1H-NMR Spectroscopy to Measure the Ki Index of Bombay Ducks During Chilling Storage as An Approach to Determine Freshness

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Abstract

The quality of fish freshness plays a crucial role in the acceptance of consumers and human health. Considering that food safety has recently become a major concern on a global scale, establishing and evaluating fish freshness is crucial for research and development. Some methods that can be used to assess fish freshness are the K-values and the Ki index. Those methods evaluate the fish freshness based on the degradation of nucleosides in fish that occurred during the deterioration process. This study aimed to assess quality freshness (Ki index) in Bombay duck (*Harpodon nehereus*), one of the tropical fisheries products, using concentration nucleosides in chilling temperature storage conditions for 12 days. Analysis of nucleosides in this study was conducted using a 1 H-NMR (*Nuclear Magnetic Resonance*) metabolomics approach. Four nucleosides (Inosine, Hypoxanthine, inosine 5' monophosphate, and ATP) were successfully analyzed during this study. This study showed Hypoxanthine was slightly fluctuated, IMP Increased until 4 days then decreased markedly, ATP decreased markedly up to 6 days, then relatively stagnant in the remaining storage and inosine decreased up to 4 days, then significantly increased from 6 days until 12 storage. Furthermore, freshness calculation showed that the Ki index of Bombay duck fish significantly increased during chilling storage conditions from 0 days to 6 days (47% - 94%). Furthermore, the Ki index showed a stable pattern from 6 – 12 days of storage (94% - 95%). The Ki index of Bombay duck value exceeded 80% after storing the fish for 4 days. Therefore, based on these calculations after 4 days storage in the chilling temperature Bombay ducks is categorized as the fish which is not fresh anymore.

Keywords: fish freshness, *harpodon nehereus*, Ki index, NMR, nucleosides

Introduction

For many coastal states and districts, especially developing nations like Indonesia, the fishing sector is a basic industry tied to international trade and is essential from a nutritional aspect. Besides that, fish is an essential source of nutrients for humans. Between 1961 and 2009, the world's fish food supply increased at an average of 3.2 per cent per year, exceeding the world's population growth of 1.7 per cent per year (Shumilina et al., 2016). Furthermore, between 2014 and 2019, the average annual per capita consumption of seafood worldwide increased slightly from 19.9 kilograms to 20.5 kilograms. However, in 2020 seafood consumption fell to multiyear lows at 19.8 kg per capita (Cai & Leung, 2022). But, since fish is one of the most perishable and delicate meals, it is crucial to keep an eye out for any changes in the quality of the fish during post-harvest treatment. Freshness is one of the most crucial fish quality factors, and it has a big impact on the quality of fish and fisheries products. Fish can be susceptible to physical, chemical, biochemical, and microbial changes that affect their freshness quality due to their intrinsic characteristics (fragile muscle tissue and activity of endogenous protease), inappropriate handling methods and storage conditions (time-temperature history, chilling or freezing, and refrigeration and preservation condition) (Cheng & Sun, 2014). So, It is crucial to maintain fish quality up until people eat it.

There are currently several officially recommended approaches for determining fish quality and freshness. Sensory, microbiological, physical, and chemical methods are used in analytical techniques to evaluate post-mortem changes (Ocaño-higuera et al., 2011). The chemical indicators are frequently used to quantify the fish deterioration process such as using volatile compound analysis. However, the bulk of them don't usually strongly relate to the sensory analysis because

they only intensify when the fish exhibits obvious alterations in the perceived sensory quality.(Tan et al., 2018). Because of this, the fish freshness product monitoring based on nucleotide degradation have received particular attention and potential to develop (Lougovois et al., 2003). The autolytic process is measured using the K value (nucleoside degradation) method right after the fish dies. This process occurs when endogenous enzymes rapidly degrade the ATP that is already present in fish muscles because of the following reaction: ATP, ADP, AMP, IMP, Inosine, and Hx (where the abbreviations are: ATP: adenosine-52 -triphosphate, ADP : adenosine-52 -diphosphate, AMP : adenosine monophosphate, IMP : inosine-52 monophosphate, AMP: adenosine-52 -monophosphate, and Hx : hypoxanthine) (Abramova et al., 2019; Ciampa et al., 2012). Therefore, a lower K value indicates fresher fish. Moreover, the K value grows at different rates depending on the species. Within a single species, the K value is impacted by fish size and storage temperature. (Hamada-sato et al., 2005).

Furthermore, Karube et al., (1984) states ATP breaks down by autolysis and bacterial degradation quickly into IMP for some fish species. The definition of the quality factor K value can be altered by omitting the adenosine phosphates, and a new method for calculating the quality factor (Ki) has been proposed. The calculation (Ki) is defined as:

$$Ki\ (\%) = ([Inosine] + [Hypoxanthine]/[IMP] + [inosine] + [Hypoxanthine]) \times 100\%$$

NMR spectroscopy has previously been used to assess fish freshness and quality. Several research groups published a review of the applications of NMR to optimize fish processing (Erikson et al., 2012). NMR is an effective tool for evaluating food quality. It enables the simultaneous characterization of numerous food sample components. High-resolution NMR is a suitable technique for calculating the K- values and Ki index since it is simple, highly reproducible, and allows for direct quantitative monitoring of compounds. Additionally, various fish metabolites that influence flavor, quality, and other aspects can be monitored simultaneously and qualitatively described, making NMR spectroscopy a reliable and, in many ways, and seems to be unique method for describing fish's metabolic profile and properties (Abramova et al., 2019; Ciampa et al., 2012; Shumilina et al., 2015, 2016, 2020).

This study aimed to demonstrate the possibility of 1 H NMR spectroscopy as a rapid technique to assess the change in nucleoside profile in Bombay duck fish (*Harpodon nehereus*) during storage at chilled temperatures for several days. Bombay duck, or Nomei fish (*Harpodon nehereus*), is a commercial fish that is widely marketed as one of the food commodities consumed by Tarakan city residents in North Borneo. Besides that, these fish are also exported to several countries, such as Malaysia, Mauritania, Bangladesh, and India. This fish has a great economic value, with production levels exceeding 10 tons per month for fresh fish and 3 tons for dried nomei fish (Utomo et al., 2022). However, Bombay duck has a moisture content that reaches more than 90% and also has the labile nature of the muscle protein (Kakatkar, Sharma, and Venugopal 2003). This condition makes the quality of Bombay duck deteriorate quickly. Moreover, based on information from the fisherman, Bombay duck fish is usually kept in chilling storage before being processed into a dry product. Therefore, evaluation of Bombay duck fish freshness using rapid techniques is very important to develop.

Materials and Methods

Materials

The raw materials used in this study were Bombay duck fish (*Harpodon nehereus*). The samples were taken from a one-day fishing vessel in Tarakan, North Borneo district. The fresh Bombay duck fish samples have an average weight of 82.35 ± 4.64 g. Chemical reagents used in this study consist of trichloroacetic acid (TCA) pro analyst (Merck) for sample extraction, potassium hydroxide (KOH) pro analyst (Merck) for adjusting the pH in extract samples, deuterium oxide (D_2O) (Sigma-Aldrich) for dissolving samples before NMR analysis was conducted, and potassium phthalate monobasic (Sigma-Aldrich) as an internal standard in NMR analysis. Moreover, the research equipment used in this study consists of a Hitachi homogenizer and centrifuge (SCANAVAC) for sample extraction and a Jeol-NMR 400 MHZ for nucleoside analysis (JNM-ECZL series NMR).

Sample Preparation

The fresh Bombay duck fish samples, which were obtained from one fishing vessel, were brought to the laboratory using a cargo plane with a cool box to maintain the fish's freshness. In the laboratory, the samples were divided into three groups of replications, and then the samples were stored at a chilled temperature of 4 ^{o} C for 12 days. During storage time, the analysis was performed at 0, 2, 4, 6, 8, 10, and 12 days. The analysis was conducted in three replications.

Figure 1. Bombay duck (*Harpodon nehereus*) or Nomei fish, a local Indonesian name.

NMR Metabolite Analysis

Statistical Analysis

The Kruskal-Walli's analysis was used to compare the differences between each metabolite variable during storage time. The Kruskal-Walli's test is a rank-based nonparametric test that can determine if there are statistically significant differences between two or more groups of an independent variable and a continuous ordinal dependent variable (Spurrier, 2010). In addition, a statistical multivariate discriminant identified metabolites formed at chilled temperatures between each day's storage time analysis. Past Statistical Software V3.08 was used for all statistical analyses (Hammer et al., 2001).

Results and Discussion

1 H-NMR Metabolite Quantification

1 H NMR analysis successfully identified four nucleosides in the Bombay duck (*Harpodon nehereus*) fillet sample during 12 days of storage duration at chilled storage, where the NMR peak signals in 0 days and 12 days of storage at chilled storage were shown in Figure 1. The following metabolites' signals were assigned: inosine, inosine 5'-monophosphate, hypoxanthine, and ATP. Furthermore, all the samples were characterized by the diagnostic signals of inosine, hypoxanthine, and inosine-5'-monophosphate, but the absence of adenosine-5'-triphosphate, adenosine-5'-diphosphate, and adenosine-5'-monophosphate signals was established through 1H NMR field spectra analysis. The results that had been published in the literature were consistent with the data that had been obtained from previous research (Abramova et al., 2019; Shouchun et al., 2010). These previous publications established that the analyzed fish contained metabolites like inosine, hypoxanthine, and inosine-5'-monophosphate following post-mortem changes. Therefore, the identification of three metabolites was used in our manuscript to estimate fish quality: hypoxanthine, inosine, and inosine-5'-monophosphate. The chemical shift of the NMR metabolite profile in Bombay duck fish is presented in Table 1. In the NMR spectra at figures 1 A and 1 B, the nucleosides (ATP, hypoxanthine, inosine, and inosine-5'-monophosphate) were shown in singlet signals. This condition means that there are no hydrogens on the adjacent atoms (Wang, Maldonado-Devincci, and Jiang 2020).

The Measurement of Metabolites to Calculate the Fish Freshness

Adenosine triphosphate (ATP) degradation and its degradation products through hypoxanthine have been extensively studied and used to monitor fish muscle freshness and shelf life. It is one of the most crucial post-mortem biochemical changes in marine organism muscle (Ocaño-higuera et al., 2011). The results of these studies showed that the Kruskal-Walli's test of the nucleosides (ATP, hypoxanthine, and inosine 5'-monophospate) that were analyzed using 1 H NMR in Bombay duck fish (*Harpodon nehereus*) showed that ATP decreased significantly ($P<0.05$) up to 6 days, then remained relatively stagnant in the remaining period of storage. Then, IMP increased up to 4 days and decreased markedly ($P<0.05$). Inosine levels were increased. However, the Kruskal-Walli's test also showed that inosine concentration increased significantly from 6 to 12 days ($p<0.05$) storage duration. Furthermore, hypoxanthine levels were slightly fluctuating or near stagnant from the beginning until the end of storage conditions (Figure 2.). Hong, et al. (2015) stated that the most common post-mortem nucleotide

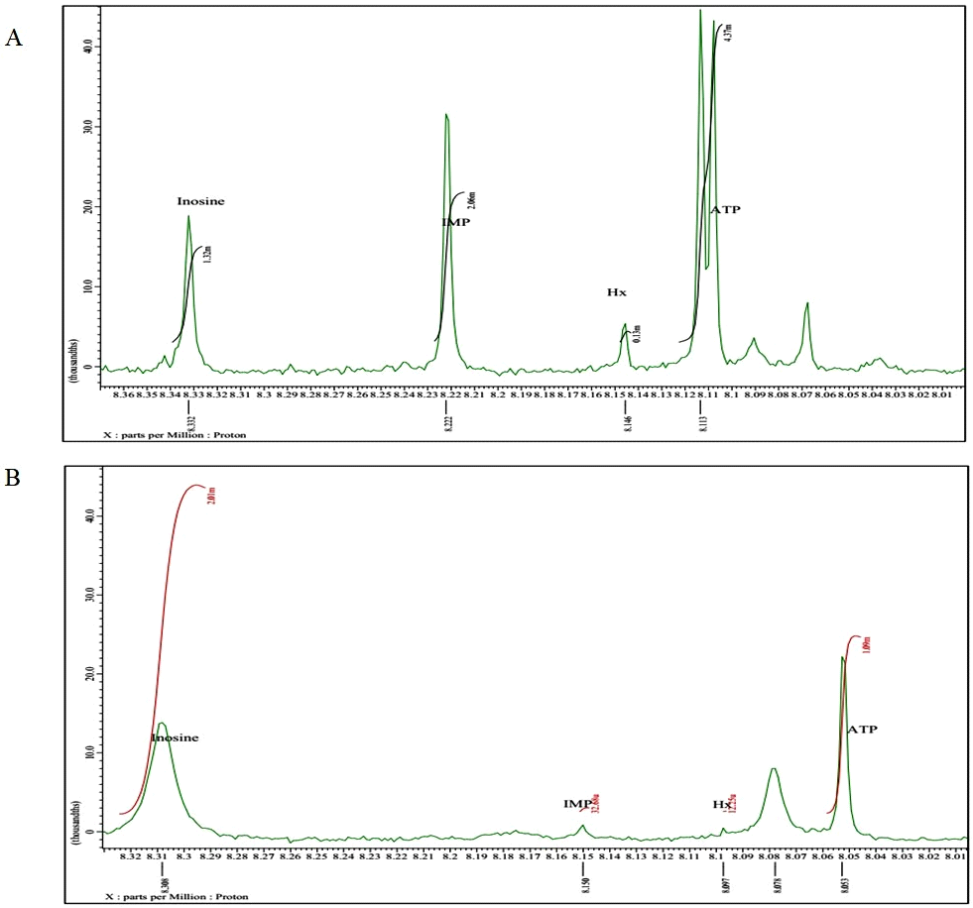
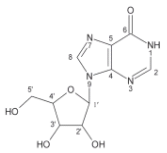
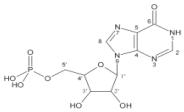
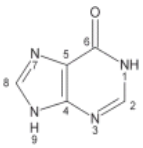
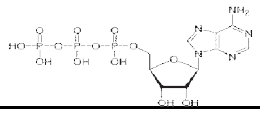


Figure 2. 1 H NMR metabolite nucleoside profile in Bombay duck fish (*Harpodon nehereus* at chilled temperature (A : 0 day storage, B : 12 day storage).

Table 1. Chemical Shifts of 1 H NMR metabolites in Bombay duck (*Harpodon nehereus*) during 12 days storage duration at chilled temperature

Metabolite	**Chemical Structures**	**Chemical Shifts**	
		Proton δH, ppm	**Multiplicity**
Inosine (Ino)		8.36	singlet
Inosin-5'-phosphate (IMP)		8.2	singlet
Hypoxanthine (Hx)		8.15	singlet
Adenosine triphosphate (ATP)		8.11	singlet

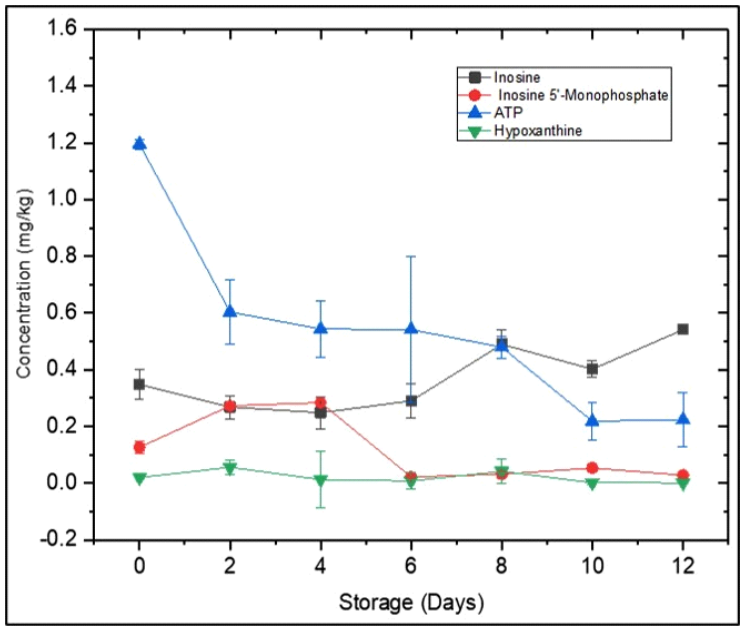


Figure 3. The change of nucleoside metabolites profiles in Bombay ducks fish during storage in chilled temperature within 12 days.

found in most fish species is IMP. Some fish species' freshness has been linked to the disappearance of IMP, which is further degraded into inosine. After that, the nucleoside phosphorylase (NP) enzyme converts inosine into Hx. The developing spoilage microflora ultimately transforms the hypoxanthine into xanthine, uric acid, and other ring cleavage products. This condition caused the hypoxanthine concentration in the samples to slightly increase until 2 days of storage, then decrease and stagnate until the end of storage. The metabolite ATP and IMP degradation of Bombay duck fish (*Harpodon nehereus*) in this study were in line with a previous study that was conducted by Shumilina et al. (2015) on Atlantic salmon and Heude et al. (2015) on brown trout fish.

Figure 4 shows the pattern of nucleoside metabolites in Bombay duck fish after it had been kept at a cool temperature for 12 days. This was done with multivariate linear discriminant analysis. A significant amount ($p<0.05$) of nucleosides was shown in each group's storage day duration. The first discriminant function revealed the primary factor (70,07%). These discriminant functions showed that in 2 days, 4 days and 6 days, the amount of IMP-characterized storage samples. Meanwhile, hypoxanthine (Hx), ATP, and inosine are moving in the opposite direction. Furthermore, the samples in the 8, 10, and 12 days storage conditions were characterized by the amount of ATP and inosine. Therefore, the linear discriminant analysis also describes that the concentration of IMP and hypoxanthine increased until 6 days of storage and decreased from 8 to 12 days. Furthermore, this analysis also describes that inosine concentration increased from 8 days to 12 days.

Several studies describe how the K value helped track the decline in fish freshness. On the other hand, it was discovered that hypoxanthine contents increased when IMP content decreased, while ATP, ADP, and adenosine monophosphate (AMP) contents decreased rapidly and nearly disappeared at 0 ° C. In these scenarios, the amounts of ADP and AMP were negligible. Therefore, the K value was simplified to Ki (Hamada-sato et al., 2005). In these studies, ADP, and AMP in Bombay duck fish (*Harpodon nehereus*) were also not detected in NMR metabolite analysis. It was indicated that the ADP and AMP in samples decreased rapidly during the post-mortem. So, the freshness calculation used in this study is Ki index freshness. According to the data in this study, the Ki index of Bombay duck fish increased significantly ($p<0.05$) from 0 to 6 days of storage duration.

Furthermore, the Ki index showed a stable pattern from 6 to 12 days of storage (Figure 5). The Ki index of Bombay duck fish samples was under 80% until 4 days of storage at chilled temperatures. Garca and Ferez-rubio (2022) stated that high-grade individuals had K-values or Ki-index values of less than 20% or 30%, depending on the fish species. Fish with a correlation between medium grades have K-values or

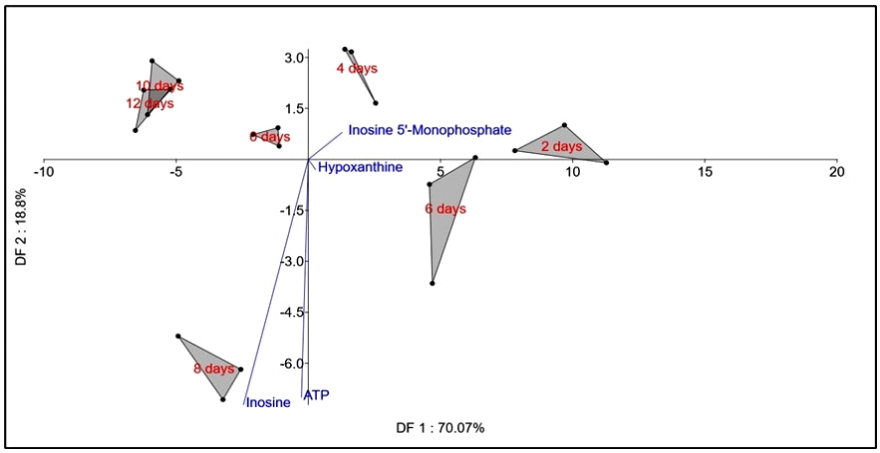


Figure 4. Linear discriminant analysis (LDA) of nucleoside metabolite in Bombay duck (*Harpodon nehereus*) within storage duration group samples

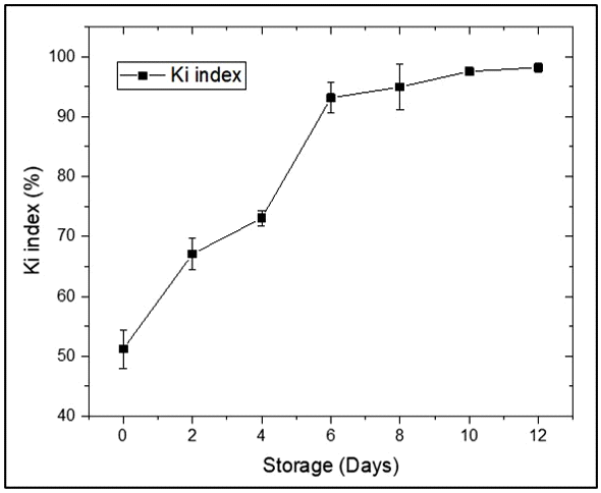


Figure 5. The Ki index freshness of Bombay duck fish (*Harpodon nehereus*) during 12 days storage duration in chilled temperature

Ki-index values of up to 50%. Finally, low-grade samples had a K-value greater than or equal to 75%. Therefore, based on the Ki index calculation, the Bombay duck fish had medium-grade freshness until 2 days of storage and low-grade freshness until 4 days of storage. After 4 days of storage, the samples were categorized as not being fresh. This condition was in line with the study that was conducted by Mehri et al. (2023), which showed that evaluation of postmortem changes in rainbow trout (*Oncorhynchus mykiss)* in room temperature storage conditions using the Ki index. Based on nucleoside calculation could explain that the Ki index in live storage was lower than 8%. Moreover, the Ki index of the fish of the fish climbed to more than 50% at 24 h. After 28 h, the Ki-index of the fish increased to 73% and gradually increased till the end.

Conclusion

The quality factor Ki, which is figured out by analyzing 1H-NMR spectra and measuring the concentrations of inosine, hypoxanthine, and inosine-5'-monophosphate produced while fish is stored, can be used to describe the fish freshness quality. Based on the study, the profile of nucleosides during the storage conditions at a chilled temperature for 12 days was different and fluctuated. Furthermore, freshness calculations showed that the Ki index of Bombay duck fish increased during chilling storage conditions for 12 days. The Ki index of Bombay ducks exceeded 80% after storing the fish for four days. Based on the Ki index calculation, this condition indicates that Bombay duck fish are not fresh anymore after 4 days of storage

in chilling temperatures. Therefore, the 1H-NMR method has proven effective, with advantages for both the environment and the economy. It may also be proposed as a potential rapid method in the future.

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Supplementary Materials

Supplementary materials is not available for this article.

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