國立交通大學

生醫工程研究所

碩 士 論 文

植基於 EEM 技術的魚鮮度指標

Evaluation index for fish freshness based on EEM analysis

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摘　　要

本篇論文主要是利用光學螢光量測來探討並分析魚肉之新鮮度並找出光學的指標，期許對於食品及工業上希望能有所貢獻與發展。相較於傳統新鮮度檢測方法，化學性判讀時間過長、易破壞魚體而造成漁業及食品業的損失，此種光學方法具有非侵入式檢測特性，並期望能達到快速檢測之目的。實驗選用兩種魚類，八隻澎湖縣養殖場的海鱸，平均重量約為 5±1.0 公斤，以及八隻新竹縣活魚餐廳的紅鮭，平均重量約 1±0.2 公斤。檢測部分與方式係將活魚處理切成相同大小的魚肚及魚背，利用自行架設的螢光光譜量測系統來進行實驗，觀察死亡後 24 小時內魚體光譜的時序變化。同一時間亦進行魚新鮮度的傳統檢測法，利用商用高效能液相層析儀(HPLC)來進行定性及定量分析，以獲得傳統方法的新鮮度標準 K 值作為魚肉新鮮度標準。由螢光的激發放射矩陣光譜之結果顯示，在激發光 330~360 nm、放射光 400~500 nm 之間有明顯的螢光訊號。此訊號的最大值位在螢光波長 470 nm 及 430 nm 處，與菸鹼醯胺腺嘌呤二核苷酸(NADH)及第一型和第五型膠原蛋白在激發光 330~360 nm 所產生的螢光光譜位置雷同。而根據正規化處理而獲得光學指數數值，以變異數分析此數值與死亡後 24 小時之關聯性，發現此數值隨

著死亡後時間逐漸地降低，且具顯著性差異(p *value*<0.05)，這表示可將此一數值視為另外一種新鮮度指標。由 HPLC 結果顯示，每隻紅鮭的代謝變化速度皆不相同，推測原因可能是不同魚體差異性造成、環境及處理過程的差異性，因此會影響 K 值變化，推測也會影響魚體裡某些螢光成分的代謝。但整體結果來說光學指標與 K 值的數值大小大致呈負相關。而光學指標的高低結果也代表著魚死後肌肉所進行的僵直化及嫩化效應所造成的。是故，以目前的研究結果，此光學指標是可以用來判斷剛死亡的魚肉及生魚片的品質。

Evaluation index for fish freshness based on EEM analysis

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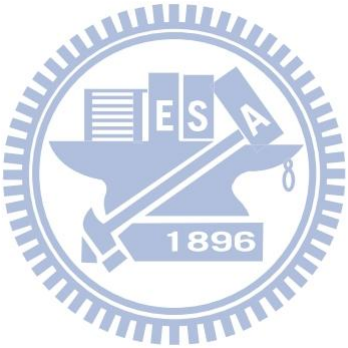
ABSTRACT

In this study, the autofluorescence emitted from fish tissue is measured as an optical evaluation index for freshness identification. Because of the time-consuming and invasive nature of the traditional method, it causes property loss in the fishery and food industries. A quick and noninvasive method is necessary. The result can be applied to the fishery and food industries for quality control. With the quickness of the fluorescence technique, it could improve the procedure of detection. The species chosen are eight cobias, which weigh 5±1.0 kilograms, from a marine products farm in Penghu and eight Seriola dumerili, which weigh 1±0.2 kilograms, from a seafood restaurant near Hsinchu Science Park. The fish were sliced at the abdomen and dorsum. Then, the fluorescence from the tissue was measured within 24 hours after the fish had died by using Y-type fiber. Meanwhile, traditional detection was employed by extracting ATP degradation products and calculating K value to confirm fish freshness. From the results of the Excitation-Emission matrix (EEM), the two peaks in excitation wavelength were 330 to 360 nm, and emission wavelengths were 400 to 500 nm. The two peaks were at the 470 nm and 430 nm emission wavelengths, respectively. It has been shown that the fluorescence indicates that the major contributors are from Nicotinamide adenine dinucleotide (NADH), collagen type I, and collagen type V. Analysis of variance (ANOVA) showed that the intensity of fluorescence decreases with refrigeration time (*p value* < 0.05). Also, the chemical result presents that there are different metabolism rates between

different fish species. It is known that with different treatments and growing environments of fish can affect the K value and even the fluorophores in fish tissue. On the whole, the optical index is negatively correlated to the K value index. The value of the optical index is that it can measure the process of rigor mortis and the tenderization of fish muscle. The optical index can be applied to quality control for the freshness of fish fillet and sashimi.



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My VBM Lab is full of challenges and brings me happiness every day. My team members are outstanding. It has been an honor to work with them, not only to due to their partnership in our high-end research, but also due to the positive ways that they have affected my life. I absolutely trust that everything will go well in the future.

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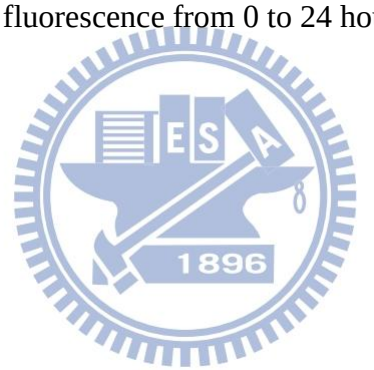

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I. Introduction

1.1. Background

1.1.1. Fishery issues in Taiwan

Taiwan is an island country, so fishing is an important industry. But compared to other marine countries with an interest in aquaculture, the Taiwan government is more focused on the domestic market. Moreover, the quality of aquatic products and processed farm products are controlled only before reaching customs, and the standard operation performance is too complicated to control. If the products are imported to another country, quality management is a big issue because the inconsistencies of laws and standards make it hard to track in the importing country. Given the trend of trade liberalization and internationalization, the enhancement of industrial competitiveness and the need for lower production costs have grown in importance for maintaining industry sustainable.

Generally, the traditional process of detecting freshness is to judge the shape, color, and smell of the fish [1, 2]. When necessary, magnifiers are used for further detection. However, if the fish products are in doubt, fishermen are required to coordinate with the Fisheries Department or its delegate agencies for sampling and are supposed to notify the inspection bodies to carry out biological tests and extraction and colorimetric measures. Tests for volatile basic nitrogen (total volatile basic nitrogen: TVB-N) and trimethylamine nitrogen (trimethylaminenitrogen: TMA-N) are frequently used.

In detecting drug residue, enzyme-linked immunosorbent assay (ELISA), thin layer chromatography (TLC), high-performance liquid chromatography-ultraviolet (HPLC-UV) detection, liquid chromatography-Mass (LC-MS) spectrometry, or liquid chromatography/tandem mass spectrometry (LC-MS/MS) have been applied for a long time.

Although these chemistry type methods are precise and accurate, it is hard to meet the business requirement nowadays by executing the complicated and time-consuming procedures. When encountering a large number of fish, the inspector may make an error and spend more time confirming the report. It is inevitable that a depletion of fish products occurs during the process of detection.

1.1.2. Biochemistry metabolism of fish tissue

From Table 1.1, it can be seen that the process of anaerobic respiration begins just after the fish start dying. In this time, metabolic glycolysis carries on with degrading creatine phosphate to generate adenosine triphosphate (ATP). Creatine phosphate breaks down into creatine and creatinine. Then, because of the anaerobic respiration, lactic acid accumulates and the pH value starts to decline, causing other enzyme activity. After that, the muscle starts the period of rigor mortis, which results from the complete consumption of ATP and the structural changes in myosin and actin. Almost at the same time, the muscle structure starts to break down following the resolution of rigor, as microbial activity multiplies quickly in the muscles. In the next step, proteins, lipids, and nucleotides start to decompose; e.g., proteins are degraded to peptides and amino acid; lipids are degraded to aldehyde, ketone, peroxide, and lipid acid; ATP is degraded to adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR), and hypoxanthine (Hx). Finally, more and more accumulation of the above metabolites makes the muscle tissue putrefied.

Table 1 The metabolism of fish tissue in the postmortem period [3]

Stage	Period	Biochemical meaning
1st	death of fish	1. anaerobic glycolysis: ADP → creatine phosphate → ATP 2. formation of lactic acid: pH value descends

2nd	rigor mortis	1. rigor mortis: acting & myosin → actomyosin 2. complete consumption of ATP: rigor mortis
3rd	resolution of rigor	muscle structure change: the degradation of z disc, myosin and actin
4th	autolysis	1. protein: protein → peptide → amino acid 2. lipid: lipid → acetone → aldehyde, ketone, peroxide, and lipid acid 3. nucleotides: ATP → ADP → AMP → IMP → HxR → Hx
5th	putrefaction	1. protein: accumulate many amino acids 2. lipid: accumulate many aldehydes, ketones, peroxides, and lipid acids 3. nucleotides: accumulation of HxR and Hx 4. low-molecule-weight compounds: microbiological degraded

Fish freshness can be detected by investigating the metabolites above. The processes are based on color, texture, flavor, and the metabolites of tissue [4], e.g., T-VBN, TMA-N, and histamine, which are the metabolites of protein [5]. Also, measuring the pH value in fish tissue or calculating ATP degradation products for K value are alternative procedures [6, 7]. Though they are accurate and precise, these traditional methods waste many times. Moreover, the complicated detection procedures waste resources.

Table 2 The traditional methods for measuring fish freshness and their meanings

	operation (Media)	**meaning**	**drawback**
sensory organ	coloring, smelling, touching	be smelly, stiff, and less red fish muscle for several hours after fish died	know-how
pH value	pH meter (glass electrode)	pH value is changed by glycolysis and other metabolism processes	invasive, time consuming
T-VBN	total VBN	main metabolite of amino acid	invasive, time consuming, complicated
TMA-N	trimethylaminenitro-gen	main metabolite of amino acid	
Histamine	histamine concentration	the metabolite of glycine	
K value	1. ATP, ADP, AMP, Hx, and hypoxanthine 2. K value calculation	ATP is not generated and the fish muscle will be stiff.	

1.1.3. The definition of K value

In this study, the K value is used as a freshness standard to compare with the optical index. ATP is the energy used directly in the creature. After fish die, ATP is degraded into ADP, AMP, IMP, HxR, and Hx during the process of storing of fresh fish or preserving seafood. IMP is formed by AMP deaminase, which is an autolytic enzyme, and spoilage bacteria result in HxR and Hx formation. IMP has a flavorful taste, whereas Hx has a bitter taste, which indicates spoilage of the fish. However, the metabolism rate from ATP to Hx is different from fish to fish. The K value was suggested as an index of fish freshness in 1959 by Japanese researchers. A K value of 40 represents the spoilage of fish, which indicates that the product should not be supplied to customers, whereas a K value of 20 can be used to restrict consumption as raw fish or sashimi. Following are the metabolism of ATP and the equation of K value:

$$\text{ATP} \rightarrow \text{ADP} \rightarrow \text{AMP} \rightarrow \text{IMP} \rightarrow \text{HxR} \rightarrow \text{Hx}$$

$$K\ value = \frac{[HxR] + [Hx]}{[ATP] + [ADP] + [AMP] + [IMP] + [HxR] + [Hx]} \times 100 \quad (1)$$

where [*ATP*], [*ADP*], [*AMP*], *[IMP*], [*HxR*], and [*Hx*] indicate the concentration of the metabolites. K value is calculated by the concentration of the six ATP degradation products. In most fish, K values increase linearly during the first days of refrigeration storage, and it is an index of freshness detection. When the K value is higher, it means the fish meat is less fresh.

1.1.4. The history of developing freshness detection

The food detection techniques have been developed for many years. In the early stage, the researchers attached importance to the accuracy (direct detection and multi-index combination) of detection techniques. The first modern method used dependent parameters (the Torry scheme) for freshness [8]. Later, it was modified by combining many characteristic features to calculate a score called "QIM." This gave a single numerical value to a broad range of characteristics. In 1962, the standard method was named Conway's method; it distills off volatile amines (or the microdiffusion of an extract) and includes measurements of di-methylamine, tri-methylamine, ammonia, and other volatile basic nitrogenous compounds associated with meat spoilage. Afterwards, more and more detection instruments were developed to establish better performance. One of the most popular methods, established in 1985, is utilizing K value calculation by High Performance Liquid Chromatography (HPLC). This K value is decided by six components, which are the metabolites of ATP in muscles, i.e., ATP, ADP, AMP, IMP, HxR, and Hx [9, 10]. HPLC is more accurate and precise than ion-exchange chromatography (IEC), which was used previously.

Nowadays in the food industry, researchers are focused on developing a low-cost system for monitoring freshness. Some of the previously used methods have been modified. Gil developed an electronic tongue by using metallic potentiometric electrodes for fish freshness analysis in 2008 [11]. Yapar measured the refractive index of eye fluid to determine changes of fish freshness in 2004 [12]. Okuma and Nanjyo combined enzyme electrodes sensors with an injection flow device to develop a system for measuring fish deterioration in 2002 [13, 14]. In 2008, Barbri produced a portable electronic nose system to measure peculiar fish smells produced over time to evaluate differences in fish freshness [15]. Kroeger adopted machine vision analysis of whole fish and fillets with respect to freshness in 2003 [16]. Also, visible and near-infrared spectroscopy has been used to analyze the quality of fish [17]. Several new detection techniques have been developed that can simplify the detection process. However,

there are many limitations for these techniques, including detecting limitations in storage time and limited applicability to certain fish species.

Amino acids, proteins, and enzymes are well-known fluorescent molecules, and the potential to use fluorescence spectroscopy to serve as an analytical tool in the chemical, biological, biomedical, and food sciences has increased in recent years. For example, the fluorescence spectra of tryptophan (amino acids), collagen (protein), and NADH (coenzyme) with linear multivariate analysis methods have been adopted to differentiate differences in normal and dyplasia tissues in humans [18]. It is also known that changes in intrinsic fluorophores can be measured in fish muscle. Also, the fluorescence spectra can be described during refrigeration storage. The destruction of aromatic amino acids, the deposition of protein, and the action of metabolic enzyme can be treated as biomarkers for fish freshness.

1.2. Literature review

1.2.1. The principle of fluorescence technology

The vibration level of electrons for most molecules is generally lowest at room temperature. When one molecule is excited by a specific wavelength, the electron energy level moves from a ground state (S1) up to an excited state (S2). After that, most electron activity drops quickly to the lowest level of vibration excitation. In the process, similar molecules hit each other and lose energy, which is called vibration relaxation or internal conversion. Then after about 10^{-9} second, the electrons drop on every vibration level to a ground state. In this time, the energy released with light is called fluorescence.

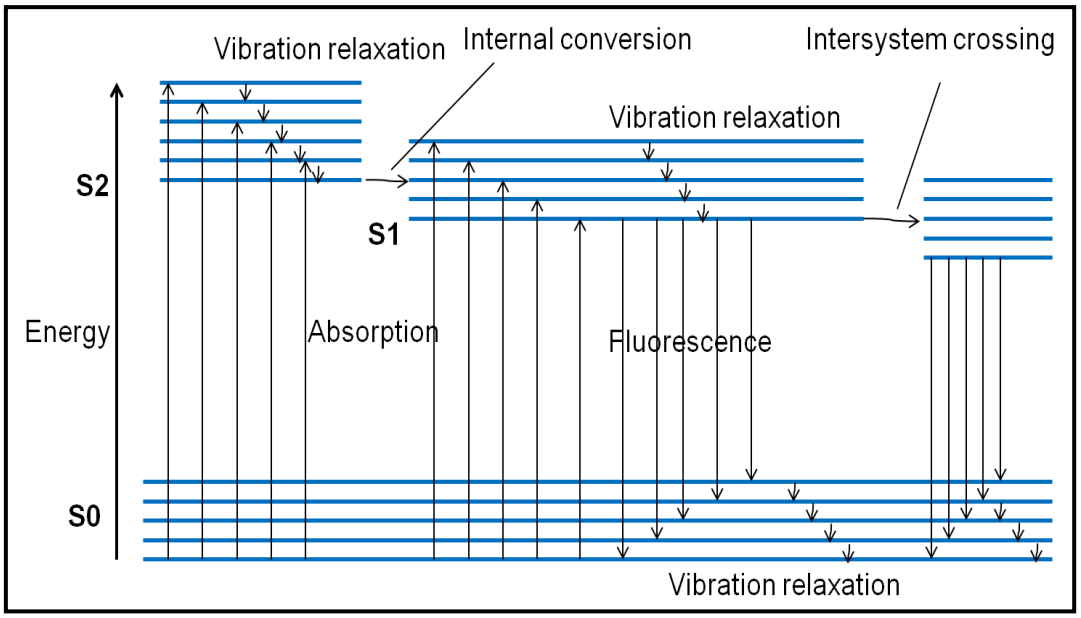


Fig. 1 The electron energy level moves from a ground state to an excited state by excited power, and the energy conversion by vibration relaxation, internal conversion, and electron jumping from excited state to ground state will emit heat and fluorescence [19].

1.2.2. Fluorescence measurement of freshness

Recently, many groups have distinguished the stages of fish freshness from fluorescence spectra. NADH, lipid oxidation, chlorophyll, tissue structure (collagen), and aromatic amino acid (tryptophan) have been investigated as the targets of fluorophores to monitor freshness. For example, Aubourg and Duflos measured the fluorescence spectra of lipid oxidation products as an index of fish freshness in 1999 [20, 21]. During refrigeration storage, fish will deteriorate with lipid degradation, which causes the proteins of the muscle to denature and change texture [22, 23]. Thus, Aubourg investigated free fatty acids, the thiobarbituric acid index (TBA-i), and fluorescence formation. The results showed that the lipid damage rate varied with difference species and storage temperatures. Dufour found that the fluorescence spectra of tryptophan and NADH could be used as fingerprints of fish. Fresh and aged fish were classified by spectra of tryptophan and NADH using Principle Component

Analysis (PCA) and Flexible Discriminant Analysis (FDA). Karoui adopted PCA and detrended fluctuation analysis (DFA) methods to extract NADH fluorescence information for determining a precise classification [24]. In addition, Andersen discussed the auto fluorescence of collagen type I and type V, which are detectable from the fluorescence spectra of salmon and cod muscle [25]. The spectra at fluorescence wavelengths 470 and 430 nm from fish tissue were assumed as collagen type I and type V, which were considered related with resolution of rigor.

Table 3 The related study on fish fluorescence spectral analysis

Authors	**Journal**	**Excitation (nm)**	**Emission (nm)**	**Target**	**Analysis Method**	**Result**
E. Dufour, J.P. Frencia, E. Kane	*Food research International 36,415–423 (2003)*	250 290 336	280-480 305-400 360-600	amino acid nucleic acid tryptophan NADH	PCA	PC1 and PC2 shows discrimination of day1, day 3 and day 5
C. M. Anderson J. P. Wold	*J. Agric. Food Chem. 51, 470-476 (2003)*	332	350-600	collagen NADH	PCA	The spectrum could be the fluorophore of collagen
R. Karoui, E. Thomas, E. Dufour	*Food research International 39 349-355 (2006)*	290 340	305-400 360-570	tryptophan NADH	PCA	NADH can predict frozen and thawed fish 100%
J. Christensen, L. Nørgaard, R. Bro, S. B. Engelsen	*Chemical review 106 (2006)*	332	350-600	amino acid chlorophyll collagen NADH	PCA FDA PARAFAC model	Summary of the research of fluorophores in fish tissue for food study
J. F. Hunt T. Ohno	*J. Agric. Food Chem. 55, 2121-2128 (2007)*	240-400	300-500	tryptophan tyrosine	PARAFAC model	2^{nd} component is related to freshness
J. SádeCká J. TóThoVá	*Czech J. Food Sci. 25, 159–173 (2007)*	250 290 336	280-480 305-400 360-600	tryptophan nucleic acid NADH	PCA FDA	Summary of the research of the fluorophores of nucleo acid, amino acid, and NADH in fish tissue

Above all, the fluorescence spectra have been analyzed for many kinds of foods. Fish freshness detection by using fluorescence spectra is an ongoing research issue and has been

studied for a long time. However, there was almost no study focusing on fresh fish that discussed fluorophore changes as a quick detection technique. Investigating the fluorophores in fresh fishes is necessary because of the importance of maintaining good fish quality. After all, fishermen could enjoy better sales if they had better quality control.

To develop a portable fluorescence measurement system for monitoring fish freshness during the delivery process in various environments, the focus of this study is on fiber-optic fluorescence spectroscopy for monitoring freshness during refrigeration time. This academic investigation can provide value for consumers and suppliers by drawing a successful roadmap for establishing an unsophisticated portable device using selective excitation light to understand the quality of fish freshness.

1.3. Objective

Because of the importance of controlling the quality of fresh fish, my study will perform optical fluorescence measurements to find an optical index related to freshness within 24 hours after the fish have died. It is very important to maintain the quality of fish for fishermen and customers till the fish has been cooked. In this study, all the metabolic processes of fish tissues are observed at different stages as the fish become less fresh. The optical index is developed to find the correlation between the optical measures and the freshness in these fresh fish. Live fish were chosen as our experiment subjects to study the metabolism of fresh fish. We assume that some fluorophores in the tissue of fresh fish will degrade or degenerate in the process of becoming spoiled. The fish samples will be sliced into equal sizes to analyze the fish fillets as they change with refrigeration time. Based on this concept, the fluorophores related to freshness can be found and analyzed. After the index is developed, it can be applied as a faster method to test sashimi or fish fillets. The optical index will be validated by traditional methods to confirm fish freshness. With the establishment of the K value by

analyzing the concentration of nucleotides using HPLC, we can learn more about the freshness within 24 hours. Finally, the index can be applied as a quick-detection instrument for fish freshness.

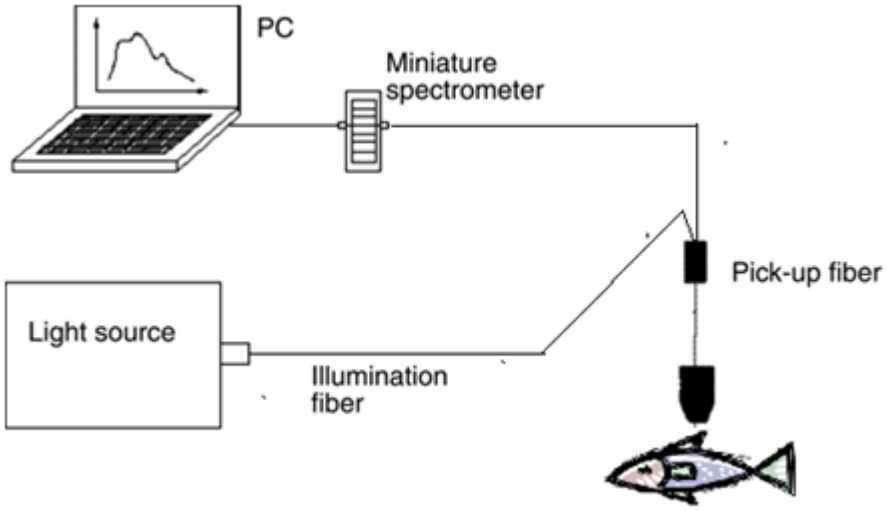


Fig. 2 depicts the measurement system, which includes a light source, illumination fiber, pick-up fiber, a miniature spectrometer, and a computer. The illumination fiber releases a specific power, and the pick-up fibers receive fluorescence to transport to the spectrometer. Finally, the signal is analyzed by a computer.

II. Material and Method

2.1. Experiment flow chart

Fig. 3 represents the flow chart of the experiment, which includes two stages. The main purpose is developing an optical technique to compare the time variant and the chemical index of different species of fish. In the first stage, cobias are used for qualitative analysis to search a spectrum area related to freshness from the Excitation-Emission Matrix (EEM). The optical index is developed by normalizing and comparing different storage times. In the second stage, we use the same spectrum area from the cobia to investigate different species of fish. Seriola dumerili are measured for narrower refrigeration time intervals to compare with the K value and to monitor the detail in the fish tissue. Statistics are developed using the optical index and these two kinds of fish species.

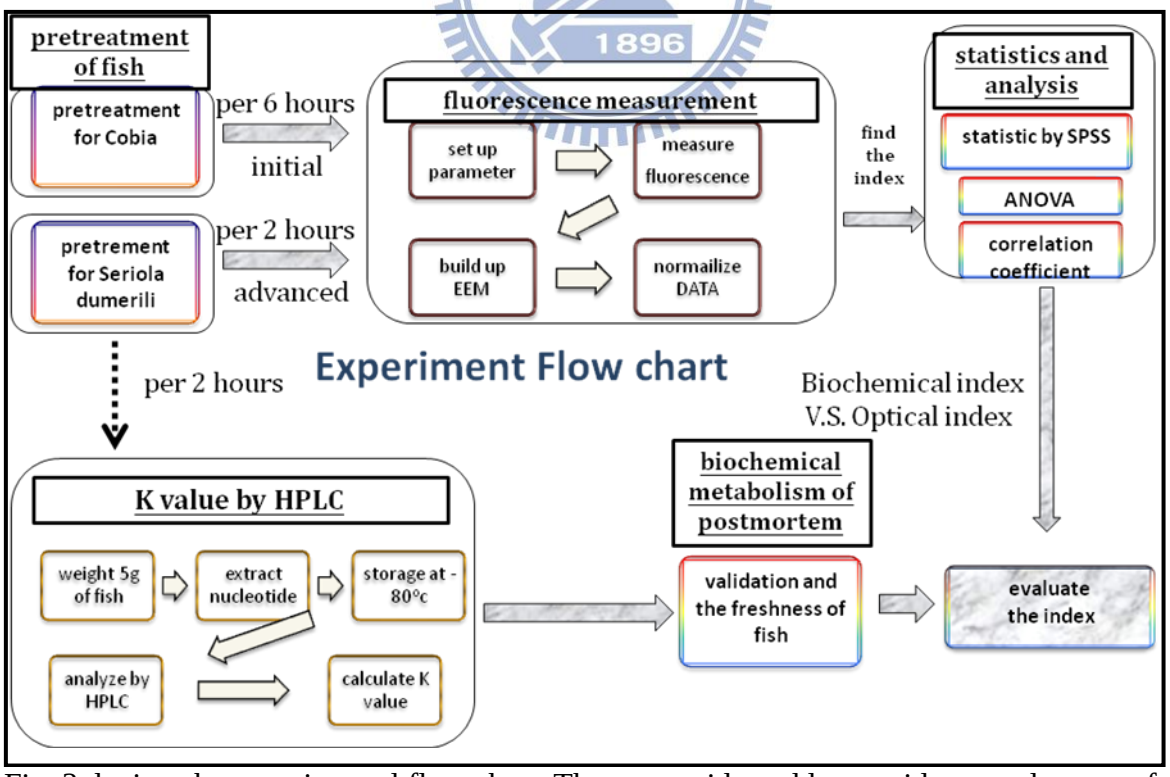


Fig. 3 depicts the experimental flow chart. The upper-side and lower-side procedures are for optical and chemical experiments, respectively.

2.2. Fish subject

2.2.1. Cobias

Cobias live in temperate and tropical zones of the sea and grow up to 6~8 kg and 1.5 m long in their first year. Cobias are mainly captured from March to May. Nowadays, cobias are the main type of fish used in offshore cage aquaculture in the world. The fish are most famous for sashimi preparation in Asia. In Taiwan, cobia is fed in a net/box in warm water in Penghu County. Since Taiwan is a desirable environment to exploit cobia offshore cage aquaculture, 5-to-7-month-old cobias, which are suitable for sashimi preparation, have been chosen as the monitoring target in this study.

2.2.2. Seriola dumerili

Seriola dumerili also live in temperate and tropical zones of the sea. The fish can grow up to 30 to 40 centimeters long. The color of the fish body is slightly brown. The meat is full of lipids and nutrients, and because of its texture and its deliciousness, it is also famous for sashimi preparation.

2.3. Pretreatment for live fish

Initial experiment

A total of eight cobias (averaged weight was 5.0±1.0 kg) in Penghu Country were adopted as experimental species. Each cobia was slaughtered and gutted by a fishmonger after the blood was washed out of the tissue by secondary water and then transported to lab as soon as possible. Equal sizes of fish fillets (4×3×1 $cm^{3)}$ had to be sliced in order to keep different widths from affecting the fluorescent intensity. Finally, the fish fillets were kept in a

refrigerator at 4 ^{o}C for 24 hours. The steps of the pretreatment process were:

I. The fishmonger stroked the fish on the head with a hammer, causing it to lose consciousness;
II. The fishmonger gutted the cobias and cleaned the blood from the tissue;
III. The fish were put into a crisper and transported with an ice bucket to the lab as soon as possible;
IV. The fish were cut into fillets of equal sizes (4×3×1 cm^3);
V. Fillets were refrigerated at 4 ^{o}C, and optical measurements were begun immediately.

Advanced experiment

A total of eight fish (averaged weight was 1.0±0.2 kg) were collected from a local seafood restaurant in Hsinchu County. We needed to measure the fluorescence spectrum in a shorter time interval compared with the cobias to analyze the differences at different time points and to ensure the freshness of the fish. The samples were validated by K value using HPLC. The steps of the pretreatment process of Seriola dumerili were similar to that applied on the cobias:

I. The chef stroked the fish on the head with a hammer, causing it to lose consciousness;
II. The chef gutted the Seriola dumerili and cleaned the blood from the tissue;
III. The chef sliced the meat in the abdomen and dorsal parts, respectively, and washed the fish again with secondary water;
IV. The fish were put into a crisper and transported with an ice bucket to the lab within 25 min;
V. The fish were cut into equal-size fillets (4×3×1 cm^3);
VI. Fillets were refrigerated at 4 ^{o}C, and optical measurements and chemical processing began immediately.

↓

Fig. 4 depicts Seriola dumerili before and after pretreatment of live fish by the chef: (a) the space for the fish in the local restaurant, (b) slaughtered fish, (c) cut fish meat, and (d) slices of abdomen and dorsum.

2.4. Optical fluorescence measurement

When the electrons of fluorophores in fish tissue are excited, these electrons jump into an excited state. When these electrons go back to a ground state, fluorescence is emitted instantaneously. The wavelength of emitted fluorescence depends on the energy level between the ground state and the excited state. For a variety of fluorophores in fish tissue, different features of fluorescence are emitted. Based on the fluorescence emitted by the fish tissue, the fluorophores can be found and identified. Finally, the optical index for freshness detection will be developed for the time points within 24 hours after the fish have died.

2.4.1. Measurement system

Fig. 5 is an overview of the Y-type fiber-optic measurement system. This system includes a CERMAX® xenon lamp (300W, Perkin Elmer, Waltham, USA), an H10

monochromator (HORIBA Jobin Yvon, Longjumeau, France), a Y-type optical fiber, a MicroHR180 spectrometer (HORIBA Jobin Yvon, Longjumeau, France), an R928 photomultiplier tube (PMT) (Hamamatsu, Shizuoka, Japan), a 1,000 voltage power supply (HORIBA Jobin Yvon, Longjumeau, France), a DataScan2 (HORIBA Jobin Yvon, Longjumeau, France), and an anti-vibration table (Newport, Taipei, R.O.C.). The xenon lamp is set up as a light source in this study for broadband wavelengths from 260 nm to 600 nm. In order to slice a narrow band of excitation light, the slit (width is 0.5mm) is put into the export of the monochromator to filter through a pure light source. The Y-type fiber, which is set up on a black platform, can transfer excitation light and emission light. After transferring the emission light, the spectrometer separates the light into mono wavelengths. Finally, the mono wavelength light is transformed into an electrical signal by PMT, and then the signal is acquired by DatasScan2 controller. The computer gathers the signal from DataScan2 by using an RS232 serial cable. All instrumentation drivers of the monochromator, spectrometer, and PMT were designed by LabVIEW (v. 7.1, National Instruments, Austin, USA). As well, the front panel of the system and the operating protocols were also designed by LabVIEW (v. 2011).

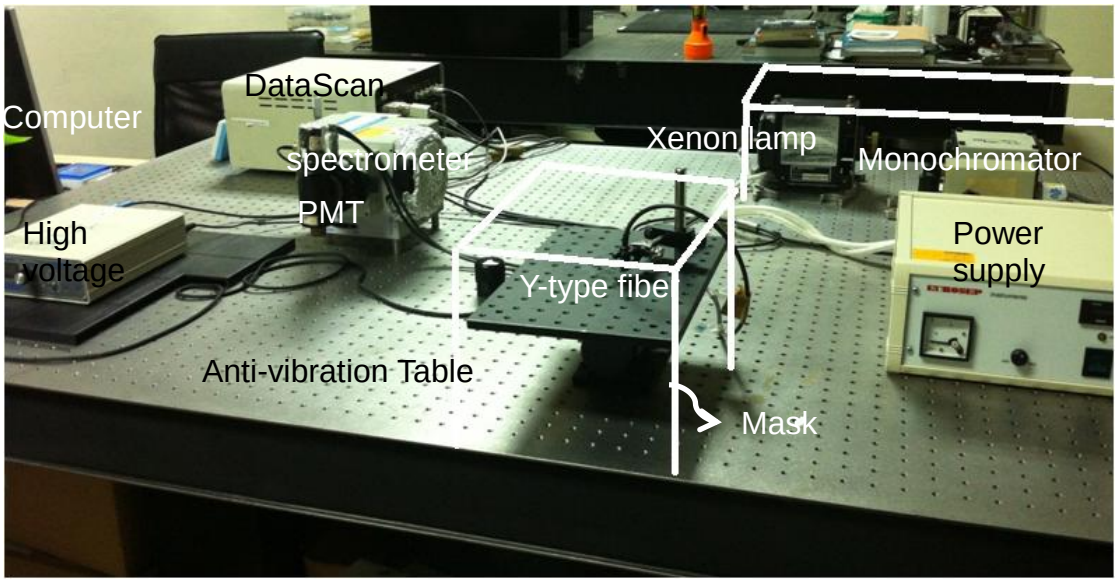


Fig. 5 shows the Y-type fiber-optic measurement system. The room lighting is turned off,

and a black mask is utilized to avoid light leakage during measurement.

2.4.2. Measurement process

The measurement environment is always kept around 25±3 ^{o}C, and samples are preserved at 4±2 ^{o}C refrigerator. In order to avoid unexpected noises, a 30 min warm-up step is necessary for the lamp and power supply. After finishing this, one fish fillet of abdomen or dorsum is put on a sample holder. Cobia fillets are taken out for measurement every 6 hours between 0 and 18 hours. As well, Seriola dumerili fillets are taken out of the refrigerator for measurement every 2 hours between 0 and 24 hours. The excitation wavelength is set from 320 to 420 nm, and the emission wavelength is set from the excitation wavelength + 30 nm (Exci+30 nm) to two times the excitation wavelength (2×Exci-80 nm) to remove second harmonic generation. For example, the emission wavelength is set from 350~560 nm at the 320 nm excitation wavelength. By the wide range of fish tissue scanning, the fluorophore regions can clearly be located. The fiber cable contains up two kinds of fibers called "Y-type fibers." One of the fibers is for the transmittal of the excitation light source, and another is for reception of the emission signal. In addition, two different sites on each fillet are measured for an average to reduce the non-homogenous effect of the fish tissue.

2.4.3. Measurement program and related parameters

Fig. 6 shows the front panel of the measurement system program. Although all instrumentation drivers were designed by LabVIEW 7.1, the front panels of the system and the block diagrams of the operating protocols were implemented modally and hierarchically at the LabVIEW 2011 environment. Before measurement, the spectrometer and monochromator were initialized. The "**Scanning…**" button is designed for automated execution until finishing

the entire procedure. The "**STOP**" button is designed for sending an emergency command from the software to the external devices immediately. The fluorescence amplitudes of every excitation wavelength are shown on the waveform graph and are saved as an American Standard Code for Information Interchange (ASCII) text file. Another drawing program is designed for the Excitation-Emission Matrix (EEM) display. Following are the measurement parameters of this system:

I. **Excitation wavelength**: from 320 to 420 nm with 10 nm interval
II. **Emission wavelength**: from (Exci+30 nm) to (2×Exci-80 nm) with 2 nm interval for each excitation
III. **Integration time**: 300 ms
IV. **High voltage**: 600 V
V. **Grating**: 1200 mm

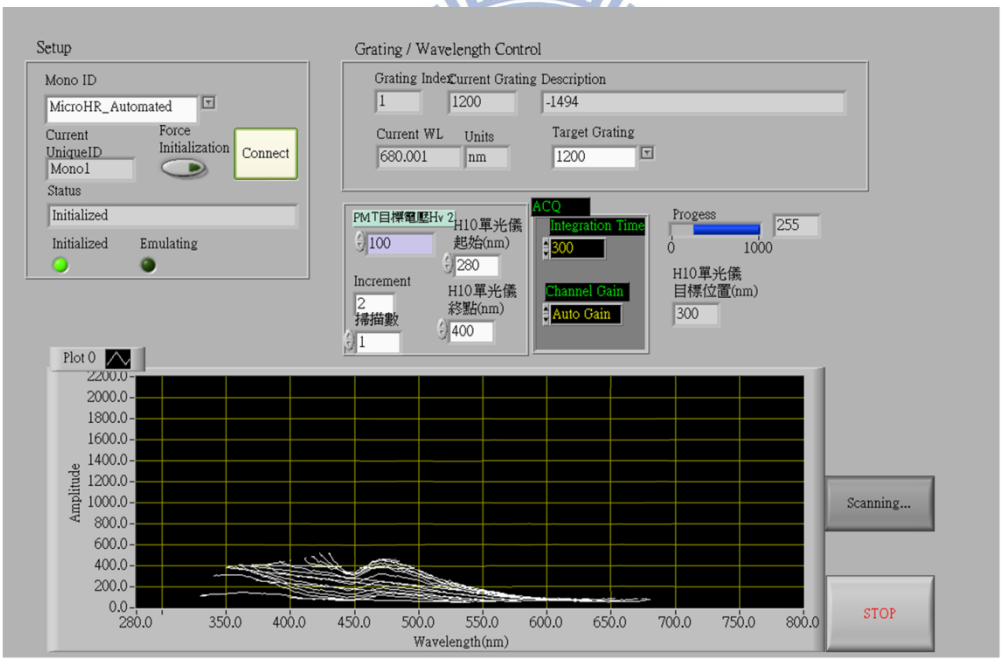


Fig. 6 shows the front panel of the measurement program.

2.4. Chemistry detection method for freshness

Fish subjects can be quite fresh even after the fish have died and 24 hours of storage. It is important to choose a chemical detection method that is appropriate for the study. K value,

which was developed by six kinds of ATP degradation products, can be a proper way to evaluate fish freshness. ATP, ADP, and AMP all exist in live fish but decompose to IMP, Hx, and HxR soon after the fish die. Based on this concept, K value calculation is used to accurately judge the freshness of fresh fish. Moreover, the result with HPLC methods can achieve K value calculation more precisely and more accurately.

2.4.1 Chemistry reagents and instruments

2.4.1.1 Instruments

A high-speed refrigerated centrifuge (6200, KUBOTA) was rented from Jen An Technology Co. Ltd. for three months. Ultrapure water was borrowed from The Department of Biochemical Science and Technology at National Chiao Tung University (NCTU). A -70 ^{o}C refrigerator was borrowed from the Department of Applied Chemistry from NCTU. An analyze column (5 μm ODS-2 4.6x250 mm, Inersil) was purchased from Vercotech Inc., HPLC (ProStar 210, liquid chromatography system, Dynamax, Thermo Finnigan, San Jose, CA, USA) and a UV-Vis Detector (ProStar 325, Dynamax, Thermo Finnigan, San Jose, CA, USA) and other tools for HPLC were borrowed from the precision instrument room at NCTU.

2.4.1.2 Chemicals and reagents

Perchloric acid (Chemical pure, KGaA, Merck) was used to extract the ATP degradation productions. Potassium hydroxide (Chemical pure, Schuchardt OHG, Merck) was used to neutralize the acidity of perchloric acid. Methanol (HPLC grade, Merck) was used as mobile phase A, and Na_2HPO_4 (HPLC grade, Merck) was used as mobile phase B for HPLC. Nucleotide standards (HPLC grade, Sigma-Aldrich) included Adenosine 5'-triphosphate

disodium salt hydrate (ATP), Adenosine 5'-diphosphate sodium salt (ADP), Adenosine 5'-monophosphate sodium salt (AMP), Inosine 5'-monophosphate sodium salt (IMP), Inosine (HxR), and Hypoxanthine (Hx), which are standards of the ATP degradation products.

2.4.2 Chemistry method procedure

During the fish fillet extraction process, it was important to keep the fillets at 4 ^{o}C temperature to prevent the ATP products from being degraded [26]. The ATP products were extracted by perchloric acid and neutralized by potassium hydroxide. To remove the crystal from the extraction solution, the samples were frozen at 0 ^{o}C to precipitate more KCl_4 crystals. The extraction process steps:

Smash 5 g fish meat by Homogenizer for 1 min
↓
Add 5 g meat into 25 mL of 6 % perchloric acid and then centrifuge for 15 min (5000 r/min)
↓
Remove 10 mL supernatant to another centrifugal tube
↓
Use potassium hydroxide to neutralize to pH 6.4 (10 mol/L solution at first and then 1 mol/L solution)
↓
Storage in refrigerator at 0 ^{o}C for 30 min
↓
Sintered filter to filtrate the Crystallization of $KClO_4$
↓
RC25 membrane syringe filter
↓
Diluted by ultrapure water to 20 mL
↓
Storage in refrigerator at -70 ^{o}C
↓
Analyze by HPLC
↓
Calculate K value and nucleotide concentrations

2.4.3 Recovery of the extraction process

In order to confirm the coefficient of recovery of the extraction process, the standards of ATP, ADP, AMP, IMP, Hx, and HxR were added into the fish fillets. We added two concentration levels of 1ml of standard solution, which contained 1,000 mg/L and 3,000 mg/L of ATP, ADP, AMP, IMP, Hx, and HxR, to compare with the fish fillet, in which the standard solutions were not added. A total of 10 g of fish meat was homogenized and divided into two groups: one was 5 g of fish meat; another was 5 g of meat plus 1 mL of standard solution to perform the extraction process. Finally, the recovery was calculated to evaluate the efficiency of extraction.

2.4.4 HPLC analysis

In the study, two kinds of mobile phases were used for HPLC analysis. Mobile phase A (0.1M Na_2HPO_4 and 6.0 pH value by H_3PO_4) and mobile phase B (Methanol) were degassed before running. The mobile phases were filtered through a 0.22 μm × 47 mm nylon membrane filter (Supelco, Bellefonte, PA, USA). After melting the extraction samples from -70 ^{o}C, the samples were centrifuged to remove redundant crystallization and then 5 μL of the sample solutions were injected into the injector. Mobile phase A and mobile phase B were used as the mobile phases at a flow rate of 0.7 mL min^{-1}. The initial composition was 98% A and 2% B for 3.0 min, then changed linearly to 50% A and 50% B for 12 mins, and then changed to 35% and 65% B for 3 mins. Finally, the composition was changed to 98% A and 2% B for 5 mins of equilibrium. Following are the gradients for phase A and B (Table 4).

Table 4 Gradient condition for mobile phase A and B

Time (min)	Flow (ml/min)	Mobile phase A (%)	Mobile phase B (%)
0	0.7	98	2
3	0.7	98	2
15	0.7	50	50
18	0.7	35	65
20	0.7	98	2
25	0.7	98	2

* Mobile phase A is 0.1M Na_2HPO_4 and 6.0 pH value by H_3PO_4
* Mobile phase B is methanol.

Table 5 lists five different concentration standards by serial dilution for calibration curve. For the higher concentrations of IMP, ATP, and ADP of fish tissue, it was necessary to heighten the concentrations appropriately.

Table 5 The standard concentration (mg/L) of six ATP degrade production

Standard	IMP	ATP	ADP	AMP	Hx	HxR
1	37.5	37.5	87.5	37.5	37.5	37.5
2	75.0	75.0	175.0	75.0	75.0	75.0
3	150.0	150.0	350.0	150.0	150.0	150.0
4	300.0	300.0	700.0	300.0	300.0	300.0
5	3,000.0	1,200.0	1,400.0	600.0	600.0	600.0

In order to avoid environmental bias, three HPLC analyses of the same standard sample within 24 hours were needed, and the relative standard deviation (RSD) was also calculated for precise confirmation of HPLC analysis. The calculation of RSD value is

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} \tag{2}$$

$$S = \left[\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1} \right]^{\frac{1}{2}} \tag{3}$$

$$\text{RSD value} = \frac{S}{\bar{x}} \times 100\ \% \quad (4)$$

where $\bar{x}$, S, and n denote as mean, standard deviation, and the number of $\{ x_i \}$, respectively.

2.4.5 K value Calculation

K value and nucleotide concentration are calculated by following equations:

$$K\ value = \frac{[HxR] + [Hx]}{[ATP] + [ADP] + [AMP] + [IMP] + [HxR] + [Hx]} \times 100 \quad (5)$$

Where [*Hx*], [*HxR*], [*ATP*], [*ADP*], [*AMP*], and [*IMP*], are treated as the concentrations of Hx, HxR, ATP, ADP, AMP, IMP, and Inosine, respectively.

2.5. Data Analysis

2.5.1. Excitation-Emission Matrix

The fluorescence amplitudes of specific excitation wavelengths were saved as an ASCII text file. The data were retrieved as a two-dimensional plot of EEM for distinguishing the variability of the fluorescence spectra clearly. Because of the line scan property of the Y-type fiber-optic measurement system and the unstable power output of the xenon lamp, every fluorescence waveform was normalized by dividing by the amplitude of (exci+30) nm emission wavelength.

2.5.2. Optical index

In order to investigate the relationship between fluorescence spectra and fish freshness,

we define an optical index for further multivariate analysis. The optical index value, $G(emi, exci)$, is:

$$G(emi, exci) = \frac{F(emi, exci)}{F(exci + 50, exci)} \quad (6)$$

where *F(emi, exci)* denotes the fluorescence amplitude of a certain excitation wavelength (*exci* nm) and emission wavelength (*emi* nm), and *F(exci+50, exci)* denotes the fluorescence amplitude of a certain excitation wavelength (*exci* nm) and emission wavelength (*exci*+50 nm).

2.5.3. Time variant analysis for optical index

One principle of ANOVA is to evaluate the experimental error. When the effect of an independent variable has much more influence than a dependent variable on an experimental error, we assume the result of dependent variable have significant difference by different independent variable. The confirmation of the optical index was made by using analysis of variance (ANOVA) of SPSS 17.0 (SPSS Inc., Chicago, USA) for evaluating the variation of fluorescence over time. To investigate all the time points, a post hoc test was performed. Sum of squares between groups (SS_B) means the variance between different groups: $SS_B = \sum_{i=1}^{k} n_i(\bar{x}_i - \bar{x})^2$, $\bar{x}_i$ is the mean of i[th] class, $\bar{x}$ is the mean of all; sum of square due to error (SS_E) means the variance in one class : $SS_E = \sum_{i=1}^{k}\sum_{j=1}^{n_j} n_i(x_{ji} - \bar{x}_i)^2$, x_{ji} is the values of number j^{th} element in number i[th] class, $\bar{x}_i$ is the mean of all elements in i[th] class. Then,

calculate Mean Square (MS): $MS_i = \frac{SS_i}{(D.F.)_i}$, where $D.F.$ means the degree of freedom. By calculating F value, the differentiation between different classes can be found out.

Following is the equation:

$$F = \frac{MS_B}{MS_E} = \frac{\frac{SS_B}{K-1}}{\frac{SS_E}{n-K}} \tag{7}$$

where MS_B is mean square between groups, MS_E is mean square due to error, K is the number of all classes, and n is the number of all elements in one class.

If F is larger, it presents the more dispersed data. When F is large enough, it presents the significant difference between two classes.

2.5.4. The relationship between optical index and K value

Pearson correlation analysis was used because of the interval scale of our experiment. Pearson correlation results show the linear correlation coefficient between two continuous variables. The correlation coefficient of r value is between -1 and 1. The negative and positive are to distinguish the negative and positive correlation. However, the result can only represent the correlation but not the cause-and-effect relationship. Following is the equation of Pearson correlation:

$$r = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2}\sqrt{\sum_{i=1}^{n}(y_i - \bar{y})^2}} \tag{8}$$

where x_i is i[th] of x variables, $\bar{x}$ is the mean of x variables, y_i is i[th] of y variables, $\bar{y}$ is the mean of y variables. We analyze every fishes sample by using LabVIEW 2011.

III. Experiment Result

3.1. Optical fluorescence results

3.1.1. The results for cobias

Fig. 7 shows the excitation-emission matrix (EEM) from a measurement of fish tissue in the abdomen. EEM consists of hundreds of measurement combinations of a single fish fillet sample, with excitation wavelength on one axis, emission wavelength on the second, and fluorescence intensity forming a third axis presented by color. With building up EEM from the fluorescence by scanning and recording a group of individual emission spectra, the fluorophores can be clearly indentified. From the results of EEM, fluorescence was observed throughout the whole collection range, with two peaks (excitation wavelength/emission wavelength) located at 340/430 nm and 340/470 nm. A ridge extended from 360/400 nm to 350/600 nm). A valley was seen between 440 and 460 nm emission wavelengths. The changes were observed between 330 and 360 nm excitation wavelengths in 12 hours, so their emission spectra was extracted to analyze the relationship between fluoresce intensities and refrigeration time.

It was the same for the dorsum samples with two peaks located at the 340/430 nm (excitation wavelength/emission wavelength) and 340/470 nm wavelengths and changes between 330 and 360 nm excitation wavelengths in several hours.

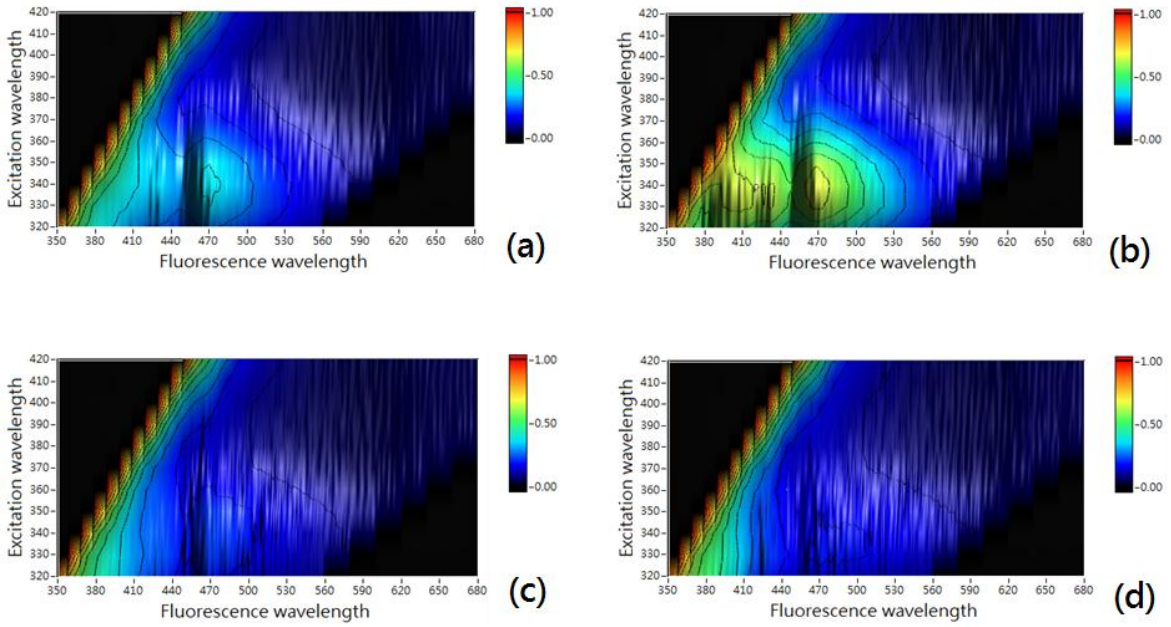


Fig. 7 depicts abdomens of cobia at different time points: (a) 0 hour, (b) 6 hours, (c) 12 hours, and (d) 18 hours.

The optical index is presented by the value of $G(emi, exci)$, which is set up by the equation (5). It presents that the indexes of *G(470,exci)* and *G(430,exci)*, where *exci* are 330 nm, 340 nm, 350 nm, and 360 nm, have relations with storage time. The labeled vertical error bars indicate the standard deviations of the mean. The results illustrated that the G value decreased by increasing refrigeration time. In addition, the large drop heights appeared between 6 and 12 hours at different excitation wavelengths. The statistical comparison of G values at different refrigeration times was also calculated through the tests of within-subject effects. The compared results indicated that the ratio values at 0, 6, 12, and 18 hours were significantly different ($p < 0.05$) under the same excitation wavelength. Furthermore, the post-hoc analysis was used to investigate the details of the relation between the refrigeration times. The results presented that the ratio values of abdomen specimens at 0 hour were no different with the ratio values of abdomen specimens at 6 hours, whereas the ratio values of abdomen specimens at 0 hour were significantly different ($p < 0.05$) at 12 and 18 hours under the same excitation wavelengths of 320, 330, 340, and 350 nm. The ratio values of abdomen

specimens at 6 hour were significantly different ($p < 0.05$) with these at 12 and 18 hours too. Nevertheless, in summary, the mean of the G value has a descending trend with refrigeration time in the eight cobias.

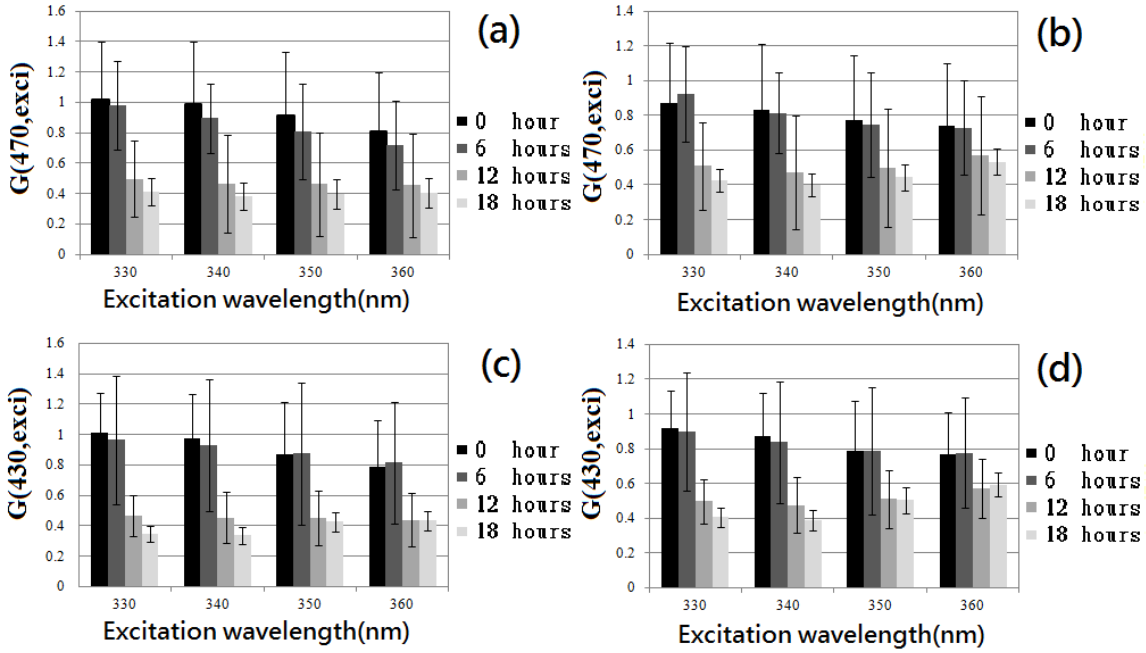


Fig. 8 shows a summary of the fluorescence ratios of eight cobias excited by 330, 340, 350, and 360 nm of excitation wavelengths: (a) *G(470,exci)* in abdomen, (b) *G(470,exci)* in dorsum, (c) *G(430,exci)* in abdomen, and (d) *G(430,exci)* in dorsum samples.

3.1.2. The results for Seriola dumerili

From fig. 9, fluorescence is observed almost at the same location as the cobias, with two peaks located at the 340/430 nm (excitation wavelength/emission wavelength) and 340/470 nm wavelengths. The descending trend of fluorescence intensity is observed between 330 and 360 nm excitation wavelengths within several hours from EEM. However, the peak intensity is weaker than for cobias. Nevertheless, the trend of fluorescence for Seriola dumerili is almost the same as for cobias. Likewise, the dorsum samples were almost the same as the

abdomen samples.

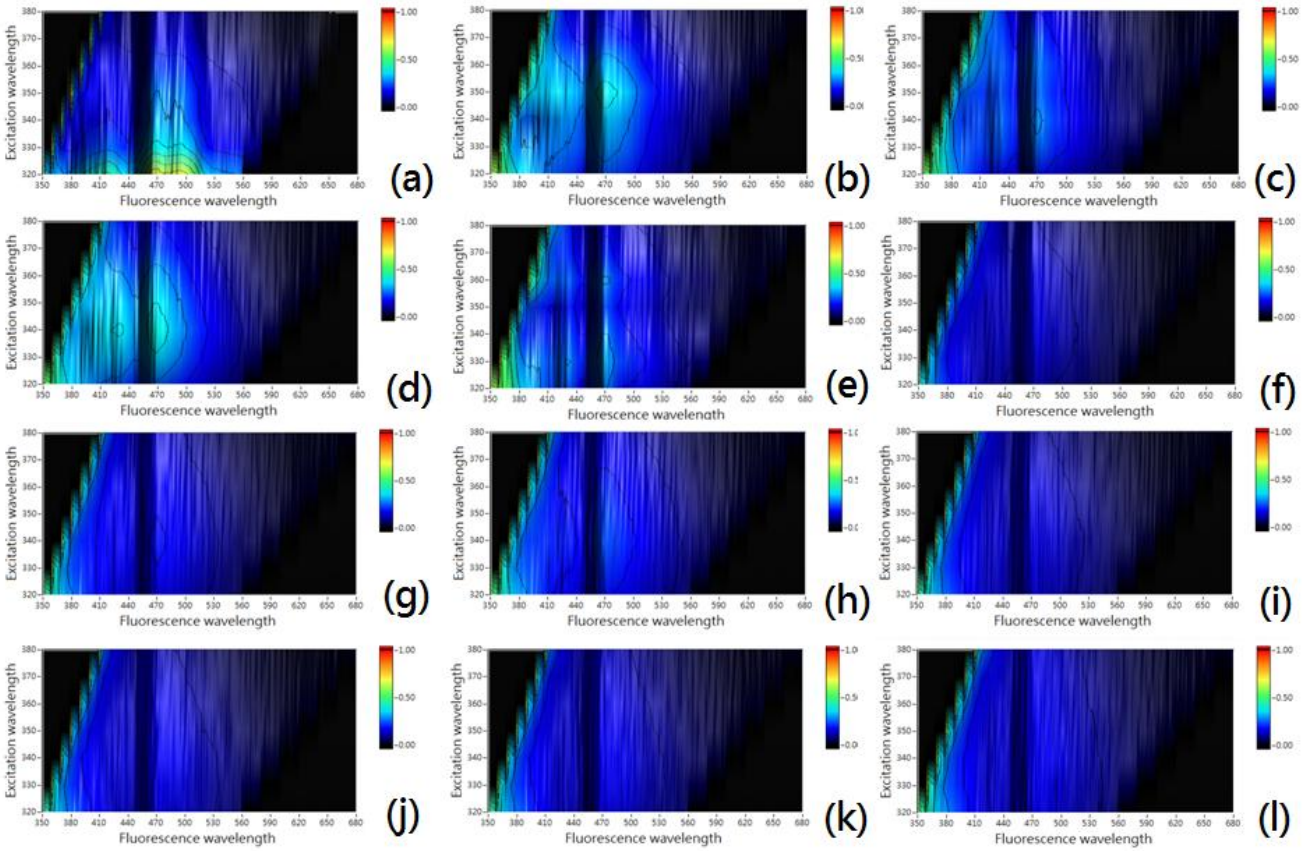


Fig. 9 shows abdomens of Seriola dumerili at different refrigeration time points: (a) 0 hour, (b) 2 hours, (c) 4 hours, (d) 6 hours, (e) 8 hours, (f) 10 hours, (g) 12 hours, (h) 14 hours, (i) 16 hours, (j) 18 hours, (k) 20 hours, and (l) 22 hours.

The optical index, as previously thought, presents that the refrigeration time (0 to 24 hours) was related to *G(470,exci)* and *G(430,exci)* at 330 nm, 340 nm, 350 nm, and 360 nm excitation wavelengths. The labeled vertical error bars indicated the standard deviations of the mean. The results illustrated that the value decreased by increasing refrigeration time. In addition, the range of G index is approximately from 1.5 to 0.5, which is approximately the same range as for the cobia. The large drop heights appeared at initial time points at every excitation wavelength. However, the trend becomes smooth after several hours.

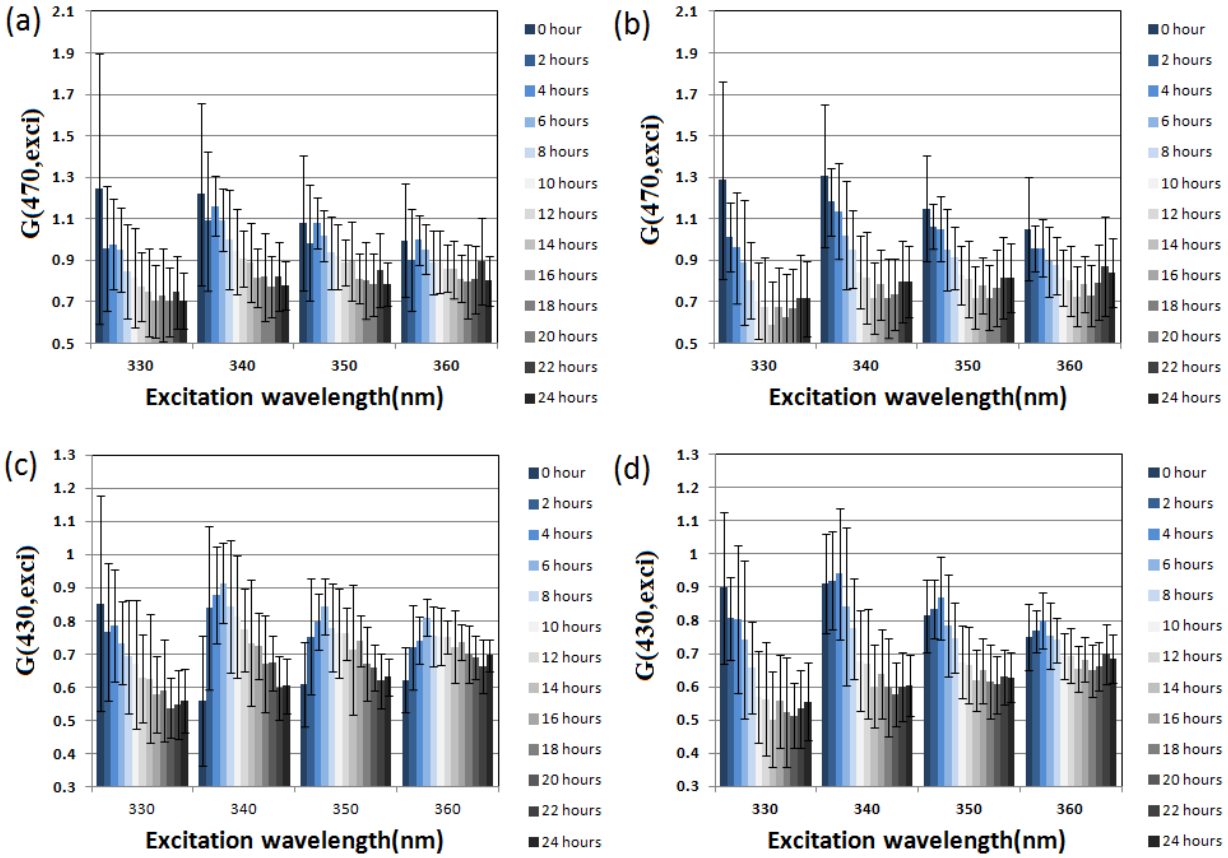


Fig. 10 depicts a summary of the fluorescence ratios of eight Seriola dumerrili excited by 330, 340, 350, and 360 nm of excitation wavelengths and for cobias, respectively: (a) *G(470,exci)* in abdomen, (b) *G(470,exci)* in dorsum, (c) *G(430,exci)* in abdomen, and (d) *G(430,exci)* in dorsum samples.

3.2. Chemical results for Seriola dumerili by HPLC

3.2.1. Recovery of ATP degradation products

The recovery of extraction samples was around 85 to 90 %, which was acceptable for our extraction process.

Table 6 The recovery (%) of six ATP degradation products

Spike level(mg/L)	IMP	ATP	ADP	AMP	Hx	HxR
1000	87.6	89.3	92.1	91.2	87.4	90.1

3000	88.7	86.4	87.6	87.3	88.9	91.6

3.2.2. The results by HPLC

Fig. 11 shows the six ATP degradation standard products by HPLC, the retention time of IMP, ATP, ADP, AMP, Hx, and HxR are approximately 6.55, 8.29, 9.0, 10.0, 10.28, and 12.4 minutes respectively. The checking-measuring curve is shown in fig. 12. It presents the linear correlation of the integral area and the concentration between the maximum and minimum standard concentration. The concentration range of fish samples are in the range of a standard curve. Also, the R^2 values are all around 0.99 to indicate the great linear regression line of the standards. The standards are executed three times in a day to confirm the stability of our HPLC detection in different periods of time. The values are around 5 ± 1 to indicate the precision of our detection.

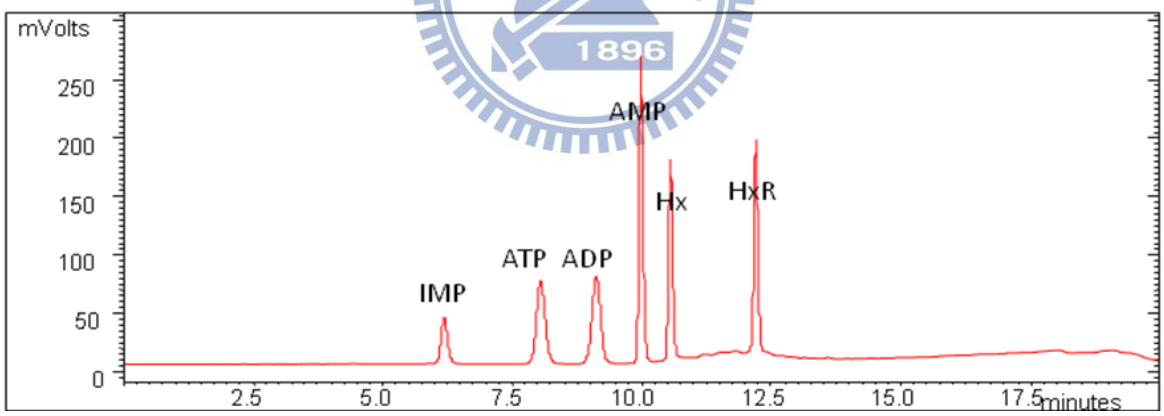


Fig. 11 shows the six nucleotide standards (ATP, ADP, AMP, IMP, Hx, and HxR) separated by HPLC.

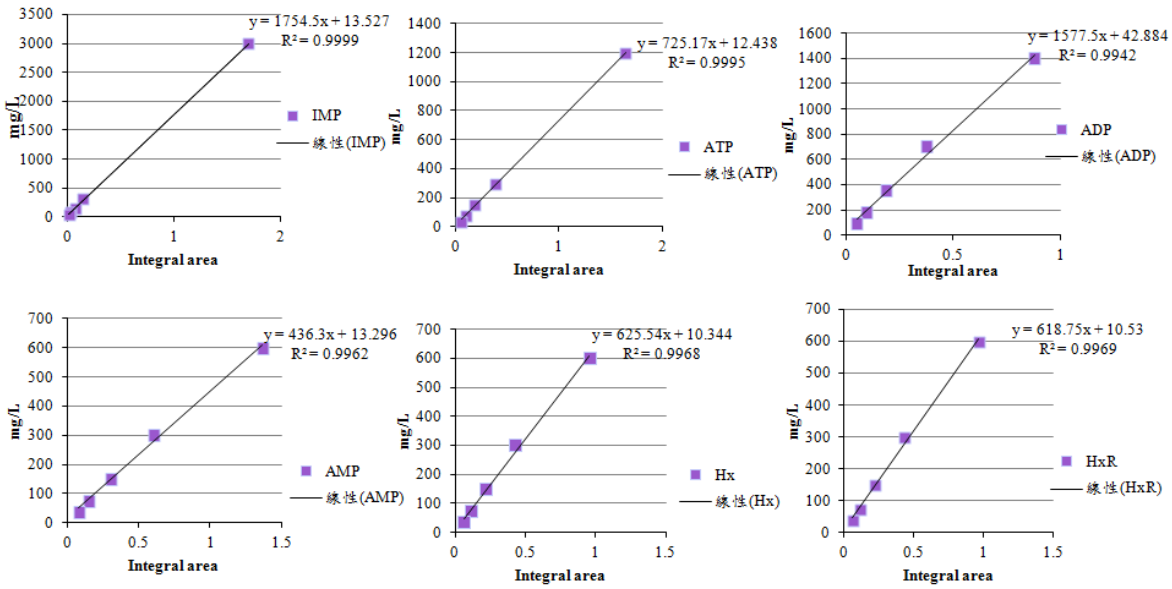


Fig. 12 depicts the checking measuring curve of the six ATP degradation products.

Table 7 The R^2 value of checking measuring curve of the six ATP degradation products

Components	IMP	ATP	ADP	AMP	Hx	HxR
R^2 value	0.9966	0.9964	0.9942	0.9962	0.9968	0.9969

Table 8 The RSD (%) of HPLC detection during one day

Compounds	mg/L	RSD(%)	Compounds	mg/L	RSD(%)
IMP	37.5	4.87	AMP	37.5	4.58
	75	3.48		75	4.67
	150	4.79		150	4.11
	300	4.72		300	4.65
	3000	4.15		600	3.21
ATP	75	5.32	Hx	37.5	4.93
	150	4.93		75	3.87
	300	3.46		150	4.67
	600	4.35		300	4.12
	1200	4.41		600	4.89

ADP	**87.5**	**4.12**	**HxR**	**37.5**	**3.74**
	175	**4.63**		**75**	**5.16**
	350	**3.69**		**150**	**5.21**
	700	**5.52**		**300**	**3.78**
	1400	**3.87**		**600**	**4.53**

3.2.3. The results for Seriola dumerili by HPLC

Fig. 13 shows the HPLC results of our Seriola dumerili samples. The six ATP degradation products can be determined by the retention times. The figure presents the high concentration of IMP; the degradation of ATP, ADP, and AMP; and the increase of Hx and HxR. In summary of all the results, we find that the eight fish subjects differ from fish to fish for the metabolism rates in postmortem (fig. 13). Some fishes started the decomposition of ATP, ADP, and AMP earlier, and it caused the earlier accumulation of IMP. Because of the accumulation of IMP, it may degrade to HxR and Hx faster after the fish die. The difference in these fishes may be caused by factors such as how they are cultivated, how they are treated, how they were slaughtered, and so on. However, the HPLC result confirms the different metabolisms between different subjects and the freshness of all the Seriola dumerili.

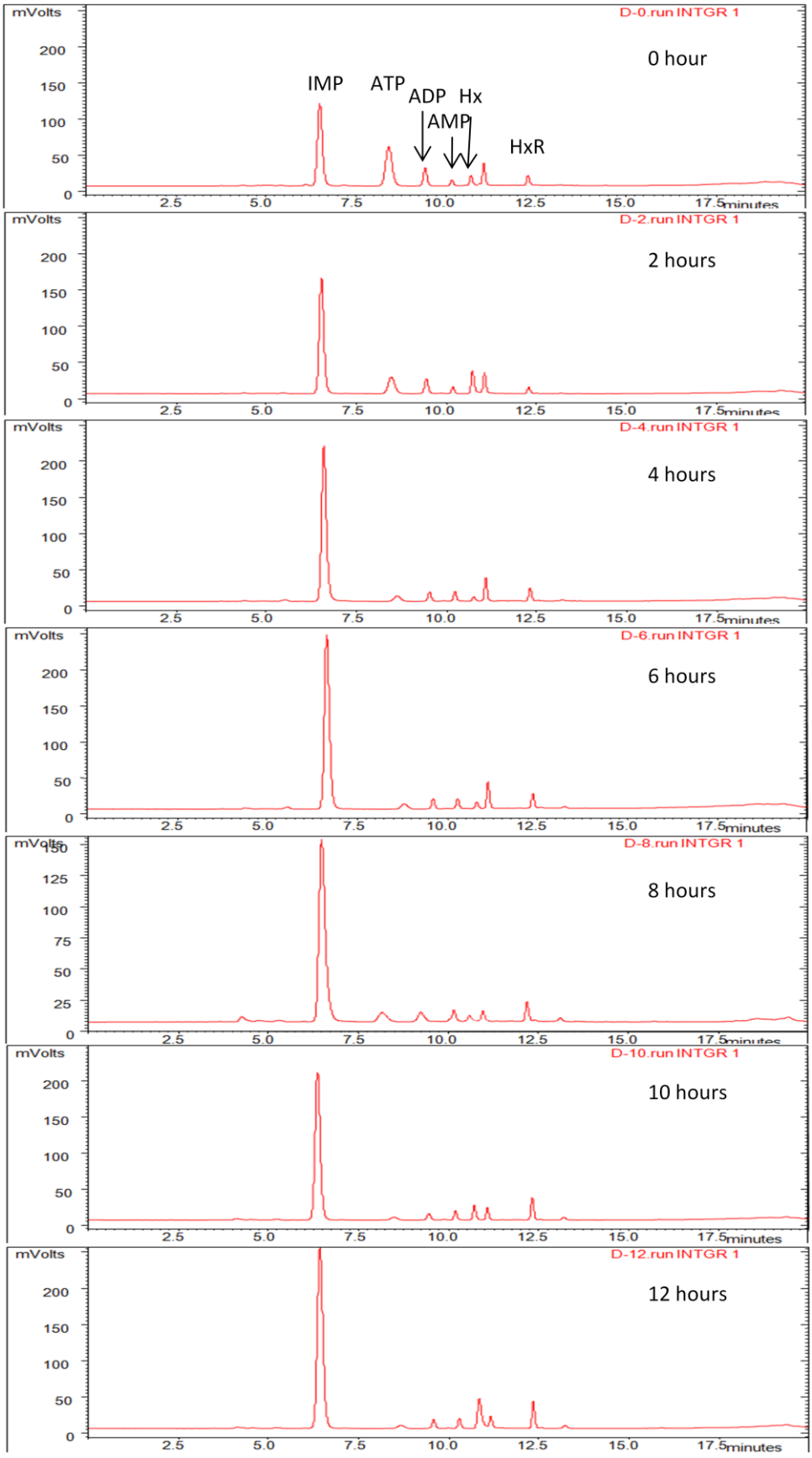


Fig. 13 shows the HPLC results of fish tissue extracts in the abdomen from 0 to 12 hours of storage time.

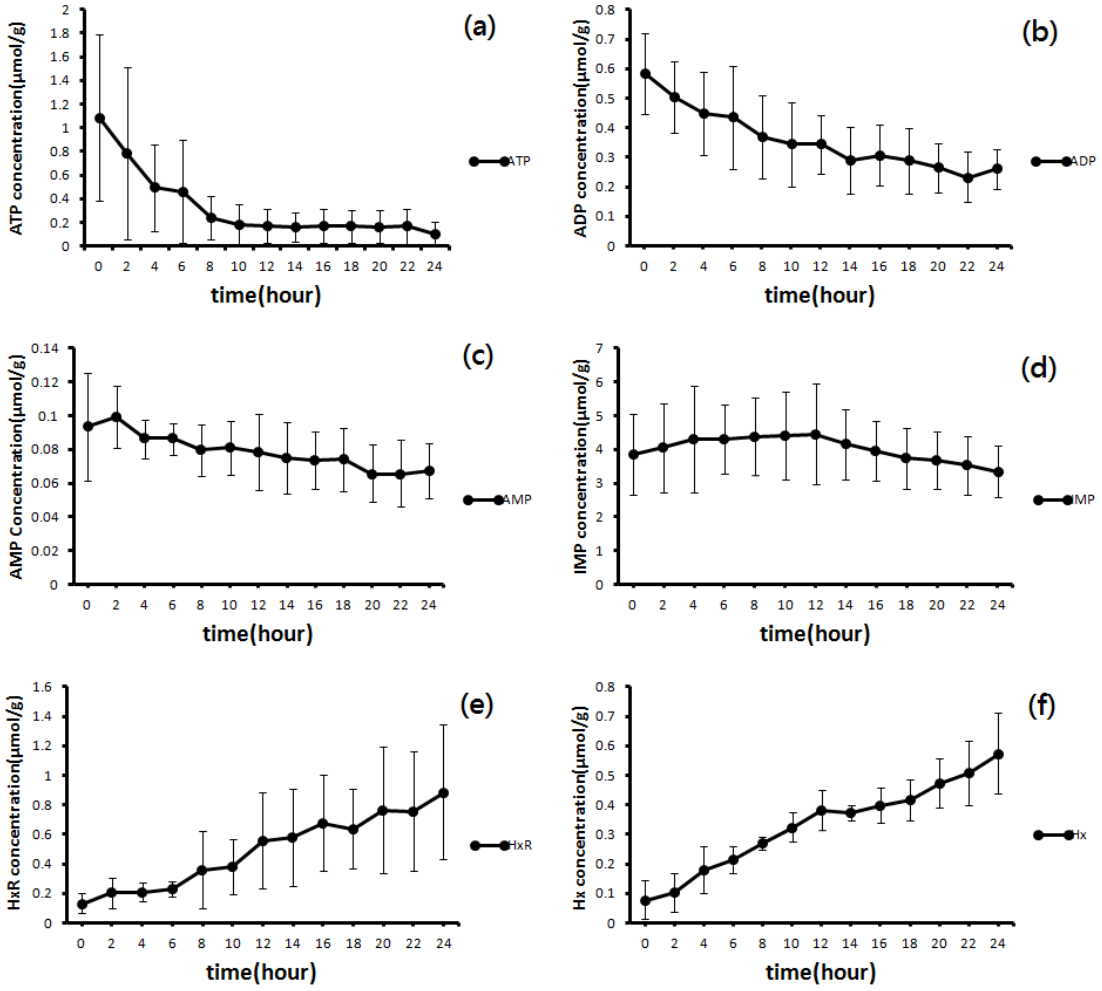


Fig. 14 summarizes the six nucleotides of eight abdomen samples of Seriola dumerili: (a) ATP, (b) ADP, (c) AMP, (d) IMP, (e) HxR, and (f) Hx, which vary with refrigeration time.

3.2.4. The K value of eight Seriola dumerili

The K value results of eight Seriola dumerili on average represent an ascending trend from 0 to 24 hours, and we find they are different for freshness. It has been proven that the different decomposition rates may affect the fish freshness, which could be caused by the earlier ATP degradation [27, 28]. Because of earlier degradation of ATP, it could cause the metabolic process of the fish muscle to proceed faster and result in earlier spoilage.

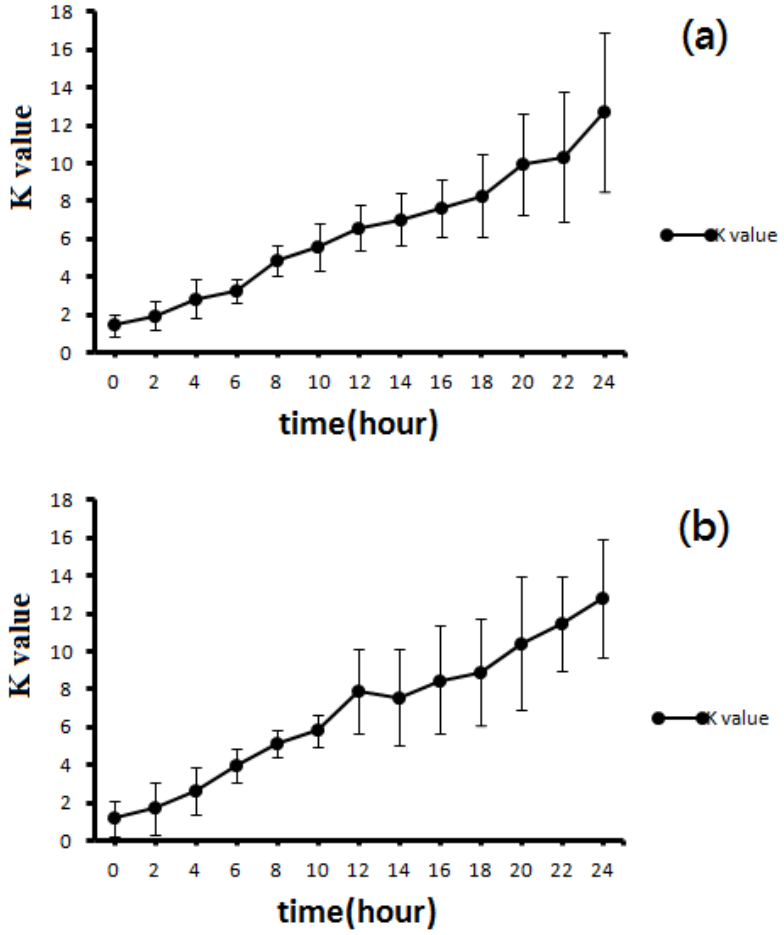


Fig. 15 summarizes the K values of eight Seriola dumerili in the (a) abdomen and (b) dorsum, which vary with refrigeration time.

3.3. The correlation between optical index and K value

For the differences in the eight samples of Seriola dumerili, the G indexes that we develop are compared with K value. Table 7 and Table 8 present the abdomen and dorsum results of the correlation coefficient between the value of optical index and K value at the same refrigeration time point (0 to 24 hours) of eight Seriola dumerili. We find that the r values of correlation coefficient represent a negative correlation between the two indexes (G indexes and K value) of approximately -0.8±0.1 for the eight Seriola dumerili. This suggests

that 340 nm excitation wavelength can be the best level for the most negative correlation coefficient with K value. For most of the linear correlations from the eight Seriola dumerili, the r^2 value is approximately in the range of 0.6 to 0.7. However, there were less clear negative correlations in some fish samples. It was assumed that they ran out of NADH and collagen in the initial stage after the fish died. The descending G index could present a process of rigor mortis and tenderization of muscle. Depending on the specific condition affecting the fishes, the descending G index may result in losing the correlation to the K value and could cause the K value to ascend faster. However, the value of G can indicate the stage of fish tissue in postmortem. The G index could confirm the early stage of fish tissue after a fish has died.

Table 9 Summary of the eight abdomens of Seriola dumerili for correlation between optical index and K value

Wavelength (nm)		**Correlation coefficient of i^{th} fish (G index V.S. K value)**							
Emi.	**Exci.**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
470	**330**	-0.755	-0.694	-0.851	-0.827	-0.564	-0.798	-0.276	-0.269
	340	-0.833	-0.792	-0.886	-0.786	-0.733	-0.813	-0.292	-0.258
	350	-0.816	-0.804	-0.866	-0.626	-0.657	-0.814	-0.006	-0.375
	360	-0.711	-0.763	-0.740	-0.565	-0.436	-0.809	0.032	-0.053
430	**330**	-0.683	-0.718	-0.834	-0.800	-0.684	-0.733	-0.495	-0.307
	340	-0.749	-0.689	-0.865	-0.725	-0.806	-0.716	-0.515	-0.536
	350	-0.686	-0.642	-0.869	-0.390	-0.797	-0.721	-0.416	-0.368

	360	-0.554	-0.586	-0.700	-0.368	-0.682	-0.678	-0.102	-0.025

Table 10 Summary of the eight dorsums of Seriola dumerili for correlation between optical index and K value

Wavelength (nm)		**Correlation coefficient of i^{th} fish (G index V.S. K value)**							
Emi.	**Exci.**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
470	**330**	-0.651	-0.771	-0.825	-0.634	-0.531	-0.749	-0.604	-0.487
	340	-0.776	-0.843	-0.883	-0.695	-0.736	-0.813	-0.651	-0.250
	350	-0.695	-0.804	-0.847	-0.680	-0.660	-0.849	-0.612	-0.024
	360	-0.380	-0.751	-0.699	-0.570	-0.451	-0.849	-0.525	0.503
430	**330**	-0.771	-0.834	-0.845	-0.613	-0.684	-0.758	-0.630	-0.503
	340	-0.859	-0.820	-0.867	-0.670	-0.834	-0.808	-0.684	-0.371
	350	-0.803	-0.799	-0.890	-0.632	-0.827	-0.817	-0.672	-0.173
	360	-0.495	-0.751	-0.804	-0.459	-0.615	-0.773	-0.594	0.473

IV. Discussion

4.1 The fluorophores of EEM spectra

We have found the spectrum that represents the concentration of components, such as collagen type I, collagen type V, and NADH [25]. NADH plays an important role for supplying and transferring energy to living creatures. When a fish dies, NADH can be generated by glycolysis, and it can maintain the NADH concentration in the fish body. After the fish tissue is acidified, which is caused by the accumulation of many lactic acids, the glycolysis of the fish muscle stops and no longer generates NADH or ATP. NADH and ATP will be oxidized to NAD^+ and ADP. During this time, the muscle starts rigor mortis, and the concentration of NADH in the fish tissue has descended, causing the ATP to degrade into ADP. We can conclude that the descending of NADH means the start of rigor mortis of fish muscle. Collagen type I and type V have been proven to participate in the tenderization of tissue in postmortem and are components of muscle structure [29, 30]. In the change of the postmortem fish muscle structure, the collagen in the connective tissue degrades and transforms. The transformation of the collagen structure participates in the process of muscle proteolysis after a fish has died. Collagen is the main constituent of this matrix, which is responsible for the integrity of the myocommata and the mechanical properties of the muscle. At the time of collagen decomposition, it softens fish tissue. However, it cannot be proven which fluorophores contribute a higher percentage to the spectrum. Regardless, for different

species (cobias and Seriola dumerili) and body parts (abdomen and dorsum), we are sure that both collagen and NADH break down in the process of fish spoilage. The NADH and collagen participate in the postmortem of rigor mortis and the tenderization of fish muscle. The spectrum that we have established can be applied as a freshness or quality index to determine the condition of fish muscle.

4.2 The optical index G

The optical index is built up by the fluorescence intensity of the 470 and 430 nm emission wavelengths at 330 to 360 nm excitation wavelengths. After the process of normalization, we have found that both *G(470,exci)* and *G(430,exci)* have a descending trend with storage time. The range of G index is approximately from 1.5 to 0.5. However, it is different from fish to fish. The optical index value has a large standard deviation in both cobias and Seriola dumerili after several hours of storage time. In some fish cases, we do not get the clear descending result. We consider that there are many factors affecting the experiment result, e.g., the growing environment, temperature, slaughter method, and experimental error [27, 28, 31]. In summary, for the eight Seriola dumerili, on the whole, the value of *G(470, exci)* and *G (430, exci)* are negatively correlated to the K value within 24 hours. The correlation can be established only when it can be ensured that the fluorophores do not initially degrade. If the fluorophores initially degrade, the descending trend of the G index with storage time is less obvious, which causes a less negative correlation with K value.

However, the lower value of G means the rigor mortis and tenderization have occurred earlier than normal, and therefore the fish is less fresh. Thus, with the value of the G index, we can confirm the condition of the fish muscle, the postmortem process period that the muscle is in, and even the freshness within 24 hours after the fish has died.

4.3 The slaughter methods for spectra

It has been proven that as a fish loses energy, the fish quality declines and results in a higher rate of spoilage [32]. It has been proven that when fish are frightened or experience tension, NADH and collagen could degrade faster [27]. My results also confirm that fish freshness is affected by these conditions. Fig. 16 shows that when fish lose energy due to treatment with ice water, the fluorophores of NADH and collagen initially degrade faster, and the G index shows a less descending trend.

There are many factors that could affect the optical index, including temperature, harvest methods, season, aquaculture condition, and so on. Some of the factors relate to the natural condition of the fish; some depend on how the fish is treated after harvesting. The value can confirm the postmortem condition of the fish tissue and can be used to validate the quality of the fish.

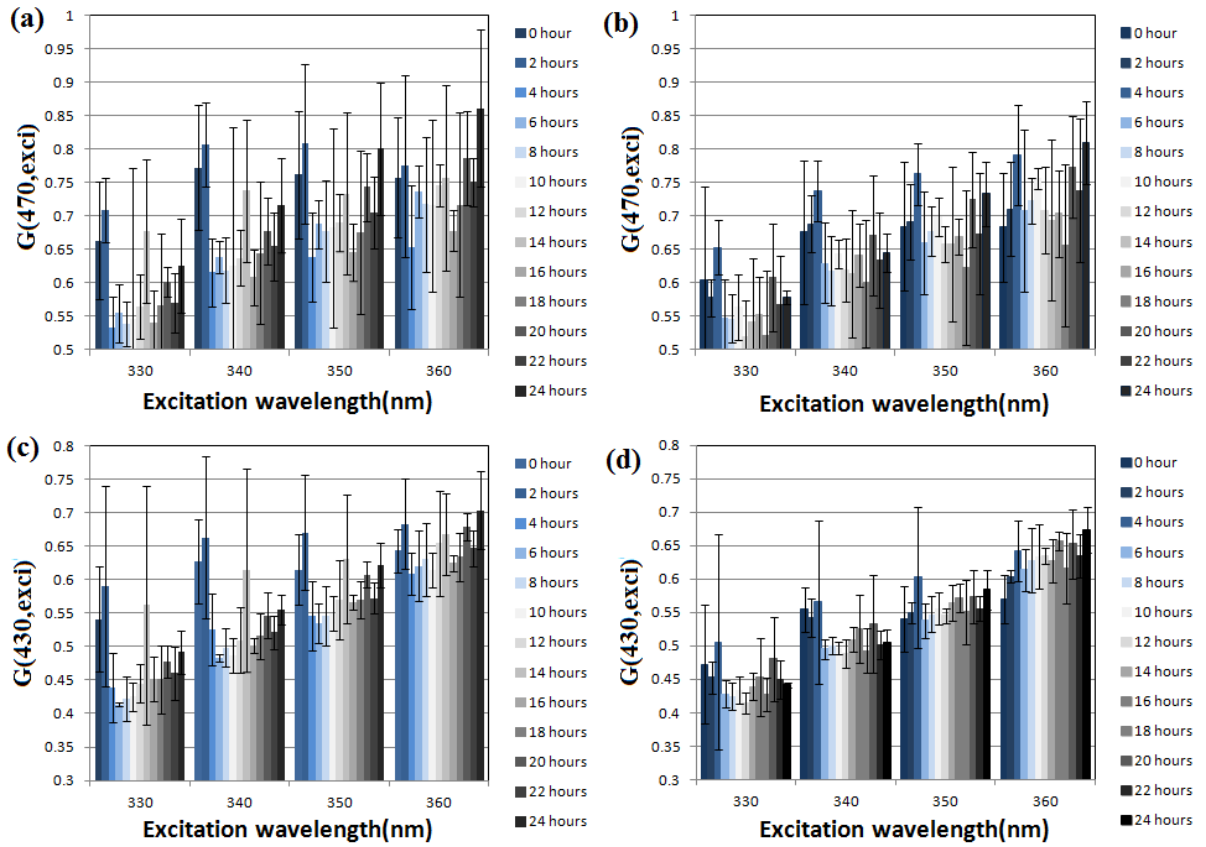


Fig. 16 summarizes the fluorescence indexes of two Seriola dumerili that experienced tension due to low temperatures induced by ice measured with 330, 340, 350, and 360 nm wavelength light sources: (a) *G(470,exci)* in abdomen, (b) *G(470,exci)* in dorsum, (c) *G(430,exci)* in abdomen, and (d) *G(430exci)* in dorsum samples.

4.4 The changes of other fluorophores in fish tissue within 24 hours of refrigeration

In addition to the direct observation from EEM, the fluorophores are also confirmed by principle component analysis of eight Seriola dumerili. Moreover, this study tries to find other fluorophores that are related to freshness by using PCA to extract the main fluorophores. Other studies have suggested that aromatic amino acid (tryptophan, tyrosine), ATP, NADH, and collagen are related to meat freshness [22]. However, for fresh fish, they needed to be investigated, which was appropriate for this study. The fluorophores of amino acid, ATP, collagen, and NADH were found by PC1 and PC2 with PCA with all the eight Seriola

dumerili. The three groups (330 nm, 280 nm, and 290 nm excitation wavelengths) of PC1 and PC2 account for more than 95 percent of all the components. Fig. 17 (a) and (b) show the main components of 470 and 430 nm emission wavelengths could be the fluorophores of NADH and collagen by 340 nm excitation wavelength, with all the fluorescence signals of Seriola dumerili loading in. Fig. 17 (c) and (d) depict the 390 nm emission wavelength, which could be the component of amino acid at 280 nm excitation wavelength, with all the fluorescence signals of Seriola dumerili loading in. In Fig. 17 (e) and (f), the 350 to 400 nm emission wavelengths could be ATP on the 290 nm excitation wavelength, with all the fluorescence signals of Seriola dumerili loading in.

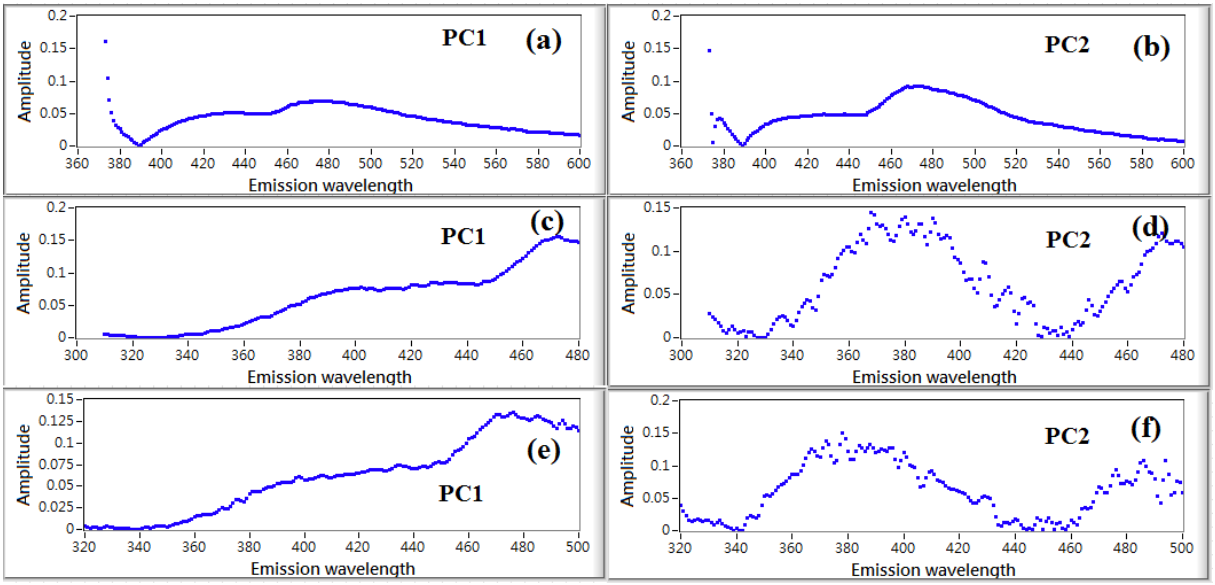


Fig. 17 depicts eight Seriola dumerili analyzed by PCA: (a) and (b) are PC1 and PC2 excited by 280 nm; (c) and (d) are PC1 and PC2 excited by 340 nm; and (e) and (f) are PC1 and PC2 excited by 290 nm.

However, it is most important to confirm which components could change to the largest degree within a short time so that the spectrum can act as a freshness or quality index. From Fig. 18, the results of PC1 show that NADH and collagen sharply decrease within 24 hours. However, at the 280 and 290 nm excitation wavelengths, it is hard to distinguish any

difference for amino acid and ATP within 24 hours. It could be that the levels of amino acid do not change a lot within a short time after the fish has died, or it could be that there are too many fluorophores in this area to balance the difference. In sum, we conclude that the fluorophores of NADH and collagen can be great biomarkers for freshness.

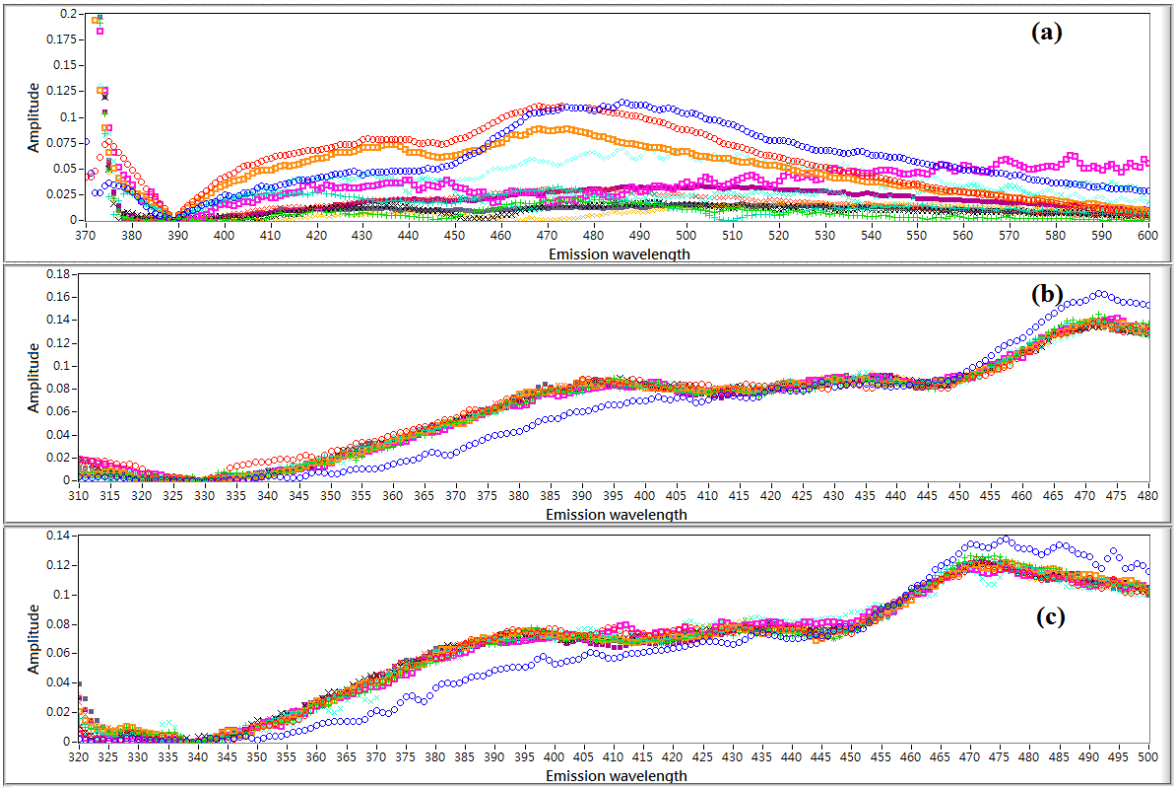


Fig. 18 shows the PC1 extracted by fluorescence from 0 to 24 hours storage time at (a) 340, (b) 280, and (c) 290 nm excitation wavelengths.

4.5 The white and red fish meats

Cobias and Seriola dumerili are white fish species, and glycolysis of the fish muscle is more severe for them than for red fish species. After white fish die, the muscles maintain the ATP concentration longer than red fish. The ATP concentration in white fish slows down the process of rigor mortis, tenderization, and even spoilage. Because of this, red fish become stale at a faster pace. In this study, cobias and Seriola dumerili were measured to analyze the spectra from tissue. However, we can speculate that the change of G index might be sharper

for red fishes because of their faster metabolism.

4.6 The problems in this study

In this study, actually, a total of 20 fish were tested. However, because of the unstable excitation power for the xenon lamp and because there were other spectra areas that we tried to test, the study was limited to eight fish subjects. In future work, we might use a laser as a power source for its stability and its concentration of energy. In addition, the delay for several minutes between slaughtering the fish and performing the initial measurements could have resulted in missed information related to the fish tissue.

Also, in this study, a Y type fiber was adopted to receive the fluorescence. Although fixing the fiber directly to the tissue surface might have prevented errors related to hand movements, the fluorescence received was only from a small point of fish tissue. This was done to reduce the non-homogeneous effect of fish tissue, but separating and measuring more parts of fish tissue could be beneficial in future research.

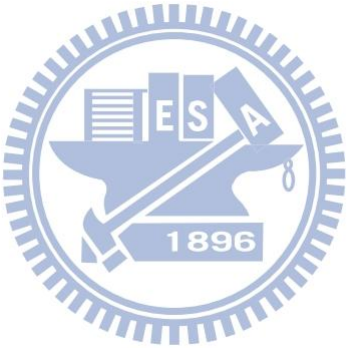
4.7 To develop a portable detector for fish freshness

4.7.1. The potential of nitrogen laser as excitation power

The 337 nm wavelength of a nitrogen laser would be a proper power source for a fish freshness detector. Nitrogen lasers have been proven to cause little destruction to tissue [10]. With the less invasive character of a nitrogen laser, it could be commercialized for the development of a portable instrument.

4.7.2. The usage and problem of G index for freshness

From my study, the sliced fishes were measured to analyze the spectrum. However, for now, it only can be applied as the freshness detector of sashimi or other fish fillets as a quick-detection method. The challenge is to investigate the change of spectra of fish muscles beyond spot measurements. By confirming the spectra change of muscle in the whole fish and developing a standard, the method can be applied as a faster and less invasive detection method.



V. Conclusion

5.1 The optical index

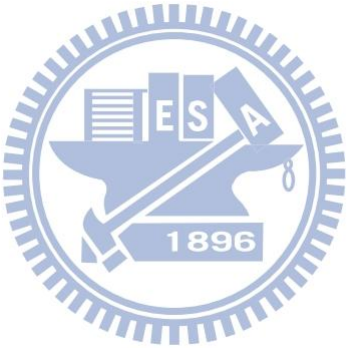
The fluorescence spectrum can present the concentration of type I collagen, type V collagen, and NADH. NADH is the energy source of the live body. When fish die, the fish muscles start glycolysis to maintain the energy of the fish muscles. After the fish muscle is acidified by the accumulation of many lactic acids from glycolysis, it stops generating NADH or ATP. At this point, the NADH may degrade. Also, it has been proven that collagen is related to the resolution of muscle. Collagen is a component of fish muscle structure and has been proven to participate in the tenderization of muscle. When collagen is transformed, the texture of fish may soften, which indicates the breakdown of muscle structure. NADH and collagen are the materials that maintain the normal metabolism and structure of muscle, and they may degrade soon after a fish dies. Thus, they play important parts in the early process of fish putrefaction. From this study, two species of fish and a total of 16 fish samples were analyzed. The G index value that we developed might descend from approximately 1.5 to 0.5 with 24 hours of refrigeration with an ascending K value. Therefore, based on the G index we developed from the fish, the condition of the fish can be confirmed. If the G index of the fish is higher, the fish could be assumed as a good quality or fresh fish which has longer expiry date. On the contrary, if the G index of the fish is lower, the fish can be assumed as a bad quality or “less fresh” fish which has shorter expiry date. In other words, we can use the G index to validate freshness in the early stages of fish putrefaction, and it can act as a measure of taste and freshness of sashimi or fish fillets.

5.2 The application of the index

For the light source for the optical index, the nitrogen laser has been commercialized for many years and is appropriate for our optical system. Also, because of the laser's less destructive wavelength to tissue [10], the index has great potential for freshness detection [33]. The index can be used to monitor the quality of sashimi in restaurants or for quality control in the aquaculture market. Restaurants and the aquaculture market have strong operation performance standards and are capable of applying these indexes. With the G indexes, they can detect the freshness of fish faster.

VI. Future Work

Up to now, we have only used 1D signals to analyze the EEM signal. However, 2D matricess of EEM may contain more information that we have not found. A 2D algorithm has faster computation and is more appropriate for 2D signal processing. It is a method to analyze the EEM spectrum for other fluorophores. Also, with a 2D algorithm, maybe more target fluorophores can be discovered. Second, the amount of fish data is not enough to prove how the index works with other fish species. Accumulating more data of other fish species is necessary. Finally, if the optical index is to be applied in the food or restaurant industries, we need to find more useful indexes and combine them to build a valuable portable instrument.



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Appendix A

Abbreviation	Full name
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
EEM	Excitation emission matrix
HPLC	High performance liquid chromatography
Hx	Hypoxanthine
HxR	Inosine
IMP	Inosine monophosphate
NADH	Nicotinamide adenine dinucleotide
PCA	Principle component analysis
PMT	Photomultiplier tube

Appendix B

Function	Description
G(emi.,exci)	$G(emi, exci) = \frac{F(emi, exci)}{F(exci + 50, exci)}$ where *F(emi, exci)* denotes the fluorescence amplitude of certain excitation wavelength (*exci* nm) and emission wavelength (*emi* nm) and *F(exci+50, exci)* denotes the fluorescence amplitude of certain excitation wavelength (*exci* nm) and emission wavelength (*exci*+50 nm). The G index is developed as a optical index for freshness.
K value	$K\ value = \frac{[Hx] + [HxR]}{[ATP] + [ADP] + [AMP] + [IMP] + [Inosine] + [Hx]} \times 100\%$ where [ATP], [ADP], [AMP], [IMP], [HxR], [Hx] mean the concentration of themselves. In most of fish, K values increase linearly during the first days of refrigeration storage and it is an index of freshness detection. When K value is higher, it means the less fresh of fish meat.
r value	$r = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2}\sqrt{\sum_{i=1}^{n}(y_i - \bar{y})^2}}$ where x_i is ith of x variables, $\bar{x}$ is the mean of x variables, y_i is ith of y variables, $\bar{y}$ is the mean of y variables. The correlation coefficient of r value is between -1 and 1 which can compare the relationship between x and y variables.
Recovery	$Recovery = \frac{the\ concentration\ from\ Analysis}{the\ real\ concentration} \times 100\ \%$ The value of recovery is used as the efficiency for our experiment process. Based on the value, it can confirm the quality of our experiment method.

RSD value	$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$, $S = \left[\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}\right]^{\frac{1}{2}}$, RSD value $= \frac{S}{\bar{x}} \times 100\ \%$ where $\bar{x}$, *S*, and *n* denote as mean, standard deviation, and the number of $\{x_i\}$, respectively. RSD is used to confirm the precision of HPLC results.