of the fish- and a small increase in group 2 (Figure 4.3) -which is probably due to the bacterial growth- were observed over time. For O₂, although there was no initial oxygen in MA packages, a very slight increase and decrease can be seen in both atmospheres. The increase can be explained by trace amounts of O₂ still exist in the pores of the fish released into the package headspace after the packaging process. The decrease that follows is probably caused by the consumption of the O₂ by aerobic bacteria, which comes in accordance with results of Morsy et al. (2016), who reported that the O₂ levels decreased during the storage period of fresh Atlantic salmon. This can be better seen in group 2, where the O₂ level reached 1.56% on the 4th day but decreased to 0.49% on the 6th day, compared to group 1. The reason for this difference is thought to be the CO₂ existence in group 1, which has a more significant inhibitory effect on mesophilic bacteria than N₂ (Milijasevic et al., 2019; Sivertsvik et al, 2002). However, in both Group 1 and 2, O₂ levels were not found statistically significant during storage period ($p>0.05$).

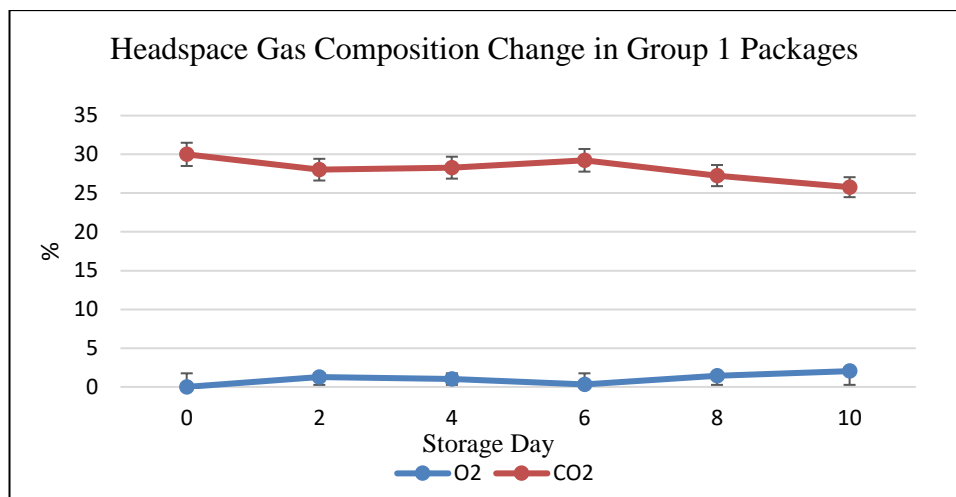


Figure 4.2. The change in CO₂ and O₂ concentration in Group 1 (30% CO₂ 70% N₂) during the storage at 4 °C for 10 days.

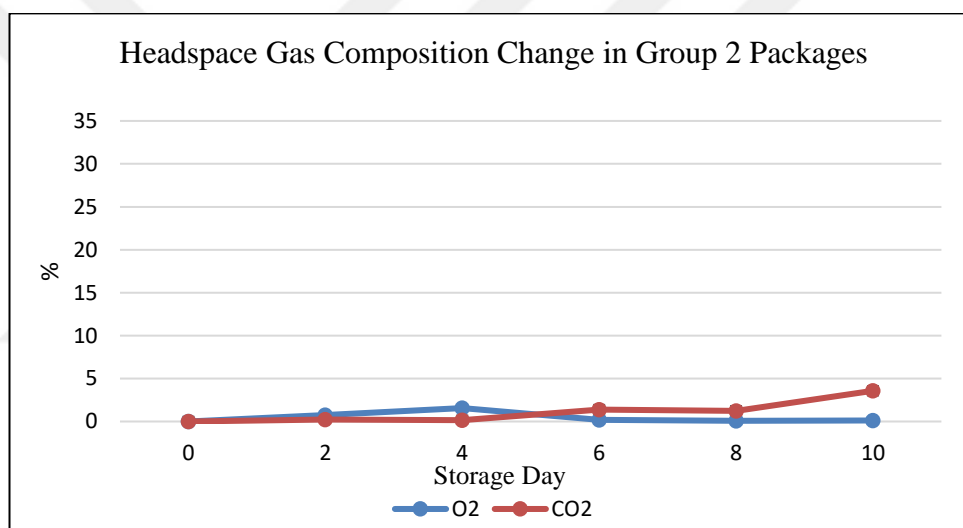


Figure 4.3. The change in CO₂ and O₂ concentration in Group 2 (100% N₂) during the storage at 4 °C for 10 days.

4.2.2. Microbial Quality

The total plate count (TPC) and *Pseudomonas aeruginosa* growth was observed during the storage of fish at 4° C for 10 days. The gradual increase over the storage period for TPC and *P. aeruginosa* is shown in Figures 4.4 & 4.5, consecutively, in the different MAP groups (30% CO₂ 70% N₂ (Group 1) and 100% N₂ (Group 2)). In group 1, TPC increased from 5.22 log cfu/g to 7.07 log cfu/g and *P. aeruginosa* increased from 3.74 log cfu/g to 6.80 log cfu/g by the 10th storage day, while in group 2, TPC increased from 5.22 log cfu/g to 7.75 log cfu/g and *P. aeruginosa* increased from 3.74 log cfu/g to 7.94 log cfu/g by the 10th storage day (p<0.05).

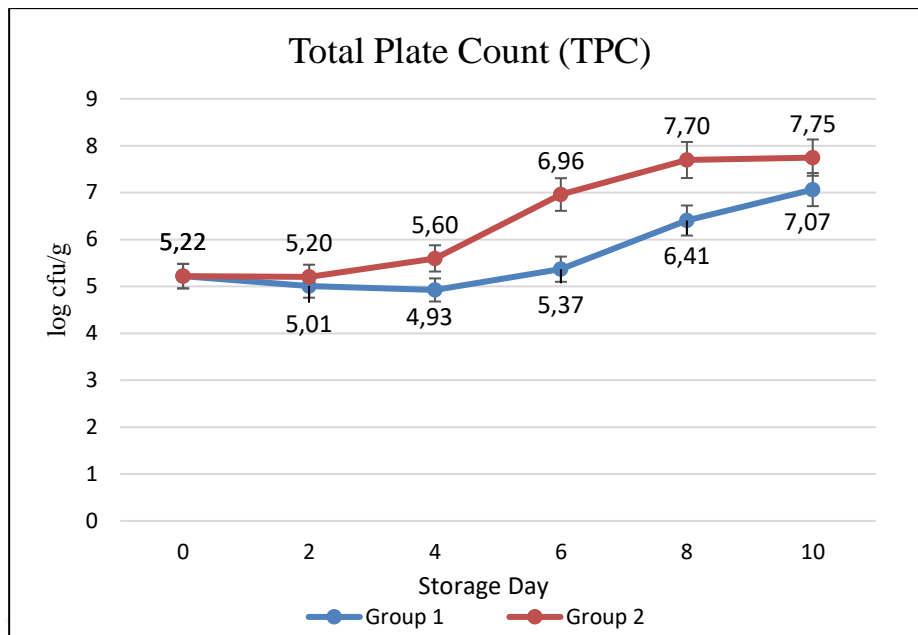


Figure 4.4. The increase in total plate count in Group 1 (30% CO₂ 70% N₂) and Group 2 (100% N₂) during the storage at 4 °C for 10 days.

The International Commission on Microbiological Specifications for Foods (ICMSF) recommends that the total mesophilic bacteria in fresh fish should not exceed 7 log cfu/g (Milijasevic et al., 2019), while the upper acceptability limit for mesophilic bacteria was reported as 6 log cfu/g for marine species (Erkan et al., 2009). However, it is thought that the exact microbial spoilage threshold is hard to define since it can widely vary depending on many factors, like the catch season, geographical location and most importantly fish species ((Pacquit et al., 2007; Pacquit et al., 2006). According to the obtained results, group 1 reached upper acceptable limit (6 log cfu/g) after 6th day (approximately on 7th day) and microbial spoilage threshold (7 log cfu/g) on the 10th day while group 2 reached upper acceptable limit after the 4th day (approximately on 5th day) and microbiological spoilage threshold at the 6th day. These results further prove the effect of MAP with CO₂ in inhibiting microbial growth, as it was reported by both Wells et al. (2019) and Morsy et al. (2016) that the same microbial spoilage threshold was reached between the 5th and 6th storage days at 4° C. As for the difference between the two groups, it is thought that the CO₂ in group 1 (30% CO₂) has contributed to the slightly longer shelf-life, since it was proven in literature that CO₂ has a better inhibitory effect compared to N₂ (Milijasevic et al., 2019; Sivertsvik et al, 2002). Interestingly, the color of the intelligent labels (all four different labels) did not show a color change related to the microbial spoilage during

the storage period in any package for both groups and the visual results (Figures 4.9 & 4.10) are in line with the chromaticity results (Table 4.2) which don't show any statistical difference between the chromaticity results for all the labels during the storage period.

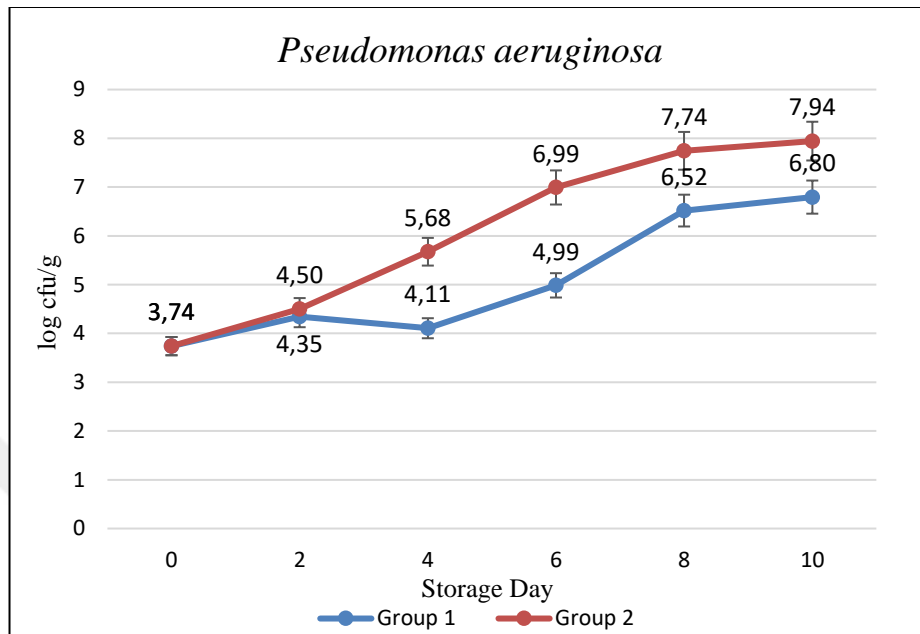


Figure 4.5. The increase in *P. aeruginosa* in Group 1 (30% CO₂ 70% N₂) and Group 2 (100% N₂) during the storage at 4 °C for 10 days.

4.2.3. pH

Figure 4.6 shows the change in pH of the packaged fish under the different MAP applications (30% CO₂ 70% N₂ (Group 1) and 100% N₂ (Group 2)). Both groups show a slight decrease (from 6.02 to 5.75 in group 1 and from 6.04 to 5.75 in group 2) followed by an increase (6.01 for group 1 and 5.94 for group 2), however, there was no significant difference in pH of the fish whether within a MAP group or between the two different MAP groups ($p > 0.05$). However, these changes can be a result of the following reasons: specific spoilage organisms thrive and produce TVB-Ns during the storage period, eventually leading to an increase in pH values, thus, many authors reported that the microbial spoilage threshold was correlated with pH 7 (Ezati et al., 2019; Morsy et al., 2016; Chun et al., 2014). On the other hand, fish under MAP shows a drop in pH due to lactic acid production since lactic acid bacteria becomes the dominant microbiota (Milijasevic et al., 2019). Another reason for this drop is when CO₂ is used in the MAP mixture, since it is soluble in fish tissues, leading to the formation of carbonic acid that also reduces the pH. The decrease in this case can be

the result of lactic acid accumulation in group 2, and a combination of lactic acid and dissolved CO₂ in group 1.

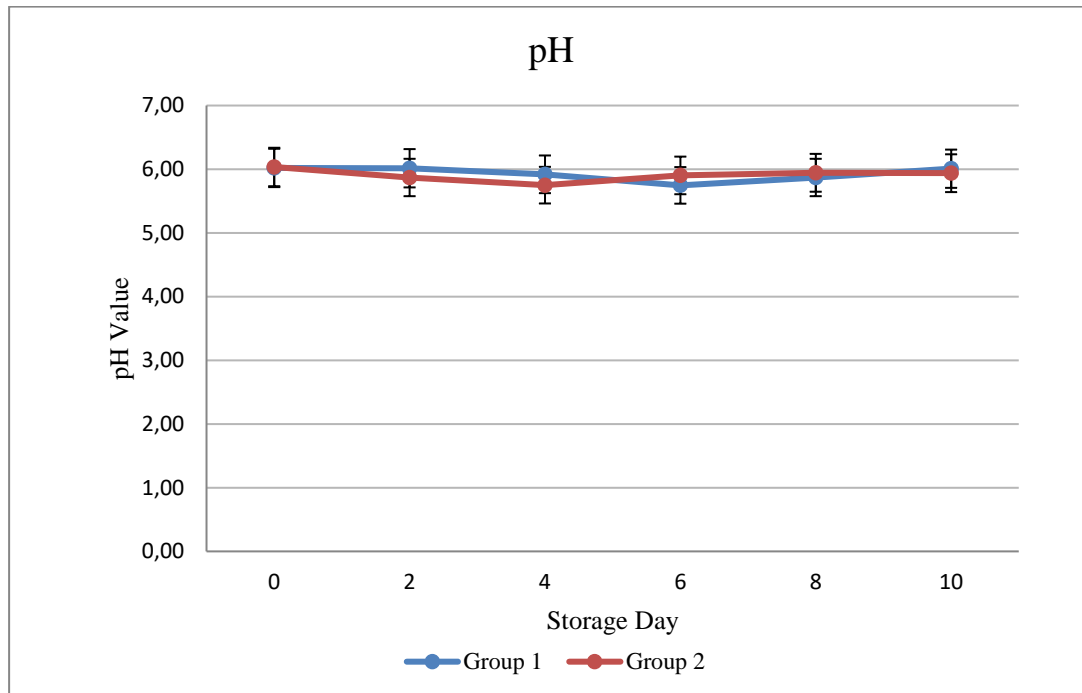


Figure 4.6. The change in packaged fish pH in Group 1 (30% CO₂ 70% N₂) and Group 2 (100% N₂) during the storage at 4 °C for 10 days.

4.2.4. TVB-N

TVB-Ns are considered an indicator of the fish freshness, since formation of TVB-N compounds (TMA, DMA, NH₃) result in unpleasant odor and flavor formation (Milijasevic et al., 2019). Figure 4.7 shows the TVB-N concentrations over the storage period. Both groups (30% CO₂ 70% N₂ (Group 1) and 100% N₂ (Group 2)) show a similar trend; a gradual increase occurs during storage.

The TVB-N level starts at 16.67 mg/100g for both groups and increases gradually until it reaches a plateau. Group 1 stabilizes around 25 mg/100 g value on the 8th day, while Group 2 stabilizes around the 6th day after reaching approximately 24 mg / 100 g TVB-N content. Liu et al. (2020) and Ezati et al. (2019) recommended a TVB-N threshold of 25 mg / 100 g, while Agustianti et al. (2021), Sariningsih et al. (2019), and Byrne et al (2002) recommended 30 mg / 100 g. Some authors even recommended 35 mg / 100 g (Morsy et al., 2016). As these values can be valid for different fish species under aerobic conditions, in MAP conditions the microorganisms responsible for the formation of volatile compounds are inhibited. Thus, some authors recommended a lower level of TVB-N level for fish packaged under MAP (i.e., 25 mg/ 100 g), or even

considered that TVB-N level is not a valid indicator for fish freshness, since even when the TVB-N level does not exceed the acceptable level, it can be considered unacceptable sensory-wise (Miliijasevic et al., 2019). Considering the acceptability threshold is 25 mg / 100 g, both MAP groups reached this value at the end of the storage between 8th and 10th days storage. TVB-N level of fish packaged with MAP including 30% CO₂ (22.30 mg /100 g) is lower than that of MAP including 100% N₂ (24.30 mg /100 g) on the 6th day of storage which is very close to threshold level. These results are in line with the microbiology results for Group 2, since Group 2 reached microbial spoilage threshold on the 6th day. On the other hand, Group 1 reached microbial spoilage threshold on the 10th day.

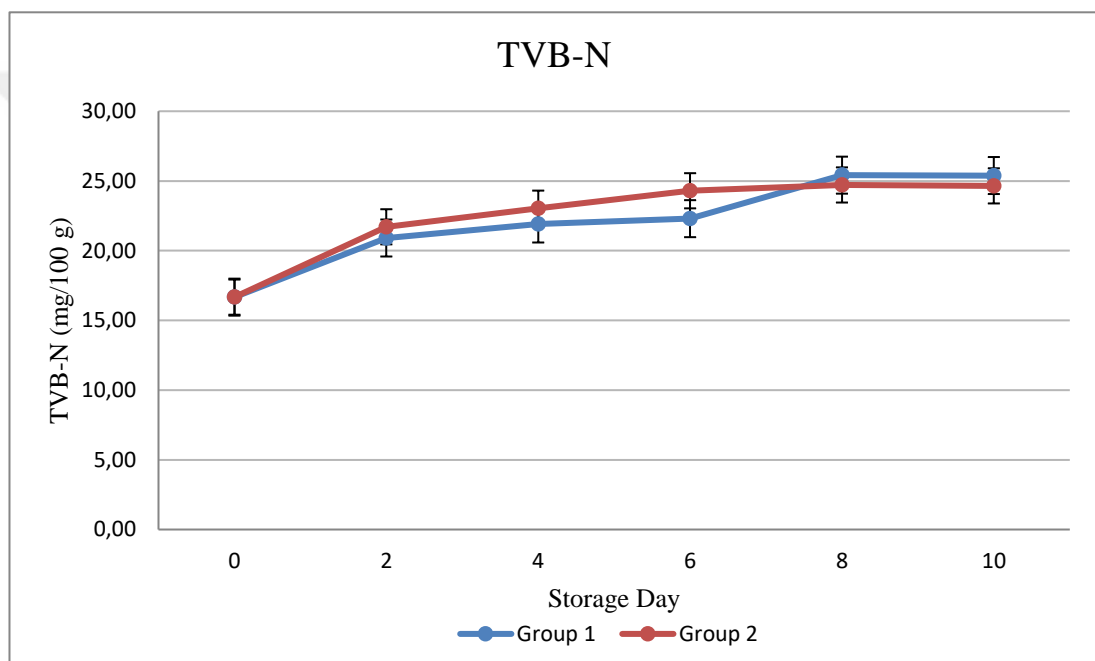


Figure 4.7. The TVB-N concentrations in Group 1 (30% CO₂ 70% N₂) and Group 2 (100% N₂) during the storage at 4 °C for 10 days.

4.2.5. TMA

Trimethylamine (TMA) alongside dimethylamine (DMA) and ammonia are the commonly measured volatile amines in fish, often used as a freshness criterion. Fresh fish has a low TMA level, reported by some authors to be as low as 10-15 ppm (Nevigato et al., 2018) or 20 ppm (Summers et al., 2016). Figure 4.8 shows TMA concentrations in the different MAP groups (30% CO₂ 70% N₂ (Group 1) and 100% N₂ (Group 2)) during the storage period. As part of the TVB-Ns, the TMA level also showed a similar gradual increase over the storage period (p<0.05), although the highest value did not exceed 6 ppm in both groups.

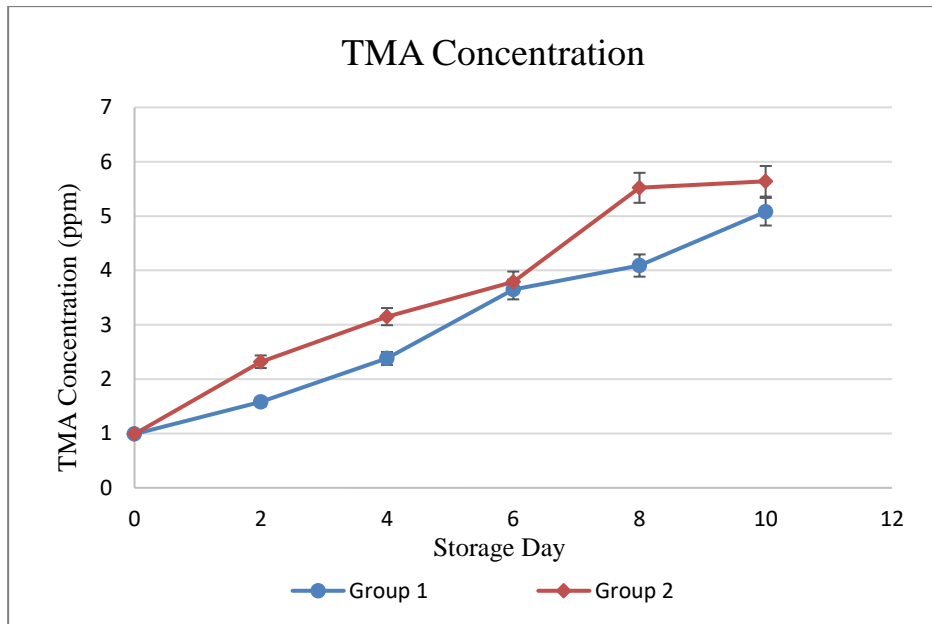


Figure 4.8. The TMA concentrations in Group 1 (30% CO₂ 70% N₂) and Group 2 (100% N₂) groups during the storage at 4 °C for 10 days.

The TMA level in Group 2 was slightly higher than Group 1, even though the final TVB-N levels in both MAP groups are very close ($p > 0.05$). This is speculated to be the result of CO₂ presence in Group 1, which has a more inhibitory effect on the specific spoilage microorganisms that contribute to the production of TMA (Milijasevic et al., 2019; Sivertsvik et al, 2002). TMA levels during the storage period did not exceed the spoilage threshold (10-15 ppm). Thus, it cannot be considered an effective indicator for the spoilage of fish under MAP conditions. Castro et al. (2006) stated that in some fish, the rise in TVB-N levels is not caused by the breakdown of TMAO to TMA, but the deamination of amino-acids. These results should be especially taken into consideration while fabricating intelligent labels for fish freshness, since the main freshness indicator often used are TVB-Ns, especially TMA. As the color change in the labels is calibrated according to TMA levels, in MAP conditions those levels are not reached, making it impossible for the labels to change color, as is the case in our study.

4.2.6. Sensory Evaluation

The sensory attributes' scores are shown in Table 4.1. Considering the score 3 as the acceptability level for all attributes, both groups (30% CO₂ 70% N₂ (Group 1) and 100% N₂ (Group 2)) are considered unacceptable by the 6th day of storage for all the sensory attributes (color, odor, texture) and overall acceptability. The different MAP

composition was insignificant in preserving the different attributes or the general acceptability of the fish packaged, and the scores for all attributes were statistically indifferent throughout the storage period for both groups. Since fish under MAP conditions have a different spoilage mechanism, the fish can reach microbial or sensorial unacceptability while TMA levels can be still below the acceptable limit (Milijasevic et al., 2019).



Table 4.1. The sensory attributes' scores of fish packaged with the different MAP during the storage at 4 °C for 10 days.

Sensory Attribute	MAP Group*	Storage Day					
		0	2	4	6	8	10
Color	Group 1	4.75±0.10 ^a	3.85±0.25 ^b	3.27±0.15 ^{bcd}	2.58±0.21 ^{de}	1.77±0.17 ^f	1.00±0.00 ^g
	Group 2	4.64±0.11 ^a	3.60±0.21 ^{bc}	3.17±0.16 ^{bcd}	2.90±0.18 ^{cd}	1.92±0.12 ^{ef}	1.00±0.00 ^g
Odor	Group 1	4.67±0.24 ^a	3.90±0.17 ^{ab}	3.10±0.17 ^{bcd}	2.69±0.18 ^{cd}	1.53±0.08 ^e	1.00±0.00 ^e
	Group 2	4.67±0.24 ^a	3.85±0.15 ^b	3.33±0.12 ^{bc}	2.46±0.26 ^d	1.45±0.13 ^e	1.00±0.00 ^e
Texture	Group 1	4.83±0.17 ^a	4.10±0.32 ^{ab}	3.21±0.10 ^{cd}	2.73±0.21 ^{de}	1.79±0.18 ^f	1.00±0.00 ^g
	Group 2	4.83±0.17 ^a	3.85±0.08 ^{bc}	3.15±0.09 ^{cd}	2.75±0.23 ^{de}	2.00±0.06 ^{ef}	1.00±0.00 ^g
Overall Acceptability	Group 1	4.85±0.10 ^a	3.73±0.34 ^b	3.10±0.05 ^{bc}	2.58±0.15 ^c	1.35±0.11 ^d	1.00±0.00 ^d
	Group 2	4.75±0.11 ^a	3.79±0.16 ^b	3.08±0.19 ^{bc}	2.44±0.20 ^c	1.56±0.12 ^d	1.00±0.00 ^d

* Group 1: 30% CO₂ 70% N₂ and Group 2: 100% N₂

**Values that do not share a same letter are significantly different ($p < 0.05$)

4.2.7. Response of intelligent labels during fish spoilage

The color change of the different labels is shown in detail for the two MAP groups (30% CO₂ 70% N₂ (Group 1) and 100% N₂ (Group 2)) in Figure 4.9 (Group 1) and Figure 4.10 (Group 2). The labels in packages are as follows: upper left: BTB-MC-HCl, upper right: BTB-CA-HCl, lower left: BTB-MC-NaOH, lower right: BTB CA-NaOH. While the results of chromaticity analysis of the four different intelligent labels for both groups are shown in Table 4.2. For Group 1, since 30% CO₂ was used in the MAP composition, the color changed from blue to green in BTB-MC-NaOH labels and from green to yellow in BTB-CA-NaOH labels immediately after packaging. This color change comes in accordance with the color change observed in these labels in the CO₂ simulation results discussed earlier, where the color changed from blue to green in BTB-MC-NaOH labels after 15% CO₂ concentration and from green to yellow in BTB-CA-NaOH labels after 20% CO₂ concentration. Looking at the chromaticity values (ΔE) for these labels in Table 4.2, the values stayed almost constant with no significant difference ($p>0.05$) in both labels since the 2nd day of storage and until the end of the storage period (10 days) at 4 °C. Since the CO₂ concentration in group 1 (Figure 4.9) stayed in the range of 25-30%, these results are expected according to the results previously obtained in the CO₂ simulation, in which the color of both labels stayed the same in this concentration range. In Group 2 (Figure 4.10), the CO₂ sensitive labels did not change color, which is only expected considering that the CO₂ level in the packages stayed in the range 0-5% during the storage period. As can be seen in Figure 4.1 BTB-MC-NaOH and BTB-CA-NaOH labels maintained the initial color at 0-5% CO₂ concentration range. This can be further be proven by the chromaticity values of these labels in Group 2, as it can be seen in Table 4.2 that the values stayed almost constant for both labels with no significant difference throughout the storage period. The results in both groups can be considered a further validation of the CO₂ simulation results discussed previously.

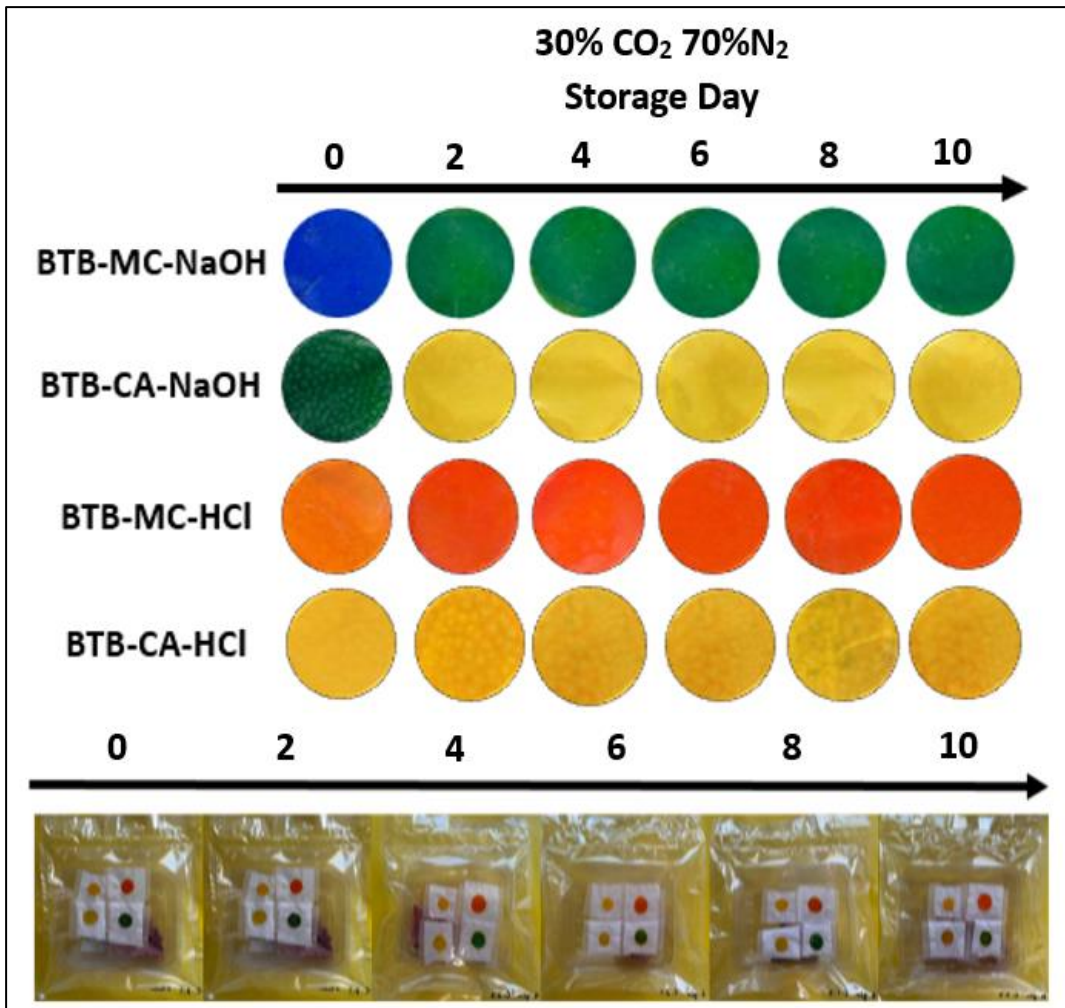


Figure 4.9. The color change of the different intelligent labels during fish storage at 4 °C for 10 days under 30% CO₂ 70% N₂ MAP group.

BTB-MC-HCl and BTB-CA-HCl labels did not show any color change during the storage period in both groups (Figures 4.9 & 4.10). These results are reflected on the chromaticity values of these labels (Table 4.2) which shows no significant differences ($p < 0.05$), whether within the same group during the storage period or between two different packaging groups on the same storage day. Considering the TMA values obtained for both groups, it was stated that the TMA values in both MAP groups did not exceed 6 ppm. In the conducted simulation (Figure 4.1), the color change in both labels (BTB-MC-HCl and BTB-CA-HCl) occurred when the TMA level is 10 ppm, which also validates the simulation results and proves that TVB-N and TMA levels cannot be used successfully to assess the freshness of fish packaged under MAP conditions. Chen et al. (2019) reported a color change from yellow to green in BTB-MC based indicators used to monitor the freshness of pork (TVB-N level 28.96 ± 1.39 mg/100g), and Hidayat et al. (2019) reported a color change from orange to blue in

BTB-based indicators (Whatman filter paper was used to entrap the dye) used to monitor the freshness of meat (TVB-N level not mentioned). On the other hand, both Lee et al. (2019) and Morsy et al. (2016) used BTB-based indicators to monitor the freshness of chicken breast & fish, respectively, but no visible color change was observed as it was the case in our study. Lee et al. (2019) used poly(ether-block-amide) (PEBA) as a binder, while Morsy et al. (2016) used silica gel. This further proves the effect of the binder used for the same pH-sensitive dye in determining the color response of the fabricated indicators.

Overall, for group 1, spoilage thresholds for both microbiology & TVB-N are reached around the 8th storage day, while for group 2, they are reached around the 6th storage day. Sensory acceptability threshold, on the other hand, was reached on the 6th day, with no significant difference in any of the attributes between both MAP groups. If the spoilage threshold for these analyses were taken into consideration, it can be said that the fish reached spoilage onset on the 6th day, however, as it was mentioned, the TMA level, on which the color change for the TVB-N sensitive labels was based, did not exceed 10 ppm in both groups, the minimum concentration required for the color change according to the simulation results. The color change of CO₂ and TMA indicators in fish under MAP without oxygen was not successfully occurred although the fish is sensorially unacceptable by the 6 days of storage since the CO₂ and TMA level did not reach the spoilage thresholds during the storage.

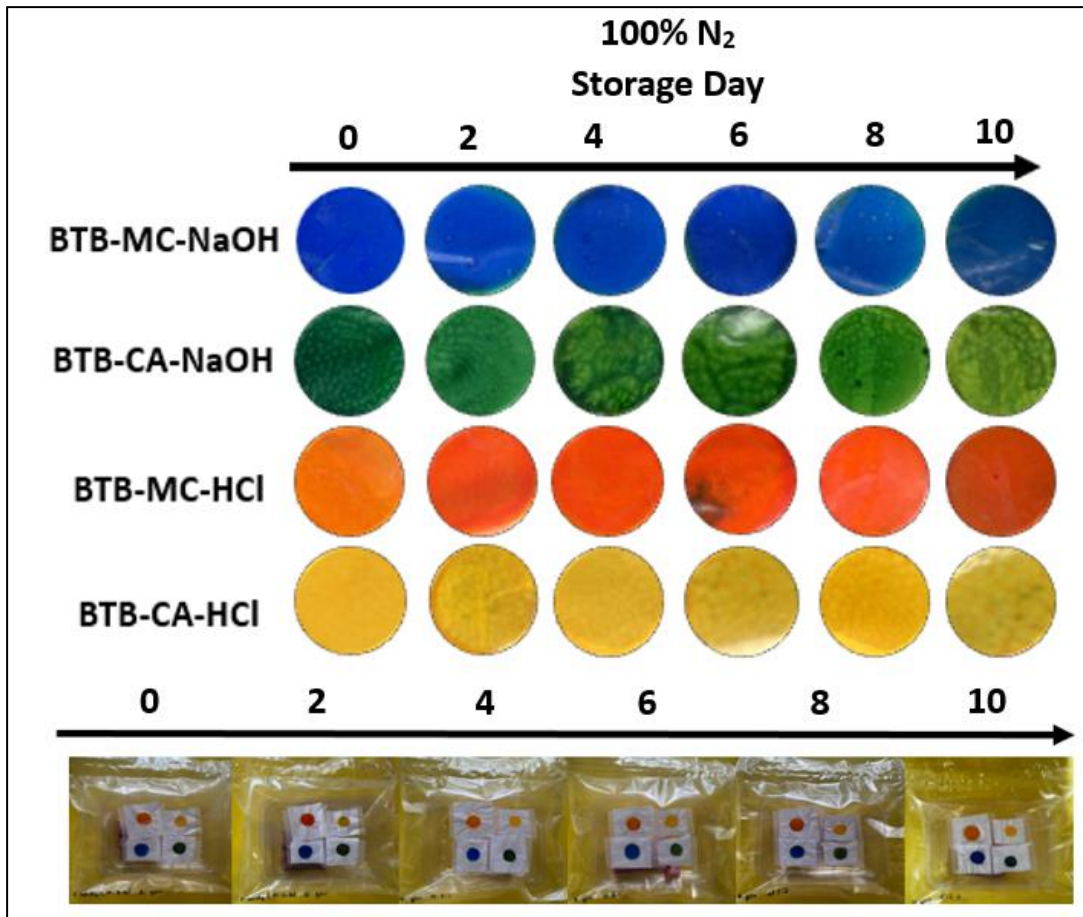


Figure 4.10. The color change of the different intelligent labels during fish storage at 4 °C for 10 days under 100% N₂ MAP group.

Table 4.2. The chromaticity values (ΔE) for the different MAP groups during the storage period of 10 days at 4 °C.

Intelligent Label	MAP Group	Storage Day				
		2	4	6	8	10
BTB-MC-NaOH	Group 1	44.34±0.64 ^a	44.95±1.05 ^a	48.01±2.34 ^a	50.55±1.32 ^a	43.97±3.23 ^a
	Group 2	4.06±0.54 ^b	5.10±0.22 ^b	5.00±1.06 ^b	4.45±0.53 ^b	3.43±0.16 ^b
BTB-CA-NaOH	Group 1	32.02±3.99 ^a	32.71±3.30 ^a	31.65±3.34 ^a	37.51±4.46 ^a	36.28±1.80 ^a
	Group 2	5.21±0.07 ^b	5.88±0.19 ^b	6.80±0.74 ^b	6.54±1.32 ^b	8.23±0.09 ^b
BTB-CA-HCl	Group 1	6.42±1.00 ^a	6.23±0.05 ^a	6.48±0.09 ^a	5.96±0.27 ^a	6.40±0.77 ^a
	Group 2	6.05±0.29 ^a	7.23±0.45 ^a	6.56±1.06 ^a	5.93±2.76 ^a	6.26±2.38 ^a
BTB-CA-HCl	Group 1	4.67±0.05 ^a	6.01±0.97 ^a	5.77±0.43 ^a	6.35±0.13 ^a	5.27±0.65 ^a
	Group 2	5.82±0.29 ^a	7.30±1.68 ^a	5.82±0.67 ^a	5.89±0.52 ^a	7.33±0.65 ^a

* Group 1: 30% CO₂ 70% N₂ and Group 2: 100% N₂

**Values that do not share a same letter are significantly different.



5. CONCLUSIONS & RECOMMENDATIONS

In this study, intelligent labels with a three-layer structure were fabricated by using BTB as a pH-sensitive dye and either MC or CA as a binder for the color changing-layer, PET as an outer layer, and white LDPE as an inner layer in a high reproducible method that is thought to be commercially applicable.

The sensitivity of the labels toward fish spoilage metabolites was tested by simulations using either CO₂ or TMA (as a representative of TVB-Ns), and applying packaging methods similar to the ones used in commercial packaging. The CO₂-sensitive labels (BTB-MC-NaOH, BTB-CA-NaOH) both showed a visible color change directly related to the increase in the CO₂ concentration: BTB-MC-NaOH labels have an initial blue color that turns to green completely when the CO₂ concentration reaches 15%, and maintains the same color until 30% CO₂. BTB-CA-NaOH labels have an initial dark green color that turns to yellowish green when the CO₂ concentration reaches 15%, and maintains a bright yellow color starting with 25% CO₂. On the other hand, TVB-N sensitive labels (BTB-MC-HCl, BTB-CA-HCl) also showed a visible color change related to the increase in TMA concentration: BTB-MC-HCl labels have an initial orange color that turns to brownish color when the TMA concentration reaches 10%, and after 15% TMA, it maintains a navy-blue color until 30% TMA. BTB-CA-HCl labels have an initial bright yellow color that turns to yellowish green when the TMA concentration reaches 10%, then maintains a green color between 15%-30% TMA. These results conclude that all the labels fabricated could successfully change color when subjected to the respective metabolite at the concentrations considered the acceptability limits for each metabolite (%15 for CO₂ and 10% for TMA), regardless of the binder used (MC or CA) in simulation study.

To evaluate the possibility of commercial use of the fabricated levels, the four different labels (BTB-MC-NaOH, BTB-CA-NaOH, BTB-MC-HCl, BTB-CA-HCl) were used for fish packaging. In addition to the different binders used, different MAP conditions that are usually used in commercial fish packaging were used. The two different MAP compositions (30% CO₂ 70% N₂ as Group 1 and 100% N₂ as Group 2) are used. The

packages were then stored at 4 °C for 10 days. In Group 1, the CO₂-sensitive labels (BTB-MC-NaOH, BTB-CA-NaOH) changed color from blue to green for BTB-MC-NaOH labels, and from green to yellow for BTB-CA-NaOH labels since the 2nd day of storage, due to the 30% CO₂ in the MAP composition. TVB-N sensitive labels (BTB-MC-HCl, BTB-CA-HCl) maintained the same color throughout the storage period of 10 days (orange color for BTB-MC-HCl labels, yellow color for BTB-CA-HCl labels). The chromaticity values (ΔE) did not show any statistical difference after the 2nd day until the 10th storage day for CO₂ sensitive labels and TVB-N sensitive labels. In Group 2, no visible color change was observed in any of the 4 labels (BTB-MC-NaOH, BTB-CA-NaOH, BTB-MC-HCl, BTB-CA-HCl). Consequentially, the chromaticity values (ΔE) showed no statistical difference during the whole storage period for all the labels in group 2 packages. These results show that regardless of the binder type used or the two different gas compositions used for MAP, no visible color change can be obtained for the different labels during storage. Headspace gas composition, microbiology, TVB-N, TMA and sensory analysis showed that packaging under MAP (in both groups) affects the spoilage mechanism of the packaged fish and that the spoilage metabolites (TMA & CO₂) present in fish packaged under atmospheric conditions in literature are produced in considerably lower amount. These metabolites -when present- cause the pH-shift and the visible color change in the intelligent labels.

In conclusion, the results of this research show that the fabricated intelligent labels - regardless of the binder used- could successfully monitor the different concentrations of spoilage metabolites (i.e., CO₂ and TMA) under simulated conditions. However, the color change of CO₂ and TMA indicators in fish under MAP without oxygen was not successfully occurred although the fish is sensorially unacceptable by the 6 days of storage since the CO₂ and TMA levels did not reach the spoilage thresholds during the storage. This is probably due to a different spoilage mechanism under MAP without oxygen than fish packaged under atmospheric conditions.

It should be kept in mind that in order to fabricate an intelligent label for commercial use, the commercial packaging condition under which the target food product is packaged play a huge role, since the intelligent labels are modeled according to the spoilage metabolites. If MAP conditions are used, the spoilage occurs differently, and

accordingly the produced spoilage metabolites change (whether the type or amount produced).





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PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

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